Stellingen behorende bij het proefschrift *Description of the kinetics and enantioselectivity of lipases* van J.B.A. van Tol

1. De algemene kritiek op de Lineweaver-Burk methode dat kleine fouten in metingen van lage snelheden te sterk doorwerken, is in feite kritiek op de gebruiker zelf, die het wegen van de fouten zoals aangegeven door Lineweaver en Burk achterwege laat.


2. De experimentele bevestiging van de lineariteit tussen de reaktiesnelheid en de hoeveelheid lipase in emulsiesystemen is niet alleen van belang voor een correcte meting van de katalytische activiteit maar ook van de enantioselectiviteit.

   *Dit proefschrift*

3. Op basis van een minimaal kinetisch schema is men er jarenlang ten onrechte vanuit gegaan dat verhoging van de omzettingsgraad bij irreversibele enzymatische resoluties altijd leidde tot toename van de optische zuiverheid van het overblijvende substraat.

   *Dit proefschrift.*

4. De dag-tot-dag variatie van enzymatische parameters zou bepaald moeten worden ten opzichte van een referentie-substraat om conclusies omtrent de enzymologie te kunnen trekken.


5. Alhoewel chiraliteit zeer belangrijk is voor geneesmiddelen, is het veel gebruikte voorbeeld van het slaapmiddel softenen onzinnig.


7. Aangezien het betrouwbaarheidsinterval van met name hoge *E*-waarden groot en asymmetrisch is, dient vergelijking van *E*-waarden op basis van de boot-strap methode te gebeuren.

8. Het gebruik van de Unifac-methode levert een schatting van de activiteitscoefficient op, die voor nauwkeurige experimenten geverifieerd dient te worden.

9. De resultaten van Meng et al. zijn alleen in overeenstemming met de conclusie van Van Bastelaere dat de lineaire vorm van glucose omgezet wordt, indien de opening van cyclisch β-glucose in het isomerase-complex de snelheidsbepalende stap is.


10. Gezien de ontwikkelingen in de reguliere geneeskunde, dient het effect van links- dan wel rechtshandigheid van alternatieve genezers, zoals handopleggers, magnetiseurs en acupressuristen, onderzocht te worden.

11. Normen en waarden zijn tijdsafhankelijke variabelen.

12. Een wetenschappelijk bewijs behoeft in theorie slechts éénmaal gepubliceerd te worden, in de praktijk is dit helaas niet voldoende.


13. Bij serieus onderzoek dient het aantal experimenten ten minste één groter te zijn dan het aantal verklaringen.

14. Om de zuiverheid van de rechtsgang te waarborgen, dient de rechter expertkennis niet alleen op onderdelen maar voor het beantwoorden van de gehele schuldvraag te gebruiken.

15. Als rood van de PvdA en blauw van de VVD het paars van het kabinet vormen, dan heeft D’66 slechts als katalysator van de formatie gefungeerd.

16. In het kader van de bezuinigingen dient men zich af te vragen of het wachtgeld van ministers, die net als AIO's aangesteld zijn voor een periode van vier jaar, betaald zou moeten worden door het eigen departement.

17. Het enthousiasme voor een nieuwe vinding is recht evenredig met de begrijpbaarheid ervan.
Description of the kinetics and enantioselectivity of lipases in various media

Proefschrift

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof. ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College van Dekanen aangewezen,
op maandag 19 september 1994 te 16.00 uur

door

Johannes Bernardus Antonius van TOL

doctorandus farmacie

geboren te Rotterdam
Dit proefschrift is goedgekeurd door de promotor:
Prof. dr. ir. J.A. Duine

This study was carried out at the Department of Microbiology and Enzymology,
Delft University of Technology, The Netherlands,
and financially supported by DSM-Andeno, Venlo, The Netherlands.
Aan mijn ouders
Voorwoord

Voor u ligt het resultaat van een actief en selectief onderzoek naar enzymen. Mede door de goede sfeer in het Kluverlaboratorium voor Biotechnologie heb ik met veel plezier aan mijn promotieonderzoek gewerkt. Ik wil de medewerkers van de sectie enzymologie, de instrumentmakerij en de technische dienst, alsmede alle overige bewoners van het lab hiervoor bedanken. Daarnaast wil ik een aantal mensen met name bedanken die bijgedragen hebben aan het onderzoek.

Het project was opgestart door Jaap Jongejan, die een enthousiaste stimulator was van nieuwe richtingen binnen het onderzoeksproject. Hans Duine was een onmisbare steun bij het afronden van het project en het schrijven van de artikelen. Hans en Jaap, dank voor de mogelijkheden en hulp die jullie mij geboden hebben.

Voor thermodynamisch inzicht en adviezen kon ik altijd terecht bij Xiao Mei Shen, Theo de Loos en Jacob de Swaan Arons van Thermodynamica. Bert van Zomeren van Statistiek wist de grondslagen van de statistiek voor mij aanschouwelijk te maken. Daarnaast heb ik ook een prettige samenwerking met Hans Kierkels van DSM gehad. De fax was hierbij ideale katalysator voor een snelle uitwisseling van ideeën en resultaten.

Natuurlijk wil ik ook Diana bedanken, die eerst als stagiaire en later als medewerker van de TU met toewijding aan het project meegewerkt heeft. Gelukkig schept zij orde in het lab, waarbij ook een nauwgezet melkhaalschema ingevoerd werd.

Aan het project is meegewerkt door een aantal afstudeerders. Hun inzet en inzichten heb ik ervaren als een bron van inspiratie. De eerste afstudeerder in het project, Jan, heeft de verkennende experimenten voor de kinetiek-in-organische-oplosmiddelen uitgevoerd. Om de wateractiviteit in de oplosmiddelen te kunnen bepalen, heeft hij uiteenlopende kleurreacties uitgevoerd. Alhoewel dit vaak tot een prachtig kleurenschouwspel leidde, bleken de dynamiek en onvoorspelbaarheid van de kleuren een hindernis voor praktische toepassing. Rob werkte buiten het langlaufseizoen enthousiast mee aan het onderzoeksproject. Door hem kwam ik tot de ontdekking dat het "lang" niet alleen op de ski's, maar ook op het seizoen slaat. Rob, nu je zelf AIO bent, hoop ik dat jouw afstudeerders net zo plezierig zijn om mee te werken als jij dat was. Als laatste afstudeerder was Willem-Jan betrokken bij het afronden van het onderzoek. Hij heeft met groot doorzettingsvermogen de losse kinetiek-in-organische-oplosmiddelen eindjes aan elkaar geknoopt.
Met kamer- en labgenoot Arie (collega Geerlof) heb ik een nuttige, maar bovenal prettige samenwerking gehad. Beste Arie, door onze gezamenlijke rondjes om, congresbezoeken en Keldertjesoverleg heb ik goede herinneringen aan mijn promotieperiode.

Han Rakels en Adrie Straathof van de "enzymengroep" van BPT stonden altijd klaar voor wandelgangenoverleg. Bas Romein, Arie Braat, Joop Houwers en Hans Kemper waren onvermoeibaar bij het oplossen van de steeds weer in een nieuwe vorm opduikende programmeerproblemen.

Hier wil ik ook een aantal mensen bedanken die buiten het onderzoek een rol in mijn leven spelen. Op de eerste plaats wil ik mijn ouders bedanken die altijd met raad en daad klaar stonden. Zoals de kaft van dit boekje al doet vermoeden, leef ik mij (na gedane arbeid) uit op de squashbaan (en squashpartner Ronald). De eerste student die meehelp met mijn onderzoek heb ik gelijk gehouden. Ingrid, onze relatie is het mooiste resultaat dat ik heb overgehouden aan mijn promotieperiode. Tevens kan ik je garanderen dat deze versie van het proefschrift echt de aller-aller-laatste is.

Bert van Tol
Contents

Chapter 1  General Introduction 9

Chapter 2  Description of hydrolase-enantioselectivity must be based on the actual kinetic mechanism: Analysis of the kinetic resolution of glycidyl (2,3-epoxy-1-propyl) butyrate by pig pancreas lipase 25

Chapter 3  Do organic solvents affect the catalytic properties of an enzyme? Intrinsic kinetic parameters of lipases in ester hydrolysis and formation in various organic solvents 47

Chapter 4  Thermodynamic and kinetic parameters of lipase catalyzed ester hydrolysis in biphasic systems with varying organic solvents 69

Chapter 5  The catalytic performance of pig pancreas lipase in enantioselective transesterification in organic solvents 89

Chapter 6  Towards intrinsic enzyme substrate affinities by using thermodynamical activities to correct ordinary kinetic parameters for substrate-water interactions 111

Summary 133

Samenvatting 135

List of Publications 137

Curriculum Vitae 139
Chapter 1

General introduction
Introduction

In preparative synthesis enzymes are applied under unnatural conditions, i.e. in media with high substrate concentrations, in biphasic systems consisting of a water and an organic solvent phase or in organic solvents with low water content. As discussed below, certain aspects of enzyme kinetics under these conditions are quite different as compared to that observed in dilute aqueous, buffered solutions. Apart from this, application of enzymes requires that know how obtained under a certain regime can be translated to other situations, i.e. that predictions can be made on enzyme performance for the conditions employed. The latter means bridging the gap that exists between enzymology and technology, the target of this thesis work. Since present academic as well as industrial research is mainly focused on kinetic resolutions with hydrolases, the need formulated above concerns in particular these conversions. However, before it is possible to derive predictive models for this, first a number of fundamental questions have to be answered: Do enzymes behave differently in organic solvents as compared to water?; if not, how can knowledge on the behavior in water be translated to that in other media?; if so, what is the underlying rationale?; are the models commonly used to describe enzyme enantioselectivity, also valid for conditions relevant for kinetic resolutions in practice? The questions formulated here will be addressed in the following sections. The present status of the field is discussed under the heading "Predictive models", in which a thermodynamic approach of kinetic models will be formulated and evaluated.

Enzyme kinetics

Enzyme kinetics in aqueous media

Since enzymic experiments are usually carried out in dilute aqueous solutions, substrate concentrations are generally used in the equations for the equilibrium constant and the reaction rate. Based on this usage, the dimensions of active sites have been traced by comparing the "affinities" ($K_m$) or specificity constants ($k_{sp}$) of a series of homologous substrates. However, if it is realized that the hydration energies may vary substantially in the series, it is clear that the "availability of the substrates for the enzyme" will also vary. A comparison based on ordinary kinetic parameters to delineate the dimensions of an active site will lead to wrong conclusions. In such a case, substrate-water interactions contribute to the overall kinetic behavior, quantified by the kinetic parameter values, while solvent-independent "intrinsic" values are needed. A similar need is manifest in comparative enzymology where it is attempted to extrapolate enzyme performance obtained in in vitro experiments to the in vivo situation. Since the extent of hydration of the substrates will be different in the two
situations, enzyme performance in the cell and that in the test tube will be different, and attempts are made to mimic cellular conditions in the test tube. The effect of substrate hydration on its binding to protein has been recognized.\textsuperscript{5-10} However, at the beginning of this thesis work, no extension to enzyme kinetics had been made, and a practical way to account for substrate-water interactions was not available.

**Enzyme kinetics in biphasic systems**

Lipase-catalyzed conversions are mostly performed in aqueous-organic, biphasic systems.\textsuperscript{2,11} The organic phase may be either an organic solvent in which the substrate is dissolved or may totally consist of the substrate. Especially in the latter case, large changes will occur in the phase ratios and compositions during a conversion process. In contrast to esterases, lipases become catalytically activated when adsorbed to the interface.\textsuperscript{12} This latter leads to a number of questions: does the lipase react with its substrate in the water phase, the organic phase or at the interface?; since the lipase will be in contact with the organic layer, does the organic phase affect its performance (in view of the changing composition of the layer during conversion or the application of different organic solvents)? It has been proposed to use interfacial substrate concentrations in rate equations,\textsuperscript{13-15} but since the interfacial state can hardly be defined,\textsuperscript{16} this has no practical relevance.

On varying the organic solvent, different catalytic activities have indeed been observed.\textsuperscript{17} To rationalize this, it has been attempted to correlate activity with various physical properties of the solvent: the Hildebrandt parameter, the dielectric constant, the hydrophobicity (log P), or combinations of these parameters.\textsuperscript{18-21} From the most frequently used plots (activity against log P), it appears that hydrophobic solvents (high log P) give the highest reaction rates (the data points suggest a sigmoidal trend).\textsuperscript{19} However, since a causal relationship is lacking, a quantitative predictive model cannot be derived from this.

Nakanishi and coworkers\textsuperscript{22} derived rate equations based on partition coefficients and assuming in this that the enzyme reacts with substrate in the aqueous phase. However, since the lipases act at the interface, and the derivation was based on an ideal biphasic system (for diluted substrates), application to the practical situation seemed questionable. Thus, at the start of the project, also no consistent model existed to describe the kinetics for lipase catalyzed reactions in biphasic systems.

The equilibrium constant in biphasic systems has been related to that in an aqueous system by introducing an apparent equilibrium constant.\textsuperscript{23,24} The latter is based on the biphasic concentrations of the substrates, that is the total amount of substrate divided by the total volume of the biphasic system. The apparent equilibrium constant depends on the partition coefficient of the substrates and the volume ratio of the organic and aqueous phase. However, in analogy to the rate equations, this relation will only be valid in the range of low substrate concentrations since otherwise the partition coefficients will vary.
Enzyme kinetics in organic media

The catalytic activity of enzymes is not restricted to aqueous mono- or biphasic systems, but is also manifest in low-water-containing media. The latter has already been noticed in the early days of enzymology. For instance, β-glycosidase dispersed in 90 % ethanol remains catalytically active. The same applies to α-chymotrypsin in methylenechloride, and to xanthine oxidase, and horseradish peroxidase and catalase suspended in various organic solvents. Klibanov and coworkers have promoted the use of enzymes in organic solvents by adding many other examples. It appears that enzymes are also catalytically active in (supercritical) gas phases.

Potential advantages of employing enzymes in organic solvents as opposed to aqueous conditions, are: the possibility to shift equilibria by selecting the appropriate solvent; reduction of water-dependent side-reactions; enhancement of thermal stability of the enzymes may occur under certain conditions; and ease of product recovery by using low-boiling solvents. However, also severe drawbacks should be mentioned: the catalytic performance of enzymes in organic solvents is generally three orders of magnitude lower than in aqueous media; organic solvents, in particular the polar ones, are sometimes detrimental for enzyme activity or stability; all kinds of effects on enzymic performance have been attributed to organic solvents, but a consistent line in this appears to be absent (the latter will be further discussed under the heading "Predictive models"). Since the first two drawbacks are very relevant for the experimental set-up in this work, aspects connected with these will be further addressed below.

Enzyme preparation

In contrast to enzymology in water, that in organic solvents has to deal with the history of the enzyme before it was lyophilized (although immobilized as well as "solubilized" variants have been described, mostly the lyophilized powder form, just suspended in the solvent, is used). Since the enzyme conformation is in fact frozen and activity is determined by this, the composition of the solvent present before lyophilization is relevant. Thus, to attain reproducible results, it is important to know the nature, concentration, and pH of the buffer, and that of any (stabilizing) agent added. This is also relevant for the reaction itself since these compounds may affect water activity, a crucial factor in the reaction and for the stability of the enzyme.

The points indicated above have not always been fully recognized in the literature. Scattered information can be found on some of them, but no general agreement exists, as illustrated for the pH memory effect. It will be clear that systematic studies on enzyme preparations with varying history are highly desirable, and attempts has been recently made. To avoid any complications, all experiments described in this thesis were carried out with the same lot of enzyme, and in case the effects observed might be due to the particle nature of
the enzyme, also the immobilized form was used (Chapter 3).

Since the enzymes do not dissolve in the solvent, catalytic activity will be directly related to the exposed surface area. For this it is crucial to know the size and the size distribution of the particles as well as the number and size of the pores. Surprisingly, hardly any attention has been paid to this. Kamat and coworkers\textsuperscript{44} reported that particles in lyophilized preparations of subtilisin are porous. In contradiction to this, all analytical methods applied to the lipase preparations used in this thesis, gave a consistent picture: massive particles without pores penetrable for substrate molecules (Chapter 3). In case our observation has general validity, the low activities of enzymes in organic solvents could be increased by increasing the surface area.

The role of water

General agreement exists that water plays a crucial role in low-water-containing systems in which enzymatic conversions take place: as a substrate,\textsuperscript{45} e.g. in ester hydrolysis; in a form bound to the enzyme, affecting its stability and catalytic performance, presumably due to the fact that the extent of hydration determines the flexibility of the protein chain.\textsuperscript{46-51} However, with respect to the latter, no consistent picture has emerged so far since it appears that each enzyme behaves differently (which may be related not only to the properties of the protein concerned, but also to the history of the preparation and to the surface area exposed to the solvent). Halling and coworkers\textsuperscript{52,53} have made important contributions to this point: by emphasizing that water activities should be used to enable comparison of the water effect in different solvents; by introducing a method with which adjustment of a constant water activity in the system can be achieved (equilibration of enzyme suspensions in the presence of hydrated salts).\textsuperscript{54,55} The many examples in which the role of water has been studied, are mentioned in several reviews.\textsuperscript{37,38,56}

Reaction conditions

The pH at which the reaction is performed in organic solvents is mostly unknown since it will depend on the history of the enzyme (state of protonation of the protein before lyophilization, the presence of buffer salts), the water content, the polarity of the solvent, and the type of (organic) acid involved in the reaction.\textsuperscript{57} However, in view of the mechanism in which the pH exerts its effects on enzyme activity in water, it can be expected that enzyme activity in organic solvents will also be strongly affected by the pH value. Experimental procedures to measure pH have been developed.\textsuperscript{57} It appears to be good practice to keep the factors (indicated above) which affect the pH constant.

It has been reported that temperature not only affects activity but also enantioselectivity, as in the case of the conversion of a racemic secondary alcohol by a thermophilic, NAD-dependent alcohol dehydrogenase.\textsuperscript{58,59} It will be clear that constant
temperatures should be used in kinetic resolutions, as has been the case in the experiments described in this thesis. Many authors claim that organic solvents affect enzyme activity as well as enantioselectivity\textsuperscript{60,61} and explanations for this assume that enzyme properties are modified by the solvents [examples can be found in several reviews\textsuperscript{37,38}]. However, can the effects really be ascribed to changes in the catalytic performance of the enzyme or are they caused by "differences in the availability of the substrate to the enzyme", originating from differences in substrate-solvent interactions? This question will be addressed in the paragraph on Predictive models.

**Enantioselectivity**

The first description of an enzymic enantioselective conversion concerns the kinetic resolution of a racemic ester by lipase.\textsuperscript{62} Nowadays, many other examples are known and lipase is still a favourite enzyme for these processes.\textsuperscript{3,5} Enantioselectivity in kinetic resolutions is indicated by the ratio of the rates with which the enantiomers are converted. In this respect, enantioselectivity is merely a special case of substrate specificity. Thus, the selectivity of enzymes obeying Michaelis Menten kinetics and catalyzing UniUni reactions, can be quantified by the ratio of the specificity constants.\textsuperscript{63,64} When it concerns different substrates, the parameter is called the selectivity constant (\(\alpha\)).\textsuperscript{65} and in the case of enantiomers, the enantiomeric ratio (\(E\)).\textsuperscript{66,67}

The concept has been applied to kinetic resolutions catalyzed by esterases\textsuperscript{62} and lipases.\textsuperscript{68} Expressions describing the progression of first order reactions have been derived by Bredig and Fajans\textsuperscript{69}. Subsequently, the process variables \(ee\) (enantiomeric excess) and \(\xi\) (degree of conversion) were introduced.\textsuperscript{70,71} Equations relating these variables have been derived for irreversible as well as reversible enzymatic resolutions by Chen and coworkers.\textsuperscript{66,67} These equations are valid for UniUni reactions, and for limiting cases of BiBi (two substrate two product) reactions. Nevertheless, they are widely used, most probably because most enzymatic reactions are of the BiBi type (e.g. hydrolases like lipase converts ester and water into alcohol and acid) but the restrictions to application of the equations are not generally realized. As will be discussed in the next section, equations should be used based on the correct mechanism and correction for substrate solvent interactions is sometimes required.
Predictive models based on thermodynamics and correct kinetic mechanisms

Equilibrium

Normally, the equilibrium of a reaction is represented by the value of the dynamic equilibrium constant \( K_{eq} \), which is based on the concentrations of the reacting species. However, since substrate-solvent interactions will vary for the reacting compounds, the value will vary with the type of solvent. To circumvent this, the thermodynamic equilibrium constant \( K_{eq}^{th} \) can be used. Since this is based on thermodynamic activities \( a \), the values are solvent independent by definition. Using \( a = \gamma \cdot c \), the following relationship between \( K_{eq} \) and \( K_{eq}^{th} \) can be formulated:

\[
K_{eq}^{th} = \frac{a_P \cdot a_Q}{a_A \cdot a_B} = \frac{\gamma_P \cdot \gamma_Q}{\gamma_A \cdot \gamma_B} \cdot K_{eq}
\]

(1)

Constant values of \( K_{eq}^{th} \) in different solvents has been obtained for many equilibria, also for enzyme-catalyzed equilibria.\(^{36,48,72,73}\) It has also been confirmed for lipase catalyzed equilibria in various biphasic systems (Chapter 4).

Kinetics

The thermodynamic equilibrium description mentioned above can also be applied to the reaction steps in the catalytic cycle. Since the thermodynamic equilibrium constant can be expressed in thermodynamic activities but also as the ratio of the rates for the forward and reverse reaction at equilibrium, it has been indicated already by several authors\(^{74}\) that thermodynamic activities can also be used in reaction rate equations.

Introduction of thermodynamic activities in rate equations can also be carried out according to the Brönsted-Bjerrum theory, using a thermodynamic instead of a dynamic equilibrium between the ground state and the transition state of the reacting species.\(^{75}\) Although no theoretical proof exists for these transformations,\(^{76}\) experimental support has been provided by the results of studies on gas phase\(^{77}\) and ionic reactions\(^{78}\). Application of thermodynamic activities has been extended to aqueous enzymatic reactions to describe the effects of salts and hydrocarbons\(^{79}\) and cosolvents\(^{80}\) on the catalytic activity of \( \alpha \)-chymotrypsin. Furthermore, thermodynamic activities were used to model the invertase-catalyzed hydrolysis at high concentrations of sucrose to correct for the non-ideality of the system.\(^{81}\)

Implementation of thermodynamic activities in rate equations for enzymes has been carried out by us\(^{82}\) for pseudo second order kinetics and by Straathof and coworkers\(^{83}\), considering the elementary reaction steps. The equations derived to describe the enzymic reaction rates are presented in Chapter 3. Recently, using a similar reasoning, Dordick and coworkers also rationalized the effects of organic solvents on the performance of horse radish peroxidase\(^{84}\) and subtilisin\(^{85}\). They concluded that perhaps the most important effect of organic
solvents concerns the ground state of the substrates. In another recent report, activity coefficients were introduced in rate equations in order to describe the effect of surface pressure on the conversion of monolayers by phospholipase. Klibanov used thermodynamic activities, in analogy to the work of Bell, to describe substrate specificity in various solvents. On considering the different approaches, it appears that the underlying theories are thermodynamically correct. However, this conclusion may not apply to the execution of the method. For instance, $\gamma$-values estimated with the Unifac method may deviate substantially from the more reliable experimentally determined ones (Chapter 3).

As shown in Chapters 3, 4 and 5, the intrinsic kinetic parameters that are corrected for solvent-substrate interactions, vary for the different solvents. The variations become much less when competitive substrate inhibition of the solvents is assumed to occur. Studies on 3-dimensional enzyme structures show indeed that organic solvents may replace structurally bound water from the active site. It is too early, however, to conclude that solvent effects remaining after correction for substrate-solvent interaction are merely due to this type of inhibition. Further research is necessary to substantiate this vision and to develop a model for the residual effects. Thus, although a complete predictive model is still awaiting, it will be clear that the incomplete one should be applied already as it is indispensable to delineate the effects that cannot be accounted for.

During our studies on enzymes in organic solvents, we realized that the concepts derived for this have impact also on classical enzymology. As shown in Chapter 6, application of this approach reveals the "intrinsic substrate specificity" of an enzyme, which concerns only enzyme and substrate. Combined with computational methods for determination of substrate-enzyme binding, this may lead to a better understanding of this process.

**Enantioselectivity**

Lipase catalyzed kinetic resolutions proceed by a ping-pong BiBi mechanism. It is well known that the alcohol moiety of the ester is removed first, after which the enzyme-acyl complex is attacked by water. The complex may, however, also react with another nucleophile, for instance the alcohol enantiomer formed during the conversion. This will affect the enantioselectivity of the resolution process, observed as a plateau effect in plots of the enantiomeric excess of the remaining substrate versus the degree of conversion (Chapter 2). From experiments in various solvents, we deduced that solvent-substrate interactions may affect the enantioselectivity as a result of this mechanistic feature of lipases. Taking this into account, the equations describing the enantioselectivity of lipases were evaluated in various solvents (Chapter 5). To find solutions for the problem of the plateau effect under preparative conditions (high concentrations of the product), the model can be used to select a solvent in which the plateau effect is minimal.
Extension of this approach to other theoretical situations has been carried out by Straathof and coworkers\textsuperscript{92}, and some of the equations were evaluated by Rakels and coworkers.\textsuperscript{93} From these and our own results it can be concluded that the equations derived are adequate and can predict enantioselective behavior of hydrolases under various conditions. Since several authors claim that certain additives (alkaloids,\textsuperscript{94-96} cosolvents,\textsuperscript{97-99} zeolites\textsuperscript{100}), affect the enantioselectivity, the models will be also useful to rationalize these findings.

The enantiomeric ratio, $E$, and the intrinsic selectivity factor, $\alpha$, that quantifies the plateau effect, of the transesterification of vinyl acetate with racemic glycidol by porcine pancreas lipase are constant for the media investigated (Chapter 5). This outcome seems to contradict the variation observed in intrinsic kinetic parameters of porcine pancreas lipase in these media. However, it is in agreement with the assumption that the solvents affect the reaction rate via competitive binding to the active site. In this context, it should be mentioned that for other enzyme substrate combinations, reversal of the enantiomer preference with the solvent has been reported.\textsuperscript{100-102} Again, a clear distinction between solvent effects on the catalytic properties of the enzyme and solvent effects on the substrates should be made.

![Figure 1](image_url)

**Figure 1.** Number of articles indexed in the chemical abstracts dealing with lipase and enantioselective catalysis.
Lipases

Lipases are attractive biocatalysts for application in industrial chemical synthesis since\textsuperscript{1,2,11}: they are available in large quantities at low cost; they are compatible with organic solvents; they show enantioselectivity for a broad range of substrates, including building blocks for production of pharmaceuticals.\textsuperscript{103} Not surprisingly, therefore, the number of reports dealing with this has steadily increased in recent years (Fig 1).

Much effort has recently been devoted to the elucidation of the 3-dimensional structure of lipases.\textsuperscript{104-107} The emerging picture is also relevant for the work presented here: lipases possess a flap which opens when the enzyme become activated by adsorption to the interface of emulsion droplets.\textsuperscript{106,107} However, our results for porcine pancreas lipase (PPL) in water (Chapter 2) and organic solvents (Chapter 5) show that the enzyme is still active in the absence of an interface, albeit with lower activity and enantioselectivity. It could be rewarding, therefore, to investigate whether the activated form of lipase could be stabilized so that better performance can be achieved in e.g. organic solvents.

Outline of the thesis

Originally, the aim of the project was to provide knowledge for the enzymatically catalyzed kinetic resolution processes carried out at Andeno-DSM. It was soon realized that in order to make reliable predictions, new concepts had to be introduced to describe PPL-catalyzed resolution of racemic glycidyl butyrate (Chapter 2). A logical step was to extend this to the enzymology of lipases in organic solvents. After further elaboration of the correction for solvent-substrate interaction, experimental verification was performed with PPL and \textit{Pseudomonas cepacia} lipase (PcL) in several organic solvents (Chapter 3). Since extensive measurements on emulsions with PcL had been carried out at DSM, it was attempted in a joint effort to model the data with the equations derived (Chapter 4). Enantioselectivity is generally regarded as a very sensitive probe for changes in enzyme performance. Thus, to study effects of organic solvents by another approach, experiments were carried out with PPL and racemic glycidol in transesterification reactions (Chapter 5). Finally, it was realized that the approach developed could contribute also to better insight into classical enzymology in water. For that purpose, binding as well as kinetic properties were modelled for porcine liver esterase, PcL and horse liver alcohol dehydrogenase (Chapter 6).
References


764.


Chapter 2

Description of hydrolase-enantioselectivity must be based on the actual kinetic mechanism: Analysis of the kinetic resolution of glycidyl (2,3-epoxy-1-propyl) butyrate by pig pancreas lipase

J. Bert A. van Tol, Jaap A. Jongejan, and Johannis A. Duine
Abstract

The kinetic resolution of R,S-glycidyl (R,S-2,3-epoxy-1-propyl) butyrate catalyzed by pig pancreas lipase (PPL) was studied in monophasic and biphasic systems. The course of the resolution at ester concentrations exceeding 0.05 M or in the presence of R,S-glycidol (R,S-2,3-epoxy-1-propanol), could not be described by the equations derived for a one substrate enzyme with a minimal kinetic scheme.\(^1\) Trivial causes like heterogeneity in activity of the (crude) PPL preparation and equilibrium phenomena due to changing phase ratios could be excluded. An equation based on the kinetic mechanism of hydrolases, in which the acyl-enzyme intermediate is allowed to react with water as well as with the produced alcohol (quantified by the selectivity factor, \(\alpha\)), was evaluated. All initial rate and conversion data could be adequately fitted with this equation, not only for PPL in the monophasic (free in solution) but also in the biphasic (adsorbed to the interface) systems where it exhibited better activity and enantioselectivity. Thus, the enantiomeric ratio (\(E\)) and \(\alpha\) are intrinsic parameters of PPL, remaining constant during the course of the reaction. The conclusions presented here applies to all hydrolases acting \(\textit{via}\) an acyl-enzyme complex.

Introduction

Discrimination between the enantiomers of a racemic substrate by an enzyme can occur at the level of recognition and/or conversion. Based on the underlying idea that this is an intrinsic property of an enzyme, enantioselectivity (represented by the parameter \(E\), the enantiomeric ratio\(^1\)) is defined as the ratio of the specificity constants (\(k_{sp}\)'s) for the enantiomers:\(^2\)

\[
E = \frac{k_{sp}^R}{k_{sp}^S} = \frac{k_{cat}^R/K_M^R}{k_{cat}^S/K_M^S} \tag{1}
\]

According to this definition, the \(E\)-value can be obtained by determining the kinetic parameters (\(k_{cat}\) and \(K_M\)) for each enantiomer. If the pure enantiomers are not available, \(E\) can be derived with methods in which initial rate measurements are carried out on samples with constant concentration of substrate but with varying ratio of enantiomers\(^3\) or by analyzing progress curves\(^4\).

Enantioselectivity of an enzyme can also be defined in an operative way, that is by relating the momentary, apparent enantiomeric ratio in the conversion process (\(E_{app}\)) to the ratio of the rates with which the enantiomers are converted at that moment:
\[ \frac{r_R}{r_S} = E_{\text{app}} \cdot \frac{c_R}{c_S} \] (2)

However, theoretical considerations have already indicated that depending on the kinetic mechanism, \( E_{\text{app}} \) and \( E \) will not always be identical.\(^5\,^6\)

By assuming that the enantiomers are mutually excluding each other on the enzyme and that a minimal kinetic scheme (reversible, uni-uni) is applicable, Chen and co-workers\(^1\) derived an equation by integrating the initial rate equations for this scheme. In this, \( E \) (defined according to eq 1) is related to the extent of conversion \((\xi)\), the equilibrium constant \((K_{eq})\) and the enantiomeric purity (indicated by the enantiomeric excess of the substrate, \(ee_s\)), as follows:

\[ E = \frac{\ln[1 - (1 + \frac{1}{K_{eq}}) \cdot (\xi - (1 - \xi) \cdot ee_s)]}{\ln[1 - (1 + \frac{1}{K_{eq}}) \cdot (\xi + (1 - \xi) \cdot ee_s)]} \] (3)

The validity of eq 3 for describing kinetic resolutions with hydrolases is so far undisputed. This is illustrated by the fact that to determine \( E \) of hydrolases in reactions that go to completion, mostly the data (for \( ee_s \) and \( \xi \)) of one sample taken during the course of the reaction are thought to suffice for that purpose. However, conversions by these enzymes occur via a ping-pong bibi mechanism\(^7\) of which it is questionable whether it may be represented by a minimal kinetic scheme. Furthermore, it is known that the acyl-enzyme complexes can react with other nucleophiles than water,\(^8\,^9\) a property which has so far not been taken seriously since eq 3 is usually also applied for conditions where water is no longer dominating (e.g. for conversions in organic solvents). Therefore, should the description for ping-pong reactions be advocated in order to obtain more reliable results on enantioselectivity of hydrolases and predictions for the kinetic resolution process? Since deviations from eq 3 will have remained largely unnoticed so far, due to common practice indicated above with respect to \( E \) determinations, the answer to this cannot a priori be given. It requires analysis of the whole conversion process catalyzed by such an enzyme under a variety of conditions.

Pig pancreas lipase (PPL) belongs to the large group of serine-type hydrolases.\(^10\) Several of these enzymes appear to be very useful since they show enantioselectivity towards the alcohol and/or acid moiety in esters.\(^9\,^11\) During our studies on kinetic resolution of glycidyl butyrate with PPL, we discovered a significant drop in \( E \)-value for samples taken during the course of the reaction.\(^5\) It seemed, therefore, an attractive model system to investigate the relevance of the precise mechanism for the remarks made above. To provide
clear evidence for the mechanism-related effect, much effort was made to examine the following possibilities as causes for the observed deviations: heterogeneity of the enzyme preparation with respect to glycicydyl butyrate hydrolysis; unreliable methods or conditions for $E$ determination;\textsuperscript{12} the changing quality (and thus the interface) and quantity (volume) of the phases during the reaction with consequences for the kinetics and thermodynamics of the system.

**Materials and Methods**

**Materials**

The following chemicals were used: racemic glycicydol (2,3-epoxy-1-propanol) (Janssen Chimica, Belgium); racemic and $R$-(-)-glycicydyl butyrate ($ee_5 = 99.5 \%$) were a gift of DSM-Andeno, The Netherlands. All other reagents were analytical grade (Merck, Germany). Porcine pancreatic lipase was obtained from Sigma Chemical Company, USA (type II, referred to as "crude PPL") and *Pseudomonas cepacia* lipase from Amano Co., Japan. Low and high molecular weight calibration kits for electrophoresis, and Coomassie brilliant blue were purchased from Pharmacia.

To screen for glycicydyl butyrate hydrolyzing activities in crude PPL and to obtain homogeneous PPL A/B, a purification procedure adapted from Verger et al.\textsuperscript{13} was applied. The system used for PPL purification consisted of a Waters HPLC-system (type 510 pump, 600 E system controller, 991 photodiode array detector, and PDA software data-acquisition). The purification procedure consisted of the following steps. Step I: 20 grams of the crude preparation was dissolved in 100 ml demineralized water and stirred gently for 1 hour at 4°C. The suspension was centrifuged for 15 min at 48,000 g and the supernatant was collected. Step II: The supernatant was applied to a Pharmacia DEAE-Sepharose (25 * 4.2 cm) column equilibrated with 5 mM Tris/HCl buffer, pH 8.0, containing 3.3 mM CaCl$_2$ and 10 mM NaCl at 4°C. After washing the column with 3 volumes of this buffer, the NaCl concentration was raised via a linear gradient in 500 ml from 10 mM to 500 mM NaCl using a flow rate of 1 ml/min. The eluate was monitored at 280 nm and fractions of 20 ml were collected. Step III: Fractions of step II with hydrolytic activity for glycicydyl butyrate were pooled and concentrated to a volume of 2.5 ml. Aliquots of this were applied to a Pharmacia Superdex 75 HR 10/30 gel filtration (30 * 1 cm) column equilibrated with 10 mM phosphate buffer, pH 7.8 and chromatographed at a flow rate of 0.2 ml/min. Homogeneity of the eluting peaks was checked by an overlay procedure using the absorption spectra taken at regular time intervals.\textsuperscript{14} Active fractions were concentrated by centrifugation in a Centriprep 10 module (Amicon, membrane cut-off 10 kDa).
Analytical procedures

GC (Hewlett Packard 5890 series II chromatograph equipped with flame ionization detection) analyses of glycidol were carried out with the aqueous phase. For glycidyl butyrate the organic phase was analyzed. Samples were obtained by extracting 0.5 ml aqueous phase with 10 ml dichloromethane and drying the organic layer with MgSO₄. The concentration of R,S-glycidol and R,S-glycidyl butyrate was determined on a HP-1 (Hewlett Packard, 1=30 m, i.d. =0.52 mm) column. The enantiomeric composition of glycidol and glycidyl butyrate was determined on a γ-TA (Astec, trifluoroacetyl derivatized γ-cyclodextrine, 1=20 m, i.d. = 0.32 mm) chiral column.¹⁵ The temperatures at which both columns were operated were: initial temperature 85 °C for 8 min, 10 °C/min to 90 °C, 10 min 90°C (allowing both glycidol and glycidyl butyrate to be determined in one run).

The molecular masses of the proteins/subunits were determined with polyacrylamide gel electrophoresis on commercially available gradient gels (8-25%) using the Pharmacia Phast system. To denature the proteins for SDS gel electrophoresis (Separation technique file 110), the protein solution was heated for 5 min at 100°C in the presence of 5% sodium dodecyl sulphate and 25% β-mercaptoethanol. Protein staining occurred with the Coomassie Brilliant blue procedure. The molecular mass of native enzyme was determined with the Superdex 75 HR gel filtration column (Step III). The column was calibrated using a mixture of Blue Dextran 2000, conalbumin, bovine serum albumin, carbonic anhydrase, chymotrypsin, cytochrome c, and ferricyanide.

The specific absorption coefficients at 280 nm were derived from the 280/205 nm absorbance ratios via the equation given by van Iersel et al.¹⁶ The ratio was determined from the spectra obtained with a Hewlett-Packard HP 8450 A photodiode array spectrophotometer. A specific absorption coefficient, A⁺₀.₁%₂₈₀, of 1.45 for PPL A/B was found (in agreement with the reported value of 1.33)¹⁷ and the final preparation consisted of 0.37 mg PPL A/B/ml. The A⁺₀.₁%₂₈₀ value of the 33 kDa enzyme in the fraction containing the minor activity was 2.37. This final preparation consisted of 0.32 mg 33 kDa enzyme/ml.

Equilibrium, dissociation and partitioning constants

The values of $\phi^X$ and $P^X$ presented in this article are based on molar fractions, indicated by the superscript X, in order to facilitate the calculation of $\phi^X$ in biphasic systems (the $\phi^X$-values have to be determined numerically). Since the term $\phi^X . P^X$ (as well as $K_{eq}^{opp}$ and $K_{eq}$ terms) is dimensionless, its value based on concentration and molar fraction is equal and is interchangeable.

$K_x$ determinations were performed by titrating solutions of butyric acid (0.85; 1.0; 1.5; 2.0; 3.0 M) with 4 M NaOH in a pH-stat apparatus. The numerical p$K_x$ values were obtained from the abscissa of log {[β(1-β)]} versus pH plots (β is the degree of dissociation, determined from pH versus NaOH titration curves of butyric acid solutions and of pure water). A
concentration-independent pKₐ value of 4.9±0.1 was obtained (Kᵦₒ = 2.26·10⁻⁷ mol/mol). Thus, since a pH of 7.8 was maintained during the conversion, practically no free butyric acid occurs in a monophasic system (fraction butyrate = 0.999).

The chemical equilibrium constant (Kₑq) for the hydrolysis of glycidyl butyrate was determined by mixing 50 mg Pseudomonas cepacia lipase with glycidol (9.25 mmol) and various amounts of butyric acid (7.48; 7.48; 7.48 and 14.96 mmol) and water (594; 661; 646; 737 mmol). After the reaction came to equilibrium, the pH of the mixture was 4.07; 4.03; 4.04; 3.96, and the amounts of glycidyl butyrate formed were 97; 78; 69; 102 µmol, respectively. After correcting for dissociation of butyric acid, a Kₑq of 1.8 ±0.1 was found. The Kₑq²-value for glycidyl butyrate hydrolysis in a monophasic system at pH 7.8 is 1.4·10³.

The partition coefficients (Pₓ) of glycidyl butyrate and water in the glycidyl butyrate - water biphasic system were calculated from their solubilities (Xₐₓ) via Pₓ = 1/Xₓₑq. The solubilities were determined by titration under stirring and observing the clouding point. The determinations were performed in 2.5 ml solvent (water or glycidyl butyrate) in a magnetically stirred, closed 4 ml vial. The titrant was added via a septum with a 100 µl syringe. The solubility of glycidyl butyrate appeared to vary with the concentration of glycidol and butyrate present in the aqueous phase. The solubility of racemic glycidyl butyrate was 39.9 g/l (Pₓ = 208), 33 g/l (Pₓ = 264), and 50.4 g/l (Pₓ = 170) at concentrations of racemic glycidol 0, 1.2, and 2.4 M and of butyrate of 0, 1.2, and 2.4 M, respectively. The solubility of water in racemic glycidyl butyrate was 21.5 g/l (Pₓ = 0.14), and no dependency on the concentration glycidol and butyrate was observed.

Partition coefficients of butyric acid in water - glycidyl butyrate biphasic systems were measured as follows. Butyric acid (400 µl) was mixed with 10 ml 0.1 M potassium phosphate buffer. The pH was adjusted with 8 M NaOH to 4.0. The butyric acid concentration in the aqueous phase, before and after extraction with 5 ml racemic glycidyl butyrate, was determined with GC (with 2-pentanone as internal standard). The partition coefficient was calculated after correcting for dissociation of butyric acid. The partition coefficient of glycidol was determined by mixing 50 µl racemic glycidol with 1 ml 10 mM potassium phosphate buffer, pH 7.8. The aqueous phase was extracted with 1 ml racemic glycidyl butyrate. 100 µl of the glycidyl butyrate phase was added to 500 µl of dichloromethane and the butyric acid and glycidol concentration was analyzed with GC (with propyl butyrate as internal standard). The Pₓ-value calculated for butyric acid was 0.9 and for glycidol 0.01.

The partition coefficient of racemic glycidyl butyrate in a 2-pentanone - water biphasic system was measured as follows. 0.5 ml 2-pentanone containing 10 µl glycidyl butyrate was extracted with 1.5 ml water. The racemic glycidyl butyrate concentration in the 2-pentanone layer was determined before and after extraction (GC with 2-octanone as internal standard).
Initial rate measurements

Crude PPL solution was prepared in the following way. 100 mg crude PPL was added to 30 ml 10 mM potassium phosphate buffer, the pH was adjusted to 7.8, and the mixture was centrifuged at 27,000 g for 10 min. To avoid unreliable results due to degradation, the preparation was prepared daily and kept on ice.

To determine the initial rates of glycicydyl butyrate hydrolysis, acid production was followed in a pH-stat apparatus (Metrohm pH stat equipment, consisting of a pH-meter type 691, a Phoenix epoxy-electrode, an impulsomat type 614, a dosimat type 655, and a 70 or 90 ml reaction vessel equipped with thermostated jacket) at pH 7.8 and 25 °C by titrating with 10.0 mM KOH. The mixtures consisted of 10 ml 0.01 M potassium phosphate buffer and various amounts of glycicydyl butyrate. The reaction was started by adding crude PPL, PPL A/B or the 33 kDa enzyme solution. For monophasic systems, 10 - 100 µl of the enzyme solutions was used. For biphasic systems, the amount of enzyme solution was varied to check whether linearity between amount of enzyme added and the observed reaction rate existed.

For determination of E from initial rates of samples having a constant concentration of glycicydyl butyrate but varying ratio of the R to the S-enantiomer, racemic ester was mixed with the R-enantiomer. The fractions of S-enantiomer were: 0.01, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5. The initial rates were measured in triplo as described above. The concentrations of lipase and glycicydyl butyrate are indicated in Tables 2 and 3.

Conversion experiments

Conversion experiments were carried out in the pH-stat apparatus by mixing 30 ml 10 mM potassium phosphate buffer, pH 7.8, (for biphasic systems, 26 ml buffer and 4 ml 2-pentanone) with R,S-glycicydyl butyrate and 1 ml crude PPL solution (prepared as indicated above, except that 0.5 g crude PPL was added to 10 ml buffer). Depending on the initial amount of R,S-glycicydyl butyrate, 0.100 or 1.00 M KOH was used for titration. The degree of conversion was calculated from the amount of KOH added and the amount of racemic glycicydyl butyrate present at the start as well as from the amount of KOH needed for complete conversion. The average of both values was used.

Conversion experiments of R-glycicydyl butyrate in the presence of racemic glycidol, and of hexyl butyrate in the presence of propanol were carried out essentially the same as the resolution experiments. The reactions were started by adding 0.6 ml crude PPL solution.

Simulation and fitting

The kinetic parameters $k'_{cat}$, $K'_M$ and $E$ (derived for mixtures with varying enantiomer ratios) were estimated from initial rate experiments by applying non-linear regression. Standard deviations of the parameter estimates were obtained from the covariance matrix.

Simulations of the kinetic resolutions were obtained after numerical integration by a
fourth order Runge Kutta procedure of the differential equation 6 \((\tau^2_A/\tau^2_s = dc^s_A/dc^s_s)\), using the stoichiometric balances \((n^R_A = n^R_{A,0} - n^P_p + n^R_{P,0}, n^S_A = n^S_{A,0} - n^P_p + n^S_{P,0}, n^R_p = n^R_{P,0} + n^R_{A,0} - n^R_A, n^S_p = n^S_{P,0} + n^S_{A,0} - n^S_A, n_b = n_{B,0} - n^R_{A,0} + n^R_A - n^S_{A,0} + n^S_A, n_Q = n_{Q,0} + n^R_{A,0} - n^R_A + n^S_{A,0} - n^S_A)\), in which \(n\) is the number of moles of the respective substrates and the subscript 0 refers to the initial number of moles). Phase diagrams relating ee\(s\) to \(\xi\) were obtained from transformation of the \(S\)- versus \(R\)-glycidyl butyrate concentration data.

Fitting of eq 6 to the combined experimental ee\(s\) and \(\xi\) data, obtained at various concentrations of substrates and products, was carried out by orthogonal non-linear regression. A standard deviation in ee\(s\) and \(\xi\) data of 0.01 (almost independent of the actual value of \(\xi\) and ee\(s\)) was determined by averaging the deviating points of Figure 3 to the fitted curves. A critical step size of 1% of the actual value of the \(E\) and \(\alpha\) estimates was taken as a stop criterion in the fitting routine. Standard deviations of the parameter values were estimated from the diagonal elements of the covariance matrix.

Simulations of the kinetic resolutions of glycidyl butyrate (Figure 3 and 4) were performed using eq 6 and assuming that \(c_Q = 0\). In the water - 2-pentanone system (Figure 4), the partitioning of glycidol, water and butyrate is neglected in the calculation of their aqueous concentrations. This is justified since \(\phi^w \approx 0.03\), their \(P^w\)-values are smaller than 1, and thus \(\phi^w.P^w < < 1\). Resolutions in water - glycidyl butyrate biphasic systems (with transient phase ratio, Figure 5) were simulated using eq 6 considering all reaction components, with calculation of the phase ratio and the aqueous concentrations of the reaction components in each integration interval of the Runge Kutta procedure. The concentrations are calculated from the stoichiometric balances and the values for the dissociation equilibrium of butyric acid (\(K_s\)), the partition equilibrium of the reaction components (\(P^X\)), the phase ratio (\(\phi^X\)), and the aqueous volume (\(V_{aq}^w\)) as follows: \(c_A = 1/(1 + \phi^X.P^A_A), n_b/V_{aq}, c_b = 1/(1 + \phi^X.P^B_B), n_b/V_{aq}, c_p = 1/(1 + \phi^X.P^P_p), n_p/V_{aq}, c_Q = 1/(1 + \phi^X.P^Q + K_s/c_{H_+}), n_Q/V_{aq}\). The \(P^X\)-value of racemic glycidyl butyrate was assumed to be constant (\(P^X = 208\)) in the simulation.

![Figure 1](attachment:image.png)

**Figure 1.** Reaction scheme for hydrolysis of an ester with a racemic alcohol moiety by a hydrolase. The symbols have the following meaning for the case of \(R,S\)-glycidyl butyrate hydrolysis by PPL: \(A^R\), \(R\)-glycidyl butyrate; \(A^S\), \(S\)-glycidyl butyrate; \(P^R\), \(S\)-glycidol; \(P^S\), \(R\)-glycidol; B, water; Q, butyric acid; E, PPL; E*, PPL-butryl complex; E'B, PPL-butryl-water complex.
Theory

Mechanism

For serine-type hydrolases, hydrolysis of an ester in which the alcohol moiety is racemic proceeds as indicated in Figure 1. Assuming pseudo steady state conditions,\textsuperscript{18} the conversion rates of all components in the scheme are related to each other, resulting in the same denominator in the rate equations of the two enantiomers. Therefore, to obtain the ratio of the rates of conversion of the enantiomers, only the numerators have to be known. These can be derived with the King-Altman method,\textsuperscript{19} and the following equation is obtained:

\[
\frac{\frac{R}{s}}{\frac{P}{A}} = \frac{k_{1}^{R} k_{2}^{R} c_{A}^{R} \cdot (k_{3}^{R} \cdot k_{4}^{R} \cdot c_{B}^{R} \cdot (k_{5}^{S} + k_{5}^{S} \cdot c_{p}^{S} \cdot (k_{3}^{R} + k_{4}^{R})))}{k_{1}^{S} k_{2}^{S} c_{p}^{S} \cdot (k_{3}^{R} \cdot k_{4}^{R} \cdot c_{Q}^{R} \cdot (k_{5}^{R} + k_{5}^{R} \cdot k_{5}^{R} \cdot c_{A}^{S} \cdot (k_{3}^{R} + k_{4}^{R})))}
\]

(4)

According to Straathof et al.,\textsuperscript{6} lumping of the elementary rate constants in eq 4 is carried out by introducing: the equilibrium constant ($K_{eq}$), the substrate selectivity factor for $A^R$ and $Q$, $\alpha^R$, indicating the relative specificity for the forward and the reverse reaction; and the enantiomeric ratio, $E$:

\[
K_{eq} = \frac{k_{1}^{R} k_{2}^{R} k_{3}^{R} k_{4}^{R}}{k_{1}^{S} k_{2}^{S} k_{3}^{S} k_{4}^{S}} = \frac{k_{1}^{S} k_{3}^{S} k_{3}^{S} k_{3}^{S}}{k_{1}^{S} k_{2}^{S} k_{2}^{S} k_{2}^{S}}
\]

(5)

\[
\alpha^R = \frac{k_{1}^{R} k_{2}^{R} (k_{3}^{R} + k_{4}^{R})}{k_{1}^{S} k_{2}^{S} (k_{3}^{S} + k_{4}^{S})} = \frac{V_{1}^{R} / K_{MA}^{R}}{V_{1}^{R} / K_{M,Q}^{R}}
\]

\[
E = \frac{k_{1}^{R} k_{2}^{R} (k_{3}^{R} + k_{4}^{R})}{k_{1}^{S} k_{2}^{S} (k_{3}^{S} + k_{4}^{S})} = \frac{V_{1}^{R} / K_{MA}^{R}}{V_{1}^{S} / K_{MA}^{S}} = \frac{\alpha^R}{\alpha^S}
\]

Substitution of the lumped parameters in eq 4 results in:\textsuperscript{6}

\[
\frac{R}{s} = \frac{c_{A}^{R} (E K_{eq} c_{B} + \alpha^R c_{p}^{S}) - c_{P}^{R} (E c_{Q} + \alpha^R c_{A}^{S})}{c_{A}^{S} (E K_{eq} c_{B} + \alpha^R c_{p}^{R}) - c_{P}^{S} (E c_{Q} + \alpha^R c_{A}^{R})}
\]

(6)

Plots of $ee_s$ versus $\zeta$ are obtained by integration of eq 6, using the stoichiometric balances.

The pH optimum of PPL for hydrolysis of glycidyl butyrate in 10 mM phosphate buffer was found to be 7.8, outside the region where chemical hydrolysis becomes significant (> pH 8.5). Since this pH-value was applied in the experiments and it is far above that of the $pK_a$-value of butyric acid, the terms with $c_{Q}$ in eq 6 are approximately zero and can be
Figure 2. Application of several kinetic models to fit the experimental data for hydrolysis of R,S-glycidyl butyrate by PPL. Panel A represents experimental data for a conversion of 4 ml R,S-glycidyl butyrate, 10 ml 10 mM potassium phosphate buffer, and 1 ml crude PPL solution. The course of the reaction was similar for crude PPL, PPL A/B, and PPL A/B combined with colipase (results not shown). Simulations were carried out using eq 6 with \( E = 3 \) (-----), \( 10 \) (-----), \( 30 \) (-----) and \( 100 \) (- - -) for the following conditions: irreversible uni-uni reaction \( c_{p}^R = c_{p}^S = 0 \) (Panel B); reversible bi-bi reaction, without back-reaction of the formed product \( (K_{eq} = 10, \alpha^R = 0.001, c_{A,0}^R = c_{A,0}^S = 1, c_{B,0} = 10, c_{P,0}^R = c_{P,0}^S = 0, c_{Q,0} = 0) \) (panel C); bibi reaction going to completion, with back-reaction of the formed product \( (K_{eq}/\alpha^R = 1, c_{A,0}^R = c_{A,0}^S = 1, c_{B,0} = 19, c_{P,0}^R = c_{P,0}^S = 0, c_{Q} = 0) \) (panel D).
ignored. On combining eq 6 with eq 2, and deleting $c_Q$ terms, the following expression is obtained for $E^{app}$:

$$E^{app} = \frac{E + \frac{\alpha^R}{K_{eq} c_B} \cdot (c_p^R - \frac{c_A^R}{c_A} c_p^R)}{1 + \frac{\alpha^R}{K_{eq} c_B} \cdot (c_p^R - \frac{c_A^R}{c_A} c_p^R)}$$  \hspace{1cm} (7)

From eq 7 it is clear that $E^{app}$ equals $E$ when no product is present. This condition applies to initial rate measurements of ester hydrolysis in the absence of other nucleophiles than water. Biphasic systems

Since an increase of the applied stirrer speed did not change the reaction rate in the initial rate experiments, and dissociation and partitioning equilibria will rapidly adapt to the changing conditions during the conversion, PPL catalyzed hydrolysis is the rate limiting step in the conversion process in biphasic systems. Therefore, the course of the reaction in these systems can be adequately described by the kinetic expression for the enzymatic reaction (eq 6). The overall equilibrium of $R,S$-glycidyl butyrate in a biphasic system is quantified by the apparent equilibrium constant ($K_{eq}^{app}$). $K_{eq}^{app}$ is related to the chemical equilibrium in a monophasic aqueous buffer for hydrolysis ($K_{eq}$), the phase ratio ($\phi^k$), the partition equilibrium of the reaction components ($P^k$), and the dissociation equilibrium of butyric acid ($K_a$). On using a biphasic system of $R,S$-glycidyl butyrate and buffer solution, changes in volume and composition ($R,S$-glycidyl butyrate and water are converted, and $R,S$-glycidol, butyric acid and butyrate are formed and distribute among the phases) occur during the course of the reaction. Since $\phi^k$ changes, $K_{eq}^{app}$ also changes during the conversion, so that the resulting transient equilibrium could limit the maximal $e_{eq}$ during the reaction.

Results

**Behaviour deviating from a minimal kinetic mechanism**

Figure 2, panel A, shows the $e_{eq}$-values as a function of $\xi$ for the kinetic resolution of racemic glycidyl butyrate in a typical biphasic conversion experiment. These data points could not be fitted with a curve simulated according to eq 3 and assuming an irreversible reaction (panel B). The $E$-value calculated according to eq 3 (which is in fact the averaged $E^{app}$-value over the conversion period) was 20 at $\xi = 0.31$ and 2.7 at $\xi = 0.95$. Application of eq 2 to fit the experimental data resulted in a drop of the $E^{app}$-value from 20 in the beginning of the conversion to 1 at degrees of conversion higher than 0.7. Also simulations based on reversible bi/bi reactions (eq 6 with $\phi^k=0$) provided curves that could not follow
the trend indicated by the experimental data (Panel C), but the simulations based on eq 6 (panel D) did. The latter indicated that the $E$-value should be between 10 and 30.

![Graph A](image1)

**Figure 3.** Enantiomeric excess values of the remaining substrate (ee$_s$) and the degree of conversion ($\xi$) for R,S-glycidyl butyrate hydrolysis in a monophasic system. Panel A shows the experimental data for conversions with the following initial concentration of racemic glycidyl butyrate: 0.017 M (●), 0.038 M (□), 0.052 M (◆). Panel B shows the effect of racemic glycidol additions at concentrations of: 0 M (Δ), 0.94 M (□), and 1.88 M (●) to a system containing 0.16 M racemic glycidyl butyrate. The data were simulated with eq 6, using the parameter values $E = 9.0$ and $C^E = 18$, the $K_{eq}$-value of 1.8, and the concentrations indicated.

For low concentrations of racemic glycidyl butyrate ($c \leq 0.052$ M, yielding a monophasic system) the experimental data could be fitted with eq 3 and an $E$-value of 9.0 ($\pm$ 0.5) was obtained (Figure 3, panel A). At a higher concentration ($c = 0.16$ M), a suboptimal plateau of ee$_s$-values was obtained. This suggested that the higher concentration of R-glycidol formed, determines whether deviations occur from eq 3, which are observed as suboptimal ee$_s$-plateaux and decreasing $E$-values (according to eq 3) during the conversion. Experimental evidence for this is provided in Figure 3, panel B, where it is shown that increasing amounts of supplementary glycidol give rise to a drop in the plateau value. The combined data presented in Figure 3, panel A and B, could be fitted adequately with eq 6, yielding an $E$-value of 9.1 ($\pm$ 0.2) and an $C^E$-value of 18 ($\pm$ 2). Inducing a biphasic system by addition of 2-pentanone, keeping the biphasic glycidyl butyrate concentration equal to that in the monophasic system ($c = 0.16$ M), and adding racemic glycidol, led also to the deviating behaviour (Figure 4). The experimental data in the biphasic system could be fitted with eq 6,
yielding an $E$-value of $20 \pm 3$ and an $\alpha$-value of $72 \pm 9$. As shown in Figure 3 and 4, an adequate fit with eq 6 was obtained for monophasic as well as for biphasic systems, also in the cases where extra amounts of glycidol were added. Thus, although higher $E$ and $\alpha$-values are observed in the biphasic system, the biphasic character does not cause the suboptimal ee$_S$-plateau.

![Figure 4](image)

**Figure 4.** Enantiomeric excess values of the remaining substrate (ee$_S$) and the degree of conversion ($\xi$) for $R,S$-glycidyl butyrate in an aqueous-2-pentanone biphasic system. The data are shown for experiments in which $R,S$-glycidol was added to 0.3 M racemic glycidyl butyrate in concentrations of: 0 M (Δ), 0.56 M (□), and 0.052 M (●) (the concentrations used are biphasic concentrations, i.e. number of moles per total reaction volume in liters). The curves shown were simulated with eq 6, using the parameter values $E = 20$ and $\alpha = 72$, the $K_{eq}$-value of 1.8, and the concentrations indicated.

### Table 1.

Kinetic parameters for the two fractions purified from crude PPL with hydrolytic activity for $R,S$-glycidyl butyrate. The two fractions, indicated as PPL A/B (52 kDa) and the Minor Activity (33 kDa), were purified to homogeneity, as judged from polyacrylamide gel electrophoresis. $R,S$-glycidyl butyrate concentrations used are indicated in Fig. 6A. The kinetic parameters were obtained from initial rate measurements, and the $k_{max}$-value represents the maximal rate observed with all PPL interfacially adsorbed. Values for mono- and biphasic systems are indicated with the subscripts m and b, respectively. The relative amount of 33 and 52 kDa enzyme was estimated from the intensity of the bands on staining the electrophoresis gel of the crude PPL preparation.

<table>
<thead>
<tr>
<th>Catalytic Parameters</th>
<th>PPL A/B (52 kDa)</th>
<th>Minor Activity (33 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k'_c$ (μmol/min/mg)</td>
<td>75 (±4) $^m$</td>
<td>22 (±2) $^m$</td>
</tr>
<tr>
<td>$K'_M$ (mM)</td>
<td>47 (±5) $^m$</td>
<td>95 (±10) $^m$</td>
</tr>
<tr>
<td>$k_{max}$ (μmol/min/mg)</td>
<td>1000 (±40) $^b$</td>
<td>45 (±3) $^b$</td>
</tr>
<tr>
<td>$E$</td>
<td>8$^m$, 17$^b$ (see Table 3)</td>
<td>17 (±3) $^{m,b}$</td>
</tr>
<tr>
<td>Relative amount of protein</td>
<td>&gt;0.95</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Excluding trivial causes

Crude PPL has been shown to contain several hydrolytic activities for certain esters. On purifying the crude PPL preparation and assaying the fractions for glycidyl butyrate hydrolysis, two active fractions were obtained in purification step III. Upon electrophoresis of the fraction showing major activity, only two protein bands were observed in a position corresponding with a protein of about 52 kDa. This pattern is similar to that obtained by Verger et al., for the isozymes of PPL, designated as PPL A and B. The fraction with minor activity (containing a 33 kDa enzyme and consisting of less than 1.5% and 0.2% of the total activity, in monophasic and biphasic systems, respectively) showed a similar E-value as the major fraction (Table 1). In agreement with this, the PPL A/B preparation showed the same enantioselectivity as the crude preparation (Tables 2 and 3).

<table>
<thead>
<tr>
<th>Glycidyl butyrate concentration (mM)</th>
<th>Crude PPL concentration (mg/ml)</th>
<th>E (± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7</td>
<td>9 (± 2)</td>
</tr>
<tr>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35</td>
<td>8 (± 1)</td>
</tr>
<tr>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22</td>
<td>6 (± 2)</td>
</tr>
<tr>
<td>38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
<td>8 (± 2)</td>
</tr>
<tr>
<td>306&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23</td>
<td>14 (± 2)</td>
</tr>
<tr>
<td>153&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16</td>
<td>20 (± 5)</td>
</tr>
</tbody>
</table>

Thus, the deviating behaviour exhibited during the course of the reaction cannot be ascribed to two or more hydrolytic activities having different selectivities and varying contributions during the course of the reaction. The isolated C-terminal domain of PPL A/B for which catalytic activity on p-nitrophenyl acetate has been reported, showed no hydrolytic activity for racemic glycidyl butyrate (data not shown). Thus it is highly likely that the activities in the monophasic and the biphasic systems, both originate from one and the same active site.
Apparently, the interfacial adsorption of PPL induces the higher activity and enantioselectivity of this.

During the conversion of glycidyl butyrate at a concentration that initially exceeds its solubility, the system shifts from biphasic to monophasic. A quite different apparent equilibrium constant exists in the biphasic system as compared to the monophasic one. In the biphasic system the fast reacting enantiomer could reach a transient equilibrium that continuously adapts to the changing phase ratio during the conversion. The parameters derived from data presented in Figure 3 and 4 and the equilibrium constants calculated in the Experimental Section were used to simulate the ee-values in such a transient system. This was carried out using eq 6 and the stoichiometric balances with correction for partitioning and dissociation of the reaction components. When the volumes of glycidyl butyrate and water are equal (7-fold molar excess of water), the effect due to this equilibrium effect becomes already vanishing small (< 0.1% ee) (Figure 5). Thus it is clear from Figure 5 that the effects resulting from the phase transition do not explain the course indicated by the experimental data points.

![Figure 5](image)

**Figure 5.** Simulation curves for ee versus ξ for systems with changing ratios of aqueous and glycidyl butyrate phases. The curves were simulated with eq 6, $E_{monophasic} = 9$, $E_{biphasic} = 20$, $K_{eq} = 1.8$, the $K$ and $P$-values given in the Experimental section, assuming that $\xi = 0$ and that the fraction interfacially adsorbed PPL equals $1-\xi$. The following initial conditions were chosen: $n_{gly} = 1$, $n_{H2O} = 7.28$ (----, the initial volume of water is half the volume of glycidyl butyrate); $n_{H2O} = 14.56$ (---, the initial volume of water equals the volume of glycidyl butyrate).

**Validity of the proposed mechanism**

To check in an independent way that PPL has different enantioselectivities for glycidyl butyrate in monophasic and biphasic systems, initial rate measurements were carried out. As shown in Figure 6, panel A, the activity of PPL for glycidyl butyrate hydrolysis increases dramatically from 75 to 1000 μmol/min/ mg PPL when the monophasic system transforms into the biphasic one. The increase not only applies to activity but also to enantioselectivity (Figure 6, panel B). As shown in Tables 2 and 3, the $E$-value did not depend on the concentration of PPL in the reaction mixture.
Figure 6. Initial rate measurements for R,S-glycidyl butyrate hydrolysis with PPL A/B in monophasic and biphasic systems. Panel A shows the effect of increasing concentrations of R,S-glycidyl butyrate (GlyBut) on the initial reaction rate (the vertical dotted line indicates the border between the monophasic and the biphasic system). The hydrolysis rate of free PPL, was simulated by using the kinetic parameters of the monophasic system ($k'_{cat} = 75 \mu$mol/min/mg PPL A/B, and $K'_M = 47$ mM). Panel B shows the initial rates on varying the enantiomeric composition of glycidyl butyrate for a monophasic (——) and biphasic (-----, ------) systems. The $E$-values calculated are 8, 12, and 16 for glycidyl butyrate concentrations of 0.024 ml (O), 0.20 ml (Δ) and 0.39 ml(□) per 5 ml buffer, respectively.

The mechanism proposed in Figure 1 predicts that the acyl-enzyme complex can either be hydrolyzed by attack of water or be converted into glycidyl butyrate by reaction with glycidol. As shown in Figure 7, transient formation of S-glycidyl butyrate is indeed observed when hydrolysis of R-glycidyl butyrate is carried out in the presence of R,S-glycidol. However, since the R-glycidyl butyrate was slightly contaminated with S-enantiomer, it was attempted to provide additional evidence. Therefore, hexyl butyrate was hydrolyzed by PPL in the presence of propanol. As appears from Figure 8, transient formation of propyl butyrate was observed during this conversion. A similar transient formation of ester has been found for Pseudomonas cepacia lipase catalyzed hydrolysis of decyl chloroacetate in the presence of n-nonanol.23

Discussion

Despite the fact that it is known that hydrolase-catalyzed kinetic resolutions proceed via a ping-pong mechanism, nevertheless an irreversible minimal mechanism is used to
describe enantioselectivity when the second substrate (water) is in large excess. As shown here, however, in the case of PPL-catalyzed hydrolysis of glycidyl butyrate this is not allowed for enantioselective conversions in which the initial concentration of the ester exceeds 0.05 M or product is initially present. As is clear from eq 7, the operative $E^{\text{app}}$ becomes equal to the intrinsic $E$ and the reaction can be described by eq 3, when the concentration of the product (alcohol) is low, the selectivity factor $\alpha^R$ is zero, or the equilibrium constant $K_{eq}$ is infinitively high. Although the glycidyl butyrate hydrolysis goes to completion under the conditions applied here (due to nearly complete dissociation of the butyric acid at pH 7.8), the equilibrium constant $K_{eq}$ of the reaction is only 1.8. On using eq 6 and the specific values for $E$ and $\alpha^R$ with respect to free and interfacially adsorbed enzyme, the enantioselective hydrolysis of racemic glycidyl butyrate by PPL can be described now for all conditions.

**Figure 7.** Progression curves for the hydrolysis of $R$-glycidyl butyrate in the presence of $R,S$-glycidol and transient formation of $S$-glycidyl butyrate in a monophasic ($\square=S-$, $\bigcirc=R$-glycidyl butyrate) and a biphasic ($\blacksquare=S-$, $\bullet=R$-glycidyl butyrate) system.

**Figure 8.** Progression curve for the hydrolysis of hexyl butyrate in the presence of propanol and the formation of transient propyl butyrate in a biphasic system. Symbols indicate hexyl butyrate (●) and propyl butyrate (●).

In view of the significant shortcoming in enantioselective performance of hydrolases in kinetic resolutions signalized here, the question can be posed whether the phenomenon has been observed before, has been overlooked in the past or is unique for this enzyme/substrate combination. Indications for its existence have been reported for glycidyl butyrate, and experimental evidence for mechanism-related effects was presented for another system. However, since $E$ is normally obtained with eq 3 and only one single determination of ee and
ξ is carried out for this, common practice is unsuited to detect the phenomenon. Thus, in general the question remains undecided, although it can be concluded that all cases where no pure enantiomer was obtained are open to suspicion. From the insight obtained, several opportunities can be indicated now for diagnosis and for determination of the genuine E and α-values: starting with a high substrate concentration, ee_s should be determined for different degrees of conversion in order to see whether a plateau value is reached; on varying the initial concentration, but keeping the extent of conversion constant, variation in ee_s is indicative; if the product enantiomer is available, ester hydrolysis in its presence will indicate whether ee_s becomes affected; if the substrate can be enriched with one of the enantiomers or if the pure enantiomers are available, initial rate measurements will provide the E-value, which can be compared with that obtained from a conversion experiment. In this connection it should be stressed, however, that for a decisive diagnosis, statistical treatment of the data should be performed so that reliable E and ee_s values with standard deviations can be obtained.12

Interfacial activation of lipases with respect to activity26 and selectivity27 has been demonstrated. When the amount of added PPL exceeds the amount that can be adsorbed to the interfacial area, the excess will dissolve in the buffer phase. This dissolved PPL fraction converts the buffer dissolved glycidyl butyrate with an activity and selectivity lower than exhibited by the interfacially adsorbed fraction. Increasing the interfacial area of such a system will result in a shift (Figure 6). As shown here, the same interfacial activation is achieved with the ester itself as with 2-pentanone, suggesting that the nature of the interface is not important in this. Also in a case where the catalytic activity of PPL, adsorbed to the interface, varied with the surface pressure of a triglyceride monolayer, the enantioselectivity hardly varied.28 It seems likely, therefore, that possible changes of the character of the interface during racemic glycidyl butyrate conversion, have no effect on the enantioselective performance of PPL. This view is in agreement with the idea of a constant E during the conversion, as was proven here.

The interfacial activation of activity as well as selectivity originates from the same catalytic centre in PPL. Present insights in the mechanism of lipases predict that the lid covering the active site is closed in the free enzyme form10,29 but opens (a form stabilized by α-helices and having an anion cavity) when an inhibitor binds or when adsorption to an interface takes places.29 According to this view, to catalyze the hydrolysis of esters in a homogeneous solution, PPL must be in an open form. If the opening of the lid would cost an equal amount of energy for both enantiomers, the opening would competitively inhibit the reaction. However, competitive inhibition does not affect the enantioselectivity12 and the E-value of the free and interfacially adsorbed PPL would be equal. Since the E-values of free and interfacially adsorbed PPL differ, it could be speculated that the conformation of the open form of free enzyme differs from that of interfacially adsorbed enzyme.

The imperfect enantioselective behaviour of hydrolases, resulting from their inherent
mechanism, has not only consequences for fundamental studies but also for practical purposes such as determining the optimal pH, temperature, organic solvent, substrate in a homologous series, and process modelling\(^3\) of kinetic resolutions of esters with either racemic alcohol or acid moieties. Contrary to general believe, continuation of hydrolysis will not always lead to higher enantiomeric purity of the residual ester since the possibility exists that a plateau was reached already. To achieve purities higher than this plateau value, several strategies can be indicated: carrying out conversions at a low concentration of substrate; continuous removal of the product enantiomer; sequential batch operations. A more mechanistic approach would be to lower \(\alpha\), i.e. to increase the preference of the acyl-enzyme complex for hydrolysis instead of alcoholysis. However, a rationale to steer this does not exist. Preliminary observations indicate that, similar to influencing \(E\), this might be attempted by adapting the reaction temperature,\(^3\) the medium,\(^3\) the nature of the active site,\(^3\) or the nature of the alcohol or acyl moiety.\(^4\)

Acknowledgements

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References

   (b) Cambou, B., and Klibanov, A.M. (1984) Preparative production of optically active esters and


see the following citations and references therein:


Hydrolysis of glycidyl butyrate by PPL 45


Chapter 3

Do organic solvents affect the catalytic properties of an enzyme?  
Intrinsic kinetic parameters of lipases in  
ester hydrolysis and formation in various organic solvents

J. Bert A. van Tol, Rob M.M. Stevens, Willem J. Veldhuizen, Jaap A. Jongejan,  
and Johannis A. Duine
Abstract

When it is assumed that organic solvents do not interfere with the binding process nor with the catalytic mechanism, the contribution of substrate-solvent interactions to enzyme kinetics can be accounted for by just replacing substrate concentrations in the equations by thermodynamic activities. It appears from the transformation that only the affinity parameters ($K_m, k_sp$) are affected by this. Thus, in theory the values of these corrected, intrinsic parameters ($K_m^{int}, k_{sp}^{int}$) and the maximal rate ($V_m$) should be equal for all media. This was verified for hydrolysis, transesterification and esterification reactions catalyzed by pig pancreas lipase and Pseudomonas cepacia lipase in various organic solvents. Correction was carried out via activity coefficients of the substrates in these solvents (calculated from values in data bases or experimentally determined ones). However, although the kinetic performances of each enzyme in the solvents became much more similar after correction, differences still remained. Analysis of the enzyme suspensions revealed massive particles with varying size distributions for the respective solvents. Although this explains the low activity of enzymes in organic solvents, no correlation was found between estimates of the amount of catalytically available enzyme and the maximal rates observed. Moreover, the solvents had similar effects on the intrinsic parameters of suspended and immobilized enzyme. The possible causes for the effects of the solvents on the catalytic performance of the enzymes, remaining after correction for solvent-substrate interactions and the amount of catalytically available enzyme, are discussed with respect to the premises on which the correction method is based.

Introduction

Enzyme catalysis and non-aqueous media are not incompatible entities, as demonstrated by the catalytic activity that enzymes have when dispersed in organic solvents\textsuperscript{1-5} or in (supercritical) gases.\textsuperscript{6-9} On the other hand, since substantial differences have been noted between reaction rate, maximal velocity ($V_{max}$) or specific activity ($k_{cat}$),\textsuperscript{1,9} substrate affinity ($K_m$), specificity constant ($k_{cat}/K_m$),\textsuperscript{10,11} and enantioselectivity ($E$)\textsuperscript{12,13} determined in water and various organic solvents, it has been attempted to demonstrate a relationship between enzyme performance and the physical properties of these media.

When plotting reaction rates of enzymes for a certain condition versus the hydrophobicity of the organic solvents applied (log P values), the scattered data points suggest a sigmoidal curve,\textsuperscript{14} but other trends can be distilled from plots in which another physical constants was considered to be relevant (e.g. the polarizability,\textsuperscript{15} or, in organic-aqueous mixtures, the viscosity\textsuperscript{16,17}). However, is this effect caused by a modification of the properties of the enzyme by the solvent? Before answering this, it is necessary to take all other effects
solvents might have on the kinetic parameters of enzymes into consideration. Solvents, including water, participate in enzyme catalysis because they interact with substrates and products (solvation, desolvation).\textsuperscript{18} In addition, since the enzymes do not dissolve in the organic solvents commonly used, heterogeneous catalysis takes place in these media, of which the efficiency depends on the amount of catalytically available enzyme. First attempts have already been undertaken by us\textsuperscript{19} and by others\textsuperscript{20-24} to correct ordinary kinetic affinity parameters for the substrate-solvent interaction. The underlying theory of our approach\textsuperscript{19} is further elaborated here and the concept tested for esterification, transesterification, and hydrolysis reactions in various organic solvents. Two enzymes, porcine pancreatic lipase (PPL) and Pseudomonas cepacia (PcL), were chosen for that purpose because it is known that they are stable in these media.\textsuperscript{5} Since water is not only a substrate in the hydrolysis and a product in the esterification reaction but also strongly affects the performance of lipases in organic solvents,\textsuperscript{1,25-29} care was exercised to condition the enzymes in an appropriate way and to maintain the water activity at constant level in the systems investigated. To correlate maximal rates with catalytically available surface, electron microscopy of the suspended enzyme particles was carried out and the enzymes were also immobilized on a solid carrier.

Materials and Methods

Chemicals and solvents

Porcine pancreatic lipase (PPL, type II) was from Sigma, and Pseudomonas cepacia lipase (PcL) from Amano. All solvents were of analytical grade and dried prior to use over 4-Å molecular sieve (Union Carbide). Propyl butyrate was prepared as follows: 3 mol propanol, 3 mol butyric acid, and 1 ml concentrated sulphuric acid were refluxed during 5 hours. The aqueous phase was removed, and the organic phase was washed twice with 250 ml 3 M sodium carbonate buffer, pH 7, dried with magnesium sulphate and distilled. Hexyl butyrate was synthesized as follows: 1.5 mol hexanol, 1 mol butyric acid and 2 ml concentrated sulphuric acid were refluxed during 4 hours. The reaction mixture was washed with 3 M sodium carbonate (pH 7) until the pH was 7, and the organic layer was distilled. Decyl acetate was prepared by refluxing 1 mol decanol and 1.5 mol acetic anhydride for 2 hours, followed by distillation. The purity of all esters obtained in this way was higher than 98 %, as judged from GC analysis.

Gas chromatography

Gas chromatography was performed with a Hewlett-Packard model 5890 series II gas chromatograph with flame ionization detection and an integrator (Hewlett-Packard 3365
Chemstation). The apparatus was equipped with a HP-1 column (30 m * 0.53 mm, film thickness 2.65 μm) (Hewlett-Packard).

**Activity coefficients**

*Methods to determine activity coefficients*

The thermodynamic activity of compound A \((a_A)\) is related to its concentration \((c_A)\), via the activity coefficient \((\gamma_A)\) according to \(a_A = \gamma_A \cdot c_A\). The activity coefficients were defined in the sense of Raoult's law, i.e. the a of a compound in its pure form is 1. When the solute and solvent are mutually poorly soluble, the activity coefficient can be determined experimentally since it equals the reciprocal of the solubility \((\gamma_A = 1/c_A^{\text{pure}})\). When the substrate and the solvent are miscible, a reference solvent is chosen in which the \(\gamma_A^{\text{ref}}\)-value is known (e.g. from the solubility), and the activity coefficient is determined from the partition coefficient \((P)\) over the solvent in which the reaction is carried out \((S)\) and the reference solvent \((\text{ref})\) via:

\[
\gamma_A^S = P_A^{\text{ref}/S} \cdot \gamma_A^{\text{ref}}
\]  

Since \(\gamma\)-values were determined under conditions similar to those applied in the kinetic experiments, and a relatively narrow concentration range was used in the kinetic experiments, the \(\gamma\)-values can be regarded as constant and can be used as such in the equations.

For correction of the kinetic data, the experimental \(\gamma^\text{exp}\) values were used by preference. However, some \(\gamma\)-values could not be determined experimentally, and these were estimated by the Unifac group contribution method,\(^{30}\) based on vapour-liquid \((\gamma^V)\) and liquid-liquid \((\gamma^L)\)\(^{31}\) equilibrium parameters.

**Determination of the activity coefficients of hexanol and tributyrine**

The \(\gamma\)-values of hexanol and tributyrin in hexane, diisopropylether and tetrachloromethane were determined experimentally according to Eqn. (1). For hexanol the reference solvent was water, the \(\gamma_{\text{water}}^{\text{hexanol}}\)-value was derived from the literature value for the solubility of hexanol in water \((c_{\text{hexanol}}^{\text{wat}} = 0.068 \text{ M})\).\(^{32}\) The \(P_{\text{hexanol}}^{\text{water}/S}\)-value was determined from the hexanol concentrations in 1 ml reaction solvent before and after extraction with 3 ml 0.1 M potassium phosphate buffer, pH 7. The total amount of hexanol in the partition experiment was 10μl, and the hexanol concentrations were determined with GC analysis.

For tributyrin the reference solvent was 1,2-ethanediol (ethylene glycol). The solubility of tributyrin in 1,2-ethanediol was determined by mixing 5, 10, 25, 50, 75, 150 and 250 μl tributyrin with 3 ml 1,2-ethanediol. After 16 hours equilibration, the tributyrin concentrations in the 1,2-ethanediol phase were measured with GC. For GC-analysis, 0.5 ml of the 1,2-ethanediol phase was mixed with 0.2 ml methanol (to reduce viscosity). It appeared that
saturation was achieved for 37 μl tributyrin/3 ml 1,2-ethanediol ($c_{\text{sat trin}} = 0.041$ M). The $P_{\text{tributyrin}}$ value was measured in the same way as indicated for hexanol, extracting 3 ml 1,2-ethanediol with 0.5 ml reaction solvent. The tributyrin concentration was measured with GC-analysis in the 1,2-ethanediol phase before and after extraction.

Activity coefficients of tributyrin were also determined with 1,2-ethanediol/water (50/50 v/v) as reference solvent. The solubility of tributyrin in the 1,2-ethanediol/water mixture was determined by adding 5, 10, 20, 50, 100 and 200 μl hexanol to 10 ml 1,2-ethanediol/water mixture. It appeared that saturation was achieved at 7.5 μl ($c_{\text{sat}} = 2.6*10^3$ mol/mol). The $P_{\text{tributyrin}}$ value was determined as indicated above, extracting 3 ml 1,2-ethanediol/water mixture with 1 ml reaction solvent. The $\gamma$-values for tributyrin that were used in the kinetic experiments were the mean values of the $\gamma$-values determined with the 1,2-ethanediol and 1,2-ethanediol/water reference solvents.

Table 1. Activity coefficients of hexanol and tributyrin in organic solvents, determined from solubility and partitioning experiments ($\gamma^p$) and with UNIFAC based on vapour-liquid equilibria ($\gamma^v$). Reference solvents for $\gamma^p$ determinations are indicated by superscripts: : water, : 1,2-ethanediol, : 1,2-ethanediol/water 50/50 (v/v). In the kinetic experiments the experimental values are used, except for 2-butanone for which the $\gamma^p$-values (based on liquid-liquid equilibria) are given in brackets. For comparison, the $\gamma^v$-values for hexanol and tributyrin in hexane, diisopropylether and 2-butanone are also given.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\gamma^p_{\text{hexanol}}$</th>
<th>$\gamma^v_{\text{hexanol}}$</th>
<th>$\gamma^p_{\text{tributyrin}}$</th>
<th>$\gamma^v_{\text{tributyrin}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane</td>
<td>2.3$^a$</td>
<td>1.6</td>
<td>1.1$^b$/1.9$^c$</td>
<td>0.6</td>
</tr>
<tr>
<td>tetrachloromethane</td>
<td>1.2$^a$</td>
<td>0.6</td>
<td>0.9$^b$/0.9$^c$</td>
<td>0.05</td>
</tr>
<tr>
<td>diisopropylether</td>
<td>0.6$^a$</td>
<td>0.6</td>
<td>1.1$^b$/0.7$^c$</td>
<td>0.2</td>
</tr>
<tr>
<td>2-butanone</td>
<td>0.2 (0.23)</td>
<td></td>
<td></td>
<td>0.05 (0.5)</td>
</tr>
</tbody>
</table>

The experimental determination of the $\gamma$-values in 2-butanone was not possible, because 2-butanone is miscible with hexanol, tributyrin and the reference solvents (water and 1,2-ethanediol). Therefore, the $\gamma$-values of hexanol and tributyrin in 2-butanone were determined with the Unifac method (for $X_{\text{hexanol}} = 0.004$ and $X_{\text{tributyrin}} = 0.002$). The $\gamma$-value used in the kinetic experiments was the mean value of $\gamma^v$ and $\gamma^p$. The experimental and Unifac $\gamma$-values for the tributyrin and hexanol transesterification reaction are presented in Table 1.

Activity coefficients of propanol, butyric acid and decyl acetate

The $\gamma^v$-values of propanol, butyric acid and decyl acetate were determined with the Unifac method using vapour-liquid equilibrium data.
Enzyme particle characterization

Surface areas of enzyme particles were determined via gas adsorption according to Brunauer, Emmett and Teller (the BET-method) in an Asap 2000 apparatus. The PPL powder was weighed and evacuated at 100 °C during 10 hours, the void volume was determined with helium and the surface area was determined with krypton. Linearity was observed in the "BET"-plots relating the amount krypton adsorbed to the relative pressure.

The surface area was also determined by applying Scanning Electron Microscopy (SEM, Joel JSM-5400) and Image Analysis. For SEM analysis, the enzyme powders were gold coated (Joel JFC-1100E ion sputter) and SEM-photo's were taken at magnifications up to 75,000 times at 25 kV. The photos of the enzyme preparations revealed details of approximately 15 nm. The Image Analyzer consisted of an inverted microscope (Olympus IMT2) equipped with a CCD video camera module (Sony XC-77CE) controlled by CUE2 (Olympus) software. The measurements were performed on samples of the reaction mixtures. Particles smaller than 1 µm (dust), and particles larger than 2000 µm (loosely connected aggregates without much connection surface), were not taken into account. The specific area was determined from the projection area assuming spherical particles. For the calculation of the specific areas (surface area per gram catalyst, m²/g), a particle density of 1.3*10⁶ g/m³ was assumed. The occurrence of internal and external diffusion limitation was investigated according to Kamat and Russel.³³

Immobilisation of PPL

PPL was immobilized on C₈ HPLC beads (Waters, Lichrosorb RP-8, d = 10 µm, mean pore size = 6 nm). 4 g PPL was dissolved in 40 ml 50 mM potassium phosphate buffer, pH 7.8, containing 3 % (w/v) t-butanol. 4 g Lichrosorb RP-8 was added to the PPL solution, the suspension was vortexed for 1 min, stored for 5 min on ice, and centrifuged for 10 min at 27,000 g. The supernatant was discarded and the pellet was frozen with liquid nitrogen. The preparation was freeze dried during 48 hours and stored under vacuum over silica-gel at 4 °C. Since PPL (the relative molecular mass is 50 kD; the diameter is approximately 4 nm³⁴,³⁵) is large compared to the pores of the beads and the incubation times were relatively short, mainly the outersurface of the beads will be coated. In order to check the efficiency of the immobilization procedure, PPL was treated similarly, except that no RP-8 particles were added.

Initial reaction rates

Hydrolysis of propyl butyrate and tributylin

Hydrolysis was carried out in a pH-stat system, consisting of a Metrohm Dosimat (model 655), an Impulsomat (model 614) and a pH-meter (model 632). 0.1 ml propyl butyrate was added to 50 ml 10 mM potassium phosphate buffer (pH 7.8). In the case of tributyrin
hydrolysis, 0.5 ml tributyrin was added to 10 ml 10 mM potassium phosphate buffer, pH 7.8. 500 mg PPL or PcL was incubated for 16 hours in 10 ml 2-butanol with an \( a_w \)-value of 0.1, 0.3, 0.5 and 0.8. The reaction was started by adding 200 \( \mu \)l equilibrated lipase suspension to the reaction mixture. The pH was kept at 7.8 by titration of 0.01 M NaOH. In the case of the tributyrine measurements, the emulsions were stirred vigorously, and the amount of PPL added was varied in order to check whether the relation between enzyme concentration and activity was linear.

**Transesterification of vinylacetate with glycidol**

400 mg PPL was incubated in 30 ml 2-butanol with water activity (\( a_w \)) of 0.01, 0.05 or 0.15, in a stoppered tube during 16 hours. The water activities were adjusted by mixing dried 2-butanol (with 4 Å zeolite) with water saturated 2-butanol, in a 10/0, 9.5/0.5 or 8.5/1.5 v/v ratio, respectively. The suspension was centrifuged at 27,000 g for 10 min and 26 ml supernatant was discarded. The sediment was resuspended in the remaining 4 ml supernatant. 1 ml suspension (containing 100 mg PPL) was added to 50 ml vials containing 25 ml 2-butanol (\( a_w = 0.01, 0.05 \) or 0.15), and 4 ml vinylacetate. The reaction was started by addition of 200 \( \mu \)l glycidol (\( R,S-2,3 \)-epoxy-1-propanol). The course of the reaction was followed by determining the concentration of glycidyl acetate with GC every 10 minutes. The particle surface area in the reaction mixture was determined with Image Analysis after 1 hour.

**Esterification of propanol and butyric acid.**

Reactions at constant thermodynamic activity were carried out at a thermodynamic activity of 0.05 for propanol and butyric acid, and of 0.5 for water. The amounts (in grams) of solvent, propanol, butyric acid and water in the reaction mixtures were, respectively; bis(2-methoxyethyl)dether (diglyme) 36.1, 2.40, 4.46, 3.92; acetone 31.5, 1.56, 5.22, 2.47; 1,2-dimethoxyethane (monoglyme) 33.9, 3.72, 4.88, 3.72; 2-butanol (methyl ethyl ketone) 34.5, 1.21, 3.97, 1.48; 2,3,4,5-tetrahydrofuran 37.3, 1.50, 4.01, 1.96; diethylether 31.6, 1.14, 2.63, 1.55; 2-pentanone 36.1, 0.94, 3.21, 0.93; dichloromethane 64.3, 0.83, 0.37, 0.11; diisopropylether 35.1, 0.31, 0.75, 0.38; tetrachloromethane 78.9, 0.13, 0.34, 0.0049; hexane 32.8, 0.056, 0.27, 0.012; isoctane 34.9, 0.056, 0.22, 0.010. Reactions at constant concentration were performed with 44 mM propanol and 36 mM butyric acid, and an \( a_w \)-value of 0.5 (obtained by mixing equal volumes of dried and water-saturated solvent).

PcL (1 g) was equilibrated with 30 ml 2-butanol, having an \( a_w \)-value of 0.5 for 16 hours under shaking in a closed vial. The sample was centrifuged at 27,000 g for 15 min. The supernatant was discarded and the resuspended in 10 ml 2-butanol, with \( a_w = 0.5 \). The reactions were started by adding 0.6 ml (60 mg PcL) of this suspension. Reaction rates were determined by taking four samples at 10 minutes intervals and determining the concentration of propyl butyrate. Experiments in each solvent were performed in duplo.
Transesterification of hexanol and tributyrine.

PPL (350 mg) and immobilized PPL (350 mg) were incubated for 16 hours in dry 2-butanol (30 ml) (the PPL suspension was sonicated prior to the incubation). The reaction mixtures typically consisted of 12 ml organic solvent (dried with 4Å zeolite). Four series of rate measurements were carried out for each solvent: at constant \( c_{\text{tribut}} \) (26 mM) and \( a_{\text{tribut}} \) (0.030) for varying \( c_{\text{hexanol}} \) (ranging from 2 to 50 mM) and at \( c_{\text{hexanol}} \) (20 mM) and \( a_{\text{hexanol}} \) (0.025) for varying \( c_{\text{tributyrin}} \) (ranging from 2 to 200 mM). The reactions were started by addition of 0.5 ml of the enzyme suspension. Four samples were taken at time intervals of 10 min, and the concentration of hexyl butyrate was determined with GC.

Hydrolysis of decyl acetate.

The reactions were performed at an \( a_{w} \)-value of 0.7 (by mixing 10 ml dry solvent with 15 ml water saturated solvent). 210 mg PceL was equilibrated for 4 hours in 30 ml solvent, having an \( a_{w} \)-value of 0.7. The PceL suspension was centrifuged at 27000 g for 10 min, 25 ml of the supernatant was rejected, the pellet was resuspended. 1 ml suspension (containing 35 mg PceL) was added to 12 ml solvent containing various amounts of decylacetate. The final decyl acetate concentrations (M) were: for diisopropylether: 0.2, 0.4, 0.7, 1, 1.2, 1.4, 1.6, for dichloromethane: 0.5, 1, 1.5, 2, 2.5, 3.0 and for 2-butanol: 0.2, 0.4, 0.6, 0.8, 1.0, 1.5. Samples were taken at time intervals of 15 min and analyzed, and the concentration of decanol was determined with GC.

Modeling

Since enzymic reactions mostly take place in dilute solutions, concentrations are used instead of thermodynamic activities in equations for equilibria and kinetics. However, since solvent molecules interact with the substrate molecule, the "availability of substrate to the enzyme" forms part of the overall kinetic parameters observed and the contribution of this will vary with the nature of the solvent. Thus, when a comparison of enzyme behaviour in different solvents has to be made, correction for these contributions should be carried out to enable assessment of real effects of the solvents on the enzyme. Theoretical considerations\textsuperscript{18,21} have already indicated that concentrations should be replaced by thermodynamic activities in the rate equations. Such an approach is presented here for an UniUni, pseudo second order reaction of a substrate (A) with enzyme (E) in a solvent (S), consisting of the following steps:

\[
E_S + A_S \xrightarrow{k_1} EA \xrightarrow{k_2} E_S + P_S \quad \text{Scheme 1}
\]
In this scheme, it is assumed that solvation occurs of E, A and product (P), as denoted with subscripts. According to the transition state theory, the reaction rate \( r_p^s \) is linear proportional to the concentration of the transition state complex \( (c_{EA^*}^s) \):

\[
r_p^s = k^* \cdot c_{EA^*}^s
\]  
(2)

in which the decomposition rate constant, \( k^* \), equals:

\[
k^* = \kappa \cdot \frac{k_B \cdot T}{\gamma}
\]  
(3)

Since \( k^* \) only depends on the Boltzmann constant \( (k_B) \), the absolute temperature \( (T) \), and Planck's constant \( (\hbar) \), and the transmission coefficient \( \kappa \) (for enzymatic reactions usually 1), it is not affected by the solvent in which the reaction takes place. In the transition state theory, the transition state complex \( (EA^*) \) is assumed to be in equilibrium with the ground state of the substrate \( (A) \) and enzyme \( (E) \). When the equilibrium is defined on basis of the thermodynamic activities of the transition state complex \( (a_{EA^*}) \) and of the enzyme and substrate \( (a_E, \text{ and } a_A) \), the corresponding thermodynamic equilibrium constant \( (K_{eq}^{th,*}) \) is independent of the solvent.\(^{36}\) Since \( a = \gamma c \) (\( \gamma \) is the activity coefficient), \( c_{EA^*}^s \) (Eqn. 2) can be expressed as a function of \( K_{eq}^{th,*} \), the activity coefficients of substrate, free enzyme and transition state complex \( (\gamma_{EA^*}^s, \gamma_E^s \text{ and } \gamma_A^s) \) and concentrations of substrate and free enzyme \( (c_A^s \text{ and } c_E^s) \):

\[
c_{EA^*}^s = K_{eq}^{th,*} \cdot \frac{\gamma_{EA^*}^s \cdot \gamma_E^s \cdot \gamma_A^s}{\gamma_{EA^*}^s} \cdot c_E^s \cdot c_A^s
\]  
(4)

Combination of Eqns. (2) and (4) results in:

\[
r_p^s = k_{sp}^{int} \cdot c_E^i \cdot a_A^i
\]  
(5)

in which \( k_{sp}^{int} \) equals:

\[
k_{sp}^{int} = k^* \cdot K^* \cdot \frac{\gamma_E^s}{\gamma_{EA^*}^s}
\]  
(6)

The parameter \( k_{sp}^{int} \) is called the intrinsic specificity constant since it is not affected by solvent-
substrate interactions. It can be argued that the value of \( k_{\text{sp}}^{\text{int}} \) (and thus the reaction rate as defined in Eqn. (5)) is independent of the solvent when the following premises are valid. First, the energy of desolvation of the active site must be independent of the nature of the solvent (i.e. Scheme (1) is applicable for all solvents). Second, all enzyme species occurring in the catalytic cycle should be identical with respect to volume and shape (including structural water), thus extent of solvation of the enzyme species should be equal (\( \gamma_{\text{E}}^{s} = \gamma_{\text{E,A+}}^{s} \)) and the substrate should be shielded from the solvent. Third, the effect of the solvent on the catalytic mechanism of the enzyme must be equal for all solvents.

The transition state assumption described above for the complete reaction scheme can also be applied for the elementary steps in Scheme (1).\(^{21}\) From this it appears that \( r_{P}^{s} \) is related to the intrinsic kinetic parameters according to:

\[
r_{P}^{s} = \frac{\kappa_{2}^{\text{int}} \cdot c_{E}^{s} \cdot a_{A}^{s}}{K_{m}^{\text{int}} + a_{A}^{s}}
\]

in which \( c_{E} \) is the total enzyme concentration. \( k_{2}^{\text{int}} \) and \( K_{m}^{\text{int}} \) are called intrinsic parameters since they are not affected by substrate-medium interactions. On dividing the numerator and denominator of Eqn. (7) by the activity coefficient of the substrate, the following expression is obtained:\(^{21}\)

\[
r_{P}^{s} = \frac{\kappa_{2}^{\text{int}} \cdot c_{E}^{s} \cdot c_{A}^{s}}{K_{m}^{\text{int}} \gamma_{A}^{s} + c_{A}^{s}}
\]

(8)

As shown by Eqn. (8), the ordinary and intrinsic Michaelis constants are related via \( K_{m} = K_{m}^{\text{int}} / \gamma_{A} \) and the ordinary value of \( k_{2} \) equals the intrinsic value, provided that the premises are valid. It should be noted that the value of \( k_{2} / K_{m}^{\text{int}} \) in Eqn. (8) equals \( k_{\text{sp}}^{\text{int}} \) in Eqn. (6).

Analogous reasoning reveals that correction for solvent-substrate interactions can be obtained by replacing the concentrations by the thermodynamic activities of the substrates, products and modulators (inhibitor and activator) in the ordinary rate equations. PPL and PcL act via a ping-pong mechanism.\(^{37}\) This implicates that the apparent kinetic parameters for a substrate A, depend on the thermodynamic activity of the second substrate B, as illustrated in the following equations:

\[
r_{P}^{s} = \frac{V_{1}^{\text{app}} \cdot c_{E}^{s} \cdot a_{A}^{s}}{K_{m,A}^{\text{app}} + a_{A}^{s}} \quad \Rightarrow \quad V_{1}^{\text{app}} = \frac{V_{1}^{\text{int}}}{1 + \frac{K_{m,A}^{\text{int}}}{a_{B}^{s}}} \quad \text{and} \quad K_{m,A}^{\text{app}} = \frac{K_{m,A}^{\text{int}}}{1 + \frac{a_{B}^{s}}{a_{B}^{s}}}\]

(9)
It is clear that when $a_b$ is not kept constant in the various solvents, the apparent maximal velocity ($V_1^{\text{app}}$) and the apparent Michaelis constant ($K_m^{\text{app}}$) will vary. However, the specificity constant ($V_{\text{nat}}^{\text{app}}/K_m^{\text{app}}$) appears to be independent of $a_b$.

**Results**

**Stability of the lipases**

To assess the reliability of the water equilibration procedure and the kinetic results, the stability of the lipases was checked in 2-butanone at $a_w$ values ranging from 0.1 to 0.8. Catalytic activity was daily assayed for the hydrolysis of propyl butyrate in aqueous buffer. No change in activity was observed for PPL at an $a_w$ of 0.1 in 4 days. However, for an $a_w$ of 0.3 and higher, a continuous decrease in activity was observed, starting after one day of incubation. Pcl remained stable at all water activities tested during 4 days of incubation. To determine the rate of $a_w$-equilibration, esterification rates for propanol with butyric acid in 2-butanone, at the same $a_w$-values at which Pcl was incubated, were measured. It appeared that equilibration took place within one day since no changes were observed after that.

**Characterization of the particles**

The specific surface area of PPL powder as determined by the BET method was 0.96 ± 0.02 m$^2$/g. A somewhat lower value (0.15 m$^2$/g, for particles ranging from 1 to 10 μm) was found with Image Analysis for suspensions of PPL in dry 2-butanone, prepared by sonication and subsequent vigourous stirring. On following transesterification of glycidol with vinyl acetate by these particles in 2-butanone with an $a_w$ of 0.01 during one hour, an increase in the specific surface area was observed from 0.15 to 0.26 m$^2$/g. SEM-photographs (75,000 ×) of gold coated PPL and PcL powder showed massive, cubic particles.38

Hydrolytic activities for propyl butyrate of immobilized PPL and of PPL treated as for immobilization, gave values of 25 μmol/min/g immobilized PPL and 5*10$^3$ μmol/min/g treated PPL, respectively. However, based on specific activities, the difference was much less (146 and 49 μmol/min/m$^2$, respectively). External diffusion limitation appeared to be absent, as determined by the methods presented by Kamat and Russel32 and as judged from the constancy of activity observed on increasing the stirrer speed in reactions catalyzed by particles and beads.

**Effects of water activity**

The effect of the water activity (0.01, 0.05 and 0.15) on the specific activity of PPL, was measured with suspended PPL particles or PPL adsorbed to carrier beads for the transesterification of glycidol with vinyl acetate in 2-butanone. Since the particles appear to
be massive and PPL is assumed to be absent from the pores of the beads, the specific activity of PPL is best related to the specific surface area. It appeared that increasing the water activity results in a lowering of the specific surface area of the particles (0.15, 0.059 and 0.048 m²/g, respectively), due to an increase in aggregation. Combined with the results of activity measurements, this revealed that a decrease in the specific activity of the PPL particles (146, 86 and 52 μmol glycidol/min/m², respectively) but also of the PPL immobilized on beads (49, 5 and 2 μmol glycidol/min/m², respectively) occurs.

**Figure 1.** Initial rates of propanol and butyric acid condensation (r₁) by PCL in various organic solvents. Experiments were carried out at aₐ = 0.5. Meaning of the symbols: O (left axis) rates at constant propanol and butyric acid activities of 0.05; ■ (right axis) rates at constant concentration of 44 mM propanol and 36 mM butyric acid. The solvents used were: dg, diglyme; ac, acetone; mg, monoglyme; bu, 2-butanone; tf, tetrahydrofuran; ee, diethyleneether; pn, 2-pentanone; dm, dichloromethane; pe, diisopropylether; ct, tetrachloromethane; hx, hexane; oc, isoctane.

**Initial reaction rates**

Initial rates were measured for esterification by PCL of propanol with butyric acid in various organic solvents. The measurements were carried out at a preset water activity, for either constant concentration or constant thermodynamic activity of the substrate. At constant substrate concentration a sigmoid curve was obtained when the rates are plotted against the log P value of the solvent (Figure 1). The difference between the rates in the various solvents can be as high as a factor 300. However, when the rates determined at constant thermodynamic activity are plotted against the log P value of the solvents (Figure 1), no such trend is observed. The data points scatter around a mean value represented by the horizontal line, an the maximal difference between the rates is a factor 5.4.
Since PPL shows high affinities for tributyrin and various alcohols, transesterification studies with this system seemed attractive to assess the effects of organic solvents by determining $V_{\text{max}}$ and $K_m$. However, substrate inhibition was observed for hexanol (Figure 2). To avoid this, measurements were carried out at low hexanol concentrations so that at least specificity constant ($V_{\text{max}}/K_m$) values could be measured accurately. Since the reactions took place at an $a_w$ of 0.01, it was not necessary to take hydrolysis into account.

![Figure 2. Transesterification rate of tributyrin and hexanol (t$_{\text{rin}}$) catalyzed by PPL in hexane at high $a_{\text{hexanol}}$-values. The concentration (top x-axis) is related to the thermodynamic activity via $c_A = a_A/y_A^s$.](image)

Transesterification rates at a constant $c_{\text{tributyrin}}$ and varying $c_{\text{hexanol}}$-values, were highest in diisopropyl ether and lowest in 2-butanol (Figure 3A). On inspecting the specificity constants (Table 2) and taking the log $P$ values of the solvents in consideration, the usual trend (exemplified by the sigmoid curve in Figure 1) appears to be at hand. On plotting the rates measured at constant $a_{\text{tributyrin}}$ against the varying $a_{\text{hexanol}}$-values (Figure 3B), it appears that the curve for diisopropyl ether is still deviating but those for the other solvents become more similar. Transesterification rates were also measured at constant $c_{\text{hexanol}}$ and $a_{\text{hexanol}}$ for varying $c_{\text{tributyrin}}$ and $a_{\text{tributyrin}}$ (data not shown). Fitting these data as well as those presented in Figure 3 with Eqn. (11), provided the specificity constants presented in Table 2. When neglecting the values obtained for diisopropyl ether but comparing those for the other solvents, it appears also here that variation is less for the intrinsic than for the ordinary values.
Figure 3. Initial transesterification rates ($r_{\text{HB}}$) of tributyryl and hexanol by PPL in various solvents. The experiments were carried out at $a_p = 0.01$. Panel A: rates as function of $c_{\text{hexanol}}$ at $a_{\text{tribut}} = 26$ mM; Panel B: reaction rate as function of $a_{\text{hexanol}}$ at $a_{\text{tribut}} = 0.032$ mol/mol. The solvents used were: O, hexane; ■, tetrachloromethane; △, diisopropyl ether; +, 2-butaneone.

Table 2. Specificity constants ($k_{sp} = V_{\text{max}}/K_m$) of PPL suspended in various solvents for the transesterification of hexanol (OH) and tributyryl (TB) on basis of concentrations and thermodynamic activities. The parameters were obtained from fitting Eqn. (9) to the experimental data, which are shown for constant TB concentrations/activities in Figure 3. The ordinary and intrinsic specificity constants are related to each other via $k_{sp} = \gamma \cdot k_{sp}^\text{ext}$.

<table>
<thead>
<tr>
<th>solvent</th>
<th>$k_{sp,\text{OH}}$ l/min/g</th>
<th>$k_{sp,\text{OH}}^{\text{ext}}$ mol/min/g</th>
<th>$k_{sp,\text{TB}}$ l/min/g</th>
<th>$k_{sp,\text{TB}}^{\text{ext}}$ mol/min/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane</td>
<td>2.8 (±0.4) × 10^{-3}</td>
<td>1.3 × 10^{-3}</td>
<td>5.4 (±0.9) × 10^{-4}</td>
<td>3.8 × 10^{-4}</td>
</tr>
<tr>
<td>diisopropylether</td>
<td>4.8 (±0.6) × 10^{-3}</td>
<td>8.6 × 10^{-3}</td>
<td>7.4 (±0.8) × 10^{-4}</td>
<td>8.6 × 10^{-4}</td>
</tr>
<tr>
<td>tetrachloromethane</td>
<td>2.5 (±0.5) × 10^{-3}</td>
<td>2.1 × 10^{-3}</td>
<td>0.6 (±0.2) × 10^{-4}</td>
<td>0.6 × 10^{-4}</td>
</tr>
<tr>
<td>2-butaneone</td>
<td>0.6 (±0.2) × 10^{-3}</td>
<td>2.8 × 10^{-3}</td>
<td>0.4 (±0.1) × 10^{-4}</td>
<td>1.4 × 10^{-4}</td>
</tr>
</tbody>
</table>

To rule out any effects of solvents on the amount of catalytically available enzyme on the results, similar experiments were carried out with immobilized PPL. When comparing the data (Figure 4) with those for suspended PPL particles (Figure 3), it appears that a similar pattern is obtained. This is also observed when the specificity constants for immobilized PPL (Table 3) are compared with those for PPL particles (Table 2), although the similarity is less since the solvent effects are reversed in some cases.
Figure 4. Initial transesterification rates ($r_{\text{TB}}$) of tributyrin and hexanol catalyzed by PPL immobilized on Lichrosorb C$_8$ in various solvents. The experiments were carried out at $a_w = 0.01$. Panel A: rates as function of $a_{\text{hexanol}}$ at $a_{\text{tributyrin}} = 0.025$ mol/mol; Panel B: rates as function of $a_{\text{tributyrin}}$ at $a_{\text{hexanol}} = 0.025$ mol/mol. The solvents used were: O, hexane; ■, tetrachloromethane; △, diisopropyl ether; +, 2-butanone.

Table 3. Specificity constants of PPL immobilized on Lichrosorb RP-8 for the transesterification of hexanol (OH) and tributyrine (TB). The parameters were obtained from fitting Eqn. (9) to the experimental data, which are partly shown in Figure 4.

<table>
<thead>
<tr>
<th>solvent</th>
<th>$k_{\text{sp,OH}}$ l/min/g</th>
<th>$k_{\text{sp,OH}}$ mol/min/g</th>
<th>$k_{\text{sp,TB}}$ l/min/g</th>
<th>$k_{\text{sp,TB}}$ mol/min/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane</td>
<td>18 ($\pm 4$) *10$^4$</td>
<td>7.8 *10$^4$</td>
<td>7.5 ($\pm 2.0$) *10$^4$</td>
<td>5.2 *10$^4$</td>
</tr>
<tr>
<td>diisopropylether</td>
<td>36 ($\pm 7$) *10$^4$</td>
<td>65 *10$^4$</td>
<td>11 ($\pm 2$) *10$^4$</td>
<td>13 *10$^4$</td>
</tr>
<tr>
<td>tetrachloromethane</td>
<td>7.5 ($\pm 2$) *10$^4$</td>
<td>6.0 *10$^4$</td>
<td>1.0 ($\pm 0.3$) *10$^4$</td>
<td>1.2 *10$^4$</td>
</tr>
<tr>
<td>2-butanone</td>
<td>8.3 ($\pm 2.1$) *10$^4$</td>
<td>32 *10$^4$</td>
<td>0.4 ($\pm 0.1$) *10$^4$</td>
<td>1.6 *10$^4$</td>
</tr>
</tbody>
</table>

Although it is generally assumed that organic solvents just affect enzymes by a mechanism related to a general physical property like log $P$, some of them might also exert a specific effect which could be related to chemical reactivity. Therefore, experiments were carried out in which the change in log $P$ is not achieved by taking a series of solvents but by making a series of mixtures consisting of two solvents with differing log $P$-value.
Figure 5. Initial transesterification rates ($r_{\text{th}}$) of tributyrin and hexanol catalyzed by PPL in hexane-2-butanol mixtures. The rates are plotted as a function of $c_{\text{hexanol}}$ (Panel A) and as a function of $a_{\text{hexanol}}$ (Panel B) at $a_{\text{tribut}} = 0.032$ in hexane/2-butanol mixtures with the following molar ratio: ●, 1/0; □, 0.7/0.3; +, 0.3/0.7; +, 0/1. The apparent kinetic parameters derived from fitting Eqn. (11) to the data are given in Table IV.

Table 4. Kinetic parameters of suspended PPL for the transesterification of hexanol at $a_{\text{tribut}} = 0.032$ in mixtures of hexane (hex) and 2-butanol (mek). The parameter values were obtained from fitting of Eqn. (7) to the experimental data (Figure 5). The applied $c_{\text{tributyrin}}$ were 26, 74, 170, 200 mM, the $\gamma_{\text{off}}$-values were 2.3, 0.7, 0.2, 0.2, the $\gamma_{\text{trib}}$-values were 1.4, 0.5, 0.3, 0.3, in the 1/0, 0.7/0.3, 0.3/0.7, and 0/1 hex/mek mixtures, respectively.

<table>
<thead>
<tr>
<th>Solvent mixture (mol/mol)</th>
<th>$V_{\text{app max}}$ $\mu$mol/min/g</th>
<th>$K_{\text{app}, \text{OH}}$ mM</th>
<th>$k_{\text{app,OH}}$ l/min/g</th>
<th>$k_{\text{app,OH}}$ mol/min/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>hex mek</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1  0</td>
<td>9 ($\pm2$)</td>
<td>3.3 ($\pm1$)</td>
<td>2.8</td>
<td>1.2</td>
</tr>
<tr>
<td>0.7 0.3</td>
<td>9 ($\pm1$)</td>
<td>7.2 ($\pm2$)</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>0.3 0.7</td>
<td>8 ($\pm2$)</td>
<td>11 ($\pm3$)</td>
<td>0.7</td>
<td>3.4</td>
</tr>
<tr>
<td>0 1</td>
<td>8 ($\pm2$)</td>
<td>14 ($\pm2$)</td>
<td>0.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

As shown in Fig. 5A, plotting of the rates against varying $c_{\text{hexanol}}$ for measurements in solvents with different log P (mixtures of 2-butanol with the more hydrophobic solvent hexane) gave a line pattern reminiscent to that observed for a series of solvents. However, plots (Figure 5B) of rates determined at constant $a_{\text{tributyrin}}$ against varying $a_{\text{hexanol}}$ showed that
the lines for different log P values are much less diverging. The same conclusions can be derived from the kinetic parameter values presented in Table 4: \( V_{\text{max}} \) values are similar but the affinity of the enzyme for hexanol, as judged from its \( K_m \) values, increases for solvent mixtures with high log P value; the intrinsic specificity constants vary less than the ordinary ones.

To determine solvent effects on PcL, hydrolysis of decyl acetate was studied in various solvents at an \( a_w \) of 0.7. Also in this case, the affinity of the enzyme for the substrate is too low so that only specificity constants could be accurately determined. As shown in Table 5, also for this enzyme the variation in intrinsic values is less than in that of the ordinary ones.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>( k_{\text{sp}} ) l/min/g PcL</th>
<th>( k_{\text{sp}}^{\text{im}} ) mol/min/g PcL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-butanolone</td>
<td>( 25 \times 10^{-6} )</td>
<td>( 12 \times 10^{-5} )</td>
</tr>
<tr>
<td>diisopropylether</td>
<td>( 7.5 \times 10^{-6} )</td>
<td>( 11 \times 10^{-5} )</td>
</tr>
<tr>
<td>dichloromethane</td>
<td>( 1.4 \times 10^{-6} )</td>
<td>( 3.3 \times 10^{-5} )</td>
</tr>
</tbody>
</table>

**Table 5.** Specificity constants of PcL for hydrolysis of decyl acetate in 2-butanolone, dichloromethane and diisopropylether at \( a_w \) of 0.7. The parameters were obtained from fitting of Eqn. (7) to the experimental data (not shown).

**Discussion**

**Effects of the media on the availability of substrate for lipase**

All methods applied to characterize the lipase powder or the suspended particles, lead to the same conclusion: the cubic particles are massive and have no pores of a size which could permit penetration by substrate. This strongly suggests that only lipase at the surface is involved in catalysis, explaining why the specific activity of enzymes in organic solvents is mostly dramatically lower as compared to that in water. Comparison of the performance of suspended PPL particles, treated in the same way as for immobilization, with that of immobilized PPL, support this view since similar specific activities are observed when these are based on the outer surface area of the particles or beads.

The experimental set-up chosen for these investigations appeared to be adequate since the enzymes were stable at the conditions employed. As reported by others,\textsuperscript{25,29} water appears to play a crucial role in the conversions carried out in organic solvents. A rise in \( a_w \) stimulated aggregation of the particles, resulting in a decrease of the specific surface area. On taking this into account, nevertheless a strong decrease in specific activity of PPL particles
was observed, suggesting that the enzyme becomes less active when the extent of hydration increases. This is confirmed with the results of PPL immobilized on beads, showing even a stronger decrease in activity when the water content of the medium increased. As a consequence of these observations, care was exercised to adjust the water activity in an appropriate way.

In the experiments discussed here, similar trends were observed for the effects of organic solvents on rates as reported by others.\textsuperscript{14,15} This is best illustrated by the sigmoid curve in Figure 1, being similar in shape to those depicted in other reports.\textsuperscript{14} Thus, the reactions and enzymes studied here, are not unusual in the sense that unfamiliar phenomena occur. Large differences exist for the various solvents between the rates at constant concentration. Not unexpected, therefore, also large differences between the specificity constants in the various solvents exist. On plotting the rates obtained at a constant a-value of the substrate against the varying a-value of the second substrate, the curves for the various solvents appear to deviate less (Figure 3). The remaining differences may originate either from incorrect $\gamma$-values or from effects exerted by the organic solvents on the enzymes. To probe the first possibility, a number of possible causes for errors are discussed.

Significant differences exist between the experimentally obtained $\gamma$-values and those calculated by UNIFAC, especially in the case for tributyrin (Table 1). In most cases (except for 2-butanone), experimentally determined values were used in the equations. Since the values of tributyrin for the two reference solvents are similar and the experimental conditions used for this mimic the actual situation during the conversions, it is likely that these values are correct. But, suppose that the $\gamma$-value of tributyrin in 2-butanone, which was determined with the Unifac method, was estimated too high by a factor 2. The tributyrin concentration in 2-butanone applied in the kinetic experiments (see e.g. Figure 3B) would then be a factor 2 too low, resulting in a reaction rate which would be maximally a factor 2 too low. However, the differences with the rate data for the other solvents would remain. It can be concluded, therefore, that the differences in rate do not originate from errors in the estimates of the $\gamma$-values alone.

The preliminary investigations presented here, show that only enzyme at the surface participates in catalysis. Since the nature of the solvent and the substrates could affect the available catalytic surface, as was demonstrated here for the water activity, differences in the amount of available catalytic surface may contribute to variation in reaction rates. Due to the enzyme/substrate combinations studied, the $k_{sp}$-values could be determined accurately but not the $k_{cat}$-values, although the curves suggest that variation in $k_{cat}$ may indeed occur. However, Image Analysis showed that the size distribution, and SEM (after incubation with the solvents) showed that the morphology of the particles did not vary substantially in the various solvents (data not shown). As shown by the results for immobilized PPL, significant differences still exist between the solvents and the trend is similar to that observed for the suspended PPL.
particles, showing that variation in catalytic available surface is not the main cause for the differences in the various solvents.

In conclusion, in order to determine the effect of organic solvents on the performance of an enzyme, correction of the kinetic parameter values for substrate-solvent interactions should take place and the intrinsic parameter values should be compared. In addition, the catalytically available amount of enzyme should be taken into account. Inaccurate \( \gamma \)-values may contribute to the differences in enzyme performance observed for the various solvents, but other causes (discussed below) seem more relevant.

**Media Effects on Lipase Performance**

As discussed already, the intrinsic kinetic parameters in various solvents will be constant provided that a number of requirements are met. However, are these requirements realistic? If the bound substrate is not completely shielded from the solvent, the correction method for substrate-solvent interactions used here will be inadequate. Although no data are available for lipase, for acyl-subtilisin complex in the ground state it was shown that approximately one third of the substrate is exposed to the solvent.\(^{50,41}\) Another assumption is that the activity coefficient of the enzyme species in the catalytic cycle is constant. This seems to be unrealistic for enzymes with large activation volumes,\(^{42}\) which may be the case for lipase (opening and closing of the lid).\(^{43}\) Of course the solvent may directly affect the catalytic mechanism of the enzyme. Indications that this may occur can be found for \( \alpha \)-chymotrypsin, for which a correlation between the dielectric constant of the solvents and the mobility of the amino acid residues was observed.\(^{44}\) This effect may be also relevant for the activity of enzymes, including lipases. In addition to this, the effect on the local polarity in the active site has been put forward as an explanation for the varying oxidation rate of phenols catalyzed by horse radish peroxidase in organic solvents.\(^{22}\) However, whether this generally occurs is questionable since the constant Hammet coefficients in organic solvents and water for subtilisin catalysis suggest that in this case the solvents do not affect the enzyme.\(^{45}\)

The most simple explanation for the remaining differences is that the solvents compete with substrate for binding to the active site. X-ray studies on \( \alpha \)-chymotrypsin and dioxane\(^{46}\) and on subtilisin and acetonitrile\(^{42}\) show that organic solvents may bind to enzymes at several places, including the active site. The high activities observed here in diisopropyl ether could mean that this solvent is less inhibitory as compared to the others. Future work will concentrate on this possibility.

**References**


Chapter 4

Thermodynamic and kinetic parameters of lipase catalyzed ester hydrolysis in biphasic systems with varying organic solvents

J.Bert A. van Tol, Jaap A. Jongejan, and Johannis A. Duine

Department of Microbiology and Enzymology, Delft University of Technology

Hans G.T. Kierkels, Erik F.T. Geladé, Frank Mosterd, Will J.J. van der Tweel, and Johan Kamphuis

DSM Research
Abstract

Equilibria and kinetics of lipase-catalyzed hydrolysis of esters were modeled for aqueous-organic, biphasic systems. By using thermodynamic activities of the substrates in ordinary rate equations, the kinetic parameters were correction for the contribution of substrate-solvent interactions and an uniform quantification of the substrates for lipase attached to the interface can be achieved. The kinetic parameters on basis of thermodynamic activities should be constant in different systems, provided that the solvents do not interfere with the binding of the substrates to the enzyme nor affect the catalytic mechanism. Experimental and computational methods how to obtain the thermodynamic activities of the substrates are presented. Initial rates were determined for Pseudomonas cepacia lipase (PcL) catalyzed hydrolysis of decyl chloroacetate in dynamic emulsions with various solvents. The thermodynamic equilibrium and corrected kinetic constants for this reaction appeared to be similar in various systems. The kinetics of PcL in an isoctane-aqueous biphasic system could be adequately described with the rate equation for a ping-pong mechanism. The observed inhibitory effect of decanol appeared to be a consequence of this mechanism, allowing the back-reaction of the decanol with the chloroacetyl-enzyme complex. The kinetic performance of PcL in three other systems could be less well described. The possible causes for this and for the remaining differences in corrected kinetic parameters are discussed.

Introduction

Biocatalysis in aqueous-organic, biphasic systems offers a number of advantages for the conversion of poorly water-soluble compounds. For instance, the yield of a condensation reaction improves substantially when the solvent added decreases the rate with which the enzyme hydrolyses the product. Moreover, in case of a lipase, the solvent enables adsorption of the enzyme to the interface, leading to interfacial activation with respect to catalytic activity and enantioselectivity.

The extent in which the effects of the solvents on the reaction rate are observed strongly varies with the type of solvent used. To find the rationale behind this, it has been attempted to establish correlations between the reaction rates for a certain enzyme/substrate combination and the physico-chemical properties of the solvents applied, e.g. with the dielectric constant, the viscosity and the hydrophobicity. However, since an underlying theory explaining the reported relationships is not available, it is unclear which of these has general validity or has just significance for the particular enzyme/substrate combination studied. This implicates that no general model exists which is able to predict the catalytic activity of an enzyme in any solvent from its catalytic activity in water.
Another complicating factor in studying lipase-catalyzed reactions in biphasic systems is which substrate concentration should be used in kinetic equations for the enzyme, that in the aqueous phase, that in the organic solvent phase, or that at the interface where the conversion take place. For water dissolved enzymes the concentration in the aqueous phase is used for that purpose.\textsuperscript{15} For lipases it has been proposed to take the concentration at the interface.\textsuperscript{16-18} However, a diffuse situation exists in the latter case, as shown for hexane-water systems,\textsuperscript{19} so that interfacial concentrations can be hardly defined.

When no diffusion limitation occurs in a system, the thermodynamic activity of a component in the system is equal in all the compartments. Thus, with the use of thermodynamic activities instead of concentrations, the problem indicated above can be circumvented. Moreover, it is also allowed to replace concentrations by thermodynamic activities in kinetic equations.\textsuperscript{20-25} With the latter, another important problem can be tackled in non-aqueous enzymology, namely the fact that substrate-solvent interactions contribute to the overall kinetic parameters of an enzyme, disenabling correct comparison of enzyme behaviour in solvents because interaction values will vary. Since thermodynamic activities account for this, application of them in kinetic equations provides the so-called intrinsic kinetic parameters.\textsuperscript{25} In principle, the values of these parameters should be equal in all solvents, provided that the bound substrate is shielded from the surrounding solvent, the activity coefficient of the enzyme conformers in that solvent are equal, and the solvents have no specific interaction with the active site. Reversibly, when differences are observed, this indicates that the assumptions are not correct and that the solvent exerts specific effects on the enzyme. Application of thermodynamic activities in kinetic studies has already been carried out for heterogenous catalysis by lipases,\textsuperscript{25} i.e. when the enzyme is suspended in organic solvents. It seemed interesting, therefore, to compare this with data for interfacially-adsorbed lipase in biphasic systems with various organic solvents.

Hydrolysis of decyl chloroacetate (DCA) with \textit{Pseudomonas cepacia} lipase (PcL) has been studied by some of us for a number of biphasic systems, revealing the physical (number and size of the emulsion droplets) as well as the kinetic characteristics.\textsuperscript{26-28} In view of this basic knowledge, the combination seemed attractive to investigate whether the organic solvents really affect the properties of PcL under this condition or whether quantitative predictions can be made with the approach outlined above. Besides these aims, it was attempted to find an explanation for the inhibiting effect of decanol (DOH) (formed during hydrolysis of DCA), observed in the previous work.\textsuperscript{28} The effect of DOH could be due to a number of reasons: e.g. inhibition of PcL; detergent action, affecting the interfacial adsorption or performance of PcL; back reaction with the acylated enzyme form.\textsuperscript{8,28} To check the probability of the latter, equations were derived in which back reaction with DOH was taken into account.
Materials and Methods

Materials

*Pseudomonas cepacia* lipase (PS-800) was purchased from Amano, dialyzed against demineralized water and lyophilized prior to use. All reagents and solvents were of analytical grade. The purity of the chloroacetic acid (Baker) was better than 99\%.

Activity coefficients for decanol and decyl chloroacetate

The activity coefficients ($\gamma$) in water of decanol (DOH) and decyl chloroacetate (DCA) were determined from their solubilities according to Eqn. (3) (see below). The solubility of DOH in water was obtained from the literature. The solubility of DCA was determined from the change in particle radius ($r_p$) due to Oswald ripening in an emulsion of DCA in water as a function of time. According to Lifshitz and Slōzov and Wagner, $r_p$ changes according to:

$$\frac{dr_p}{dt} = \frac{8. \sigma.D.s.V_m}{R.T}$$

in which $\sigma$ is the interfacial tension at the aqueous-organic interface and $D$, $s$, and $V_m$ are the diffusion coefficient, the solubility, and the molar volume of the organic phase, respectively. The $r_p$-value was determined with a fiber optic dynamic light scattering set-up. The emulsion was made using a microfluidizer.

The $\gamma$-values of DOH and DCA in the organic phases are not constant in the range in which they are applied (the $\gamma$-value of a component $A$ varies between 1 in the pure form and the value at infinite dilution of $A$ in the solvent). Therefore, $\gamma$-values were calculated for the actual concentration applied in the kinetic experiments with the Unifac group contribution method. Since the CH$_2$Cl - CH$_2$COO group interaction parameter is not included in the liquid-liquid equilibrium (LLE) parameter tables, the Unifac parameters from the vapor-liquid equilibrium (VLE) data bank were used to calculate the $\gamma$-values ($\gamma'$) for systems containing DCA. However, since the CH$_2$Cl - COO group interaction parameter is also not included in this data bank, the ester structure was 'inverted' and the activity coefficient of DCA was approximated by using that of chloroethyl decanoate. Thus, $\gamma'$-values were used for the organic solvents and $\gamma^{<\omega}$-values (based on experimentally determined solubilities) for water. To check the reliability of the calculated $\gamma'$-values, comparison was carried out with values at infinite dilution ($\gamma^{\infty}$), and the $\gamma^{\omega}$-values determined in water (Table 1). Since the aqueous phase is dilute (DOH and DCA poorly dissolve), an $a_w$ value of 1 was used in the rate experiments.
Table 1. \( \gamma \)-values for DOH, DCA and HCA in various solvents. The reference state (\( \gamma = 1 \)) are the pure compounds, for DOH and DCA the pure state being the liquid phase and for HCA the solid state. \( \gamma^0 \)-values were determined with the Unifac group contribution method (at \( X = 0.01 \), for poorly soluble compounds at their saturation) based on VLE parameters. The \( \gamma^0 \)-values of DOH, determined with the Dechema base for infinite solutions, are given in brackets. \( \gamma^\infty \)-values were determined in various ways: \(^a\), determined with Oswald ripening; \(^b\), experimental solubility data; \(^c\), experimental partitioning data; \(^d\), solubility data from literature.\(^{29}\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Solvent</th>
<th>( \gamma^\infty )</th>
<th>( \gamma^0 ) (( \gamma^0 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCA</td>
<td>Isooctane</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dibutylether</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toluene</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIBK</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DOH</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>( 7 \times 10^6 ) (^{(0)} )</td>
<td>( 2 \times 10^6 )</td>
</tr>
<tr>
<td>DOH</td>
<td>Isooctane</td>
<td>7.0 (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dibutylether</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toluene</td>
<td>2.8 (5.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIBK</td>
<td>2.0 (2.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>( 2.4 \times 10^4 ) (4.3 ( \times 10^4 ))</td>
<td></td>
</tr>
<tr>
<td>HCA</td>
<td>Isooctane</td>
<td>62(^b)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>MIBK</td>
<td>1.8(^b)/1.5(^c)</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>DOH</td>
<td>1.8(^b)/1.3(^c)</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>3.2(^c)</td>
<td>13</td>
</tr>
</tbody>
</table>

Activity coefficients for chloroacetic acid

The activity coefficient of chloroacetic acid (HCA) in water and in the organic solvents was determined via the solubility measurements in isooctane and measuring partition over the aqueous-organic solvent biphasic system, according to Eqns. (3) and (4) (see below). The solubility in isooctane was determined by adding portions of 1 ml isooctane to 200 mg of HCA until all HCA was dissolved. The portions were added under stirring and equilibration periods of 30 min were allowed before the next portion was added.

The distribution of HCA was measured by mixing 2 ml of the organic phase (saturated with water) with the aqueous 0.1 M H\(_2\)SO\(_4\) buffer (pH 2). After equilibration, the phases were separated by centrifugation at 12\( \times 10^3 \) g at room temperature. The concentration of HCA in the organic phase was determined before and after equilibration by GC analysis, using flame ionization detection (on a HP-1 column, \( l = 30 \) m., i.d. = 0.53 mm, oven temperature: \( T_{initial} = 75^\circ\)C for 6 min, 10\(^\circ\)/min 120\(^\circ\)C for 6 min, with 2-nonanon as internal standard). The partition coefficients were determined from the distribution values after correcting for dissociation of HCA.
Equilibrium constants

Equilibrium constants were determined by carrying out conversions with various amounts of DOH, HCA, water and organic solvent. The pH of the mixtures was adjusted to 4 by addition of 10 M NaOH. The reaction was started by addition of 20 mg PcL and the conversion was followed in time by measuring the amounts of DOH and DCA by GC. The amounts of water and HCA were derived from the amounts present at the start and the amount of DCA formed. When the conversion stopped (after about 60 hours) an additional amount of PcL was added in order to check whether the equilibrium had been attained. The number of moles at equilibrium of water, DOH, HCA, DCA and solvent were 2.45, 0.0139, 0.0158, 0.00154, and 0.406 methylisobutylketone; 2.54, 0.0134, 0.0146, 0.00215, and 0.391 isooctane; 3.61, 0.11, 0.108, and no solvent; 3.61, 0.127, 0.113, and no solvent. The thermodynamic equilibrium constant was calculated from the experimental data via Eqn. (6). For HCA in the organic solvents and water, and DOH and DCA in water the experimental \( \gamma^\exp \)-values were used (Table 1). The other \( \gamma \)-values were calculated with Unifac-VLE for the molar fraction of the components found at the equilibrium.

Hydrolysis experiments

Hydrolysis rates were measured in 50 mM Tris/HCl buffer pH 8 containing 4% (v/v) organic phase in a total volume of 200 ml, at 25 °C. After allowing equilibration of the droplets (sizes appeared to be stable after stirring for 5 min), the reaction was started by addition of 0.5 mg PcL. The pH was kept at 8.0 by titration of 0.1 M NaOH (Radiometer pH-stat). The rates were determined from the tangent of the initial part of the titration curve. After each experiment, the reaction vessel was successively cleaned with water, acetone and demineralized water.28

Fitting of experimental rate data

Parameter values were obtained from fitting Eqn. (8) to the experimental rate data obtained for varying thermodynamic activities of DCA and DOH (\( a_{DCA} \) and \( a_{DOH} \)). Non-linear fitting was carried out for single as well as multiple curves as indicated in the text. Estimations of the standard deviations of the parameter values were obtained from the diagonal elements of the covariance matrix. The validity of the ping-pong BiBi model for the reaction was estimated from visual inspection of the linearity of the reciprocal plots (Eqns. (10), (11) and (12)). The values of the slope and intercept were plotted, and compared with the lines obtained with the parameters resulting from the fit of Eqn. (8) to the rate data. Kinetic parameter values indicated by a superscript \( X \), were obtained from rate data versus molar fractions of the substrates, and by replacing the thermodynamic activities in Eqn. (8) by molar fractions.
Modeling

Thermodynamic activities and activity coefficients

The thermodynamic activity of compound A \( (a_A) \) is related to its molar fraction \( (X_A) \) and activity coefficient \( (\gamma_A) \) in the considered phase via:

\[
a_A = \gamma_A \cdot X_A
\]  

(2)

The thermodynamic activities are defined in this paper in the sense of Raoult's law, i.e. the activity and the activity coefficient of a component in its pure form is 1. Since at equilibrium the activity of the components is equal in all phases, the activity coefficient of poorly soluble compounds can be estimated from their solubility \( (X_A^{sat}) \):

\[
\gamma_A^{sat} \cdot X_A^{sat} = 1 \iff \gamma_A^{sat} = \frac{1}{X_A^{sat}}
\]  

(3)

The value of the activity coefficients will vary with the composition of the medium. However, when the solubility of the solute is low, the composition of the mixture hardly changes with these small amounts of solute. Therefore, the activity coefficient of poorly soluble compounds can be assumed to be constant and can be estimated from its solubility. Under phase-equilibrium conditions, the thermodynamic activity of the components in the organic phase equals that in the aqueous phase. Therefore, the partition coefficient \( (P) \) of a compound A is related to its activity coefficient via:

\[
P_A = \frac{X_A^{org}}{X_A^{aq}} = \frac{\gamma_A^{org}}{\gamma_A^{aq}}
\]  

(4)

Thus, \( \gamma_A \) can be determined from \( P_A \) when the activity coefficient of A in the other (reference) phase is known. In cases where the activity coefficient cannot be determined via solubility and partitioning, the activity coefficients can be estimated with the Unifac group contribution method.\(^{33}\)

Equilibria

The thermodynamic equilibrium constant for a reaction \( (K_{eq}^{th}) \) is defined for a certain temperature and pressure, and it is independent of the medium in which the reaction is performed.\(^{37}\) The effect of the medium on the dynamic equilibrium constant \( (K_{eq}) \) is quantified by the activity coefficients of the reactants. This means that when \( K_{eq}^{th} \) is known, the equilibrium can be calculated in any medium for which the activity coefficients are known.\(^{38}\)

For a two substrate (decyl chloroacetate, DCA, and water, W) two product (decanol, DOH,
and chloroacetic acid, HCA) reaction, the dynamic equilibrium constant is related to the thermodynamic equilibrium constant according to:

\[
K_{eq}^{th} = \frac{a_{DOH} \cdot a_{HCA}}{a_{DCA} \cdot a_w} = K_{eq} \cdot \frac{\gamma_{DOH} \cdot \gamma_{HCA}}{\gamma_{DCA} \cdot \gamma_w}
\]  

(5)

HCA will dissociate to an extent determined by the pH applied to the reaction and the reaction components will distribute over the phases of the system. Thus, the observed dynamic equilibrium constant \(K_{eq}^{obs}\) is given by: \(^{21,39,40}\)

\[
K_{eq}^{obs} = K_{eq}^{th} \cdot \frac{\gamma_{eq}^{HCA} \gamma_{eq}^{DOH} \gamma_{eq}^{org}}{(1 + \phi \gamma_{eq}^{HCA}) \gamma_{eq}^{org \cdot H}} \frac{(1 + \phi \gamma_{eq}^{DOH}) \gamma_{eq}^{org \cdot DC A}}{(1 + \phi \gamma_{eq}^{DC A}) \gamma_{eq}^{org \cdot DC A}} \frac{K_{a \cdot HCA}}{K_{a \cdot HCA}}
\]  

(6)

in which \(\phi\) is the phase ratio, defined as moles organic phase over moles aqueous phase, and \(K_a\) is the dissociation constant of HCA. The solvents used in this study, are virtually immiscible with water. Since DOH and DCA are also poorly soluble in water, they will be nearly completely dissolved in the organic phase. Therefore, under the conditions applied to the experiments, the aqueous phase can be considered as a dilute aqueous phase, i.e. \(\gamma_{eq}^{eq} = 1\).

![Figure 1](image)

**Figure 1.** Reaction scheme for the hydrolysis DCA catalyzed by PcL. The symbols have the following meaning: DOH, decanol; HCA, 2-chloroacetic acid; E, PcL; EDCA, PcL-DCA complex; ECA, chloroacetyl-PcL complex; EHCA, chloroacetic acid-PcL complex.

**Enzyme kinetics**

*Pseudomonas cepacia* lipase (PcL), which is a serine hydrolase,\(^{41}\) catalyzes the hydrolysis of DCA via a ping-pong BiBi mechanism (Figure 1). In view of the linear relationship found between the amount of added PcL and the reaction rate, the lipase will be fully adsorbed to the interface under the conditions applied and the enzymatic reaction rate
can be described by a single rate equation.\textsuperscript{28} In addition, since the system is vigorously stirred (stirring speeds higher than 600 rpm have no effect on the reaction rates\textsuperscript{28}), phase partitioning of the components is at equilibrium and the thermodynamic activity of the components will be equal in all phases. Accordingly, the enzymatic reaction is the rate determining step for the system.

In correcting for substrate-solvent interaction, the ordinary rate equations can be used,\textsuperscript{42,43} but concentrations have to be replaced by thermodynamic activities.\textsuperscript{20,21} Since at the pH applied in the experiments HCA is fully deprotonated, \( a_{\text{HCA}} \) will be virtually zero. The rate of the ping-pong BiBi reaction is then given by:

\[
r_{\text{DCA}} = \frac{V_1 \cdot a_{\text{DCA}} \cdot a_w}{K_{l, \text{DCA}} \cdot K_{m, w} \cdot a_{\text{DCA}} \cdot a_w + K_{l, \text{DCA}} \cdot K_{m, w} \cdot a_{\text{DCA}} \cdot a_w + a_{\text{DOH}} + a_{\text{DCA}} \cdot a_{\text{DOH}}} \tag{7}
\]

for which the terms of the lumped parameters are presented in Table 2. After rearranging Eqn. (7), and taking \( a_w = 1 \), a rate equation with four parameters is obtained:

\[
r_{\text{DCA}} = -\frac{\beta_1 \cdot a_{\text{DCA}}}{a_{\text{DCA}} + \frac{\beta_4 \cdot a_{\text{DOH}} + \beta_3}{a_{\text{DOH}} + \beta_2}} \tag{8}
\]

in which:

\[
\beta_1 = \frac{V_1 \cdot K_{l, \text{DOH}}}{K_{m, w}}; \quad \beta_2 = K_{l, \text{DOH}} \cdot (1 + \frac{1}{K_{m, w}}); \quad \beta_3 = \frac{K_{m, \text{DCA}} \cdot K_{l, \text{DOH}}}{K_{m, w}}; \quad \beta_4 = K_{l, \text{DCA}} \tag{9}
\]

However, for initial rate measurements where the amount of DOH formed can be neglected or for conversions in which \( a_{\text{DOH}} \) remains constant, Eqn. (8) simplifies to a Michaelis Menten type equation with 2 parameters.

In order to determine whether the effect of DOH on the reaction rate is due to the reversibility of the enzyme mechanism, the adequacy of the ping-pong model was checked from the linearized form of Eqn. (8):

\[
-\frac{1}{r_{\text{DCA}}} = \frac{1}{\beta_1} \left(1 + \frac{\beta_4 \cdot a_{\text{DOH}} + \beta_1}{a_{\text{DCA}} + \beta_2} \right) a_{\text{DCA}} \tag{10}
\]

When \( 1/r_{\text{DCA}} \) is plotted against \( a_{\text{DOH}} \) at constant \( a_{\text{DCA}} \)-values, straight lines should be obtained.
which intersect in one point. When the intercept and the slope of these lines at various DOH activities are plotted against $1/a_{DCA}$, again straight lines should be obtained according to:

$$\text{slope} = \frac{K_{m,w}}{V_1 K_{i, DOH}} + \frac{K_{m,w} K_{i, DCA}}{V_1 K_{i, DOH}} \cdot \frac{1}{a_{DCA}}$$ (11)

and:

$$\text{intercept} = \frac{1}{V_1} + \frac{K_{m,DCA}}{V_1} \cdot \frac{1}{a_{DCA}}$$ (12)

Table 2. Definition of the pseudo steady state parameters for the ping-pong mechanism depicted in Figure 1 and described by Eqn. (7) (according to the nomenclature introduced by Cornish Bowden\textsuperscript{2}).

<table>
<thead>
<tr>
<th>Lumped parameter</th>
<th>Elementary rate constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_1$</td>
<td>$k_2 k_4 c_{PP}/(k_2 + k_4)$</td>
</tr>
<tr>
<td>$K_{m,DCA}$</td>
<td>((k_1 + k_2)k_4/ [k_1 (k_2 + k_4)])</td>
</tr>
<tr>
<td>$K_{m,w}$</td>
<td>(k_3 (k_3 + k_4)/ [k_5 (k_2 + k_4)])</td>
</tr>
<tr>
<td>$K_{i,DCA}$</td>
<td>(k_1/k_1)</td>
</tr>
<tr>
<td>$K_{i,DOH}$</td>
<td>(k_2/k_2)</td>
</tr>
</tbody>
</table>

Results

Activity coefficients and equilibria

The $\gamma$-values that are used to derive the equilibrium and kinetic constants are the experimental ($\gamma^{exp}$) values, or when these were not available the Unifac-VLE ($\gamma^{U}$) values. To evaluate the reliability of these $\gamma$-values, they were compared with their $\gamma^{U}$-values in binary mixtures (Table 1). It appears that the $\gamma$-values for some compounds deviate substantially, e.g. the $\gamma^{U}_{DOH}$-value is 10 times smaller than the $\gamma^{exp}_{DOH}$-value. On the other hand, the $\gamma^{exp}_{DCA}$ and $\gamma^{exp}_{HCA}$-values in water appear to be similar to the $\gamma^{U}$-values. The solubilities of HCA in isooctane, methyl isobutyl ketone (MIBK) and DOH were 200 mg/37 ml, 1700 mg/2 ml, and 1300 mg/2 ml, respectively. The corresponding $\gamma$-values are presented in Table 1.

Since HCA will be practically completely dissociated at pH 8, the hydrolysis reaction will go to completion at the experimental conditions. Accordingly, pH 4 was chosen for the equilibrium experiments to allow accurate determination of the remaining DCA. The equilibrium constants based on molar fractions ($K_{eq}^X$) and on thermodynamic activities ($K_{eq}^a$)
are given in Table 3 for three different organic solvents. The average value of $K_{eq}^{th}$ is 0.024 ± 0.004. The $K_{eq}^{obs}$-value, calculated according to Eqn. (5) at pH 8 in an isooctane system as described in Table 3, is 284, and the corresponding equilibrium conversion is 99.998%.

<table>
<thead>
<tr>
<th>Organic solvent</th>
<th>$K_{eq}^{obs}$</th>
<th>$K_{eq}^{th}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylisobutylketone</td>
<td>0.058</td>
<td>0.027</td>
</tr>
<tr>
<td>Isooctane</td>
<td>0.036</td>
<td>0.028</td>
</tr>
<tr>
<td>Decanol (duplo)</td>
<td>0.21 (±0.02)</td>
<td>0.018 (±0.002)</td>
</tr>
</tbody>
</table>

**Table 3.** Equilibrium constants for the hydrolysis reaction of DCA in biphasic systems. $K_{eq}^{obs}$ is based on the total amount (moles) of the reacting components in the biphasic system, $K_{eq}^{th}$ is calculated according to Eqn. (6) with the $\gamma^{exp}$ and $\gamma^{v}$-values given in Table 1.

**Initial rates of DCA hydrolysis in DCA - water biphasic systems**

It appears (Figure 2) that the maximal reaction rates were reached at high substrate concentrations. At these high concentrations, both the solvent and the substrate contribute to the properties of the organic phase. At $X_{DCA} = 1$, the organic phase is pure DCA. Thus, the variation observed in the curves at this point (Figure 2) reflects the spread in the measured rates, which is probably due to variation in the PCL concentration.

![Figure 2](A and B graphs)

**Figure 2.** Initial hydrolysis rates of PCL for DCA in biphasic systems for varying $X_{DCA}^{exp}$ (Panel A) and $a_{DCA}$ (Panel B). The experimental data were fitted with Eqn. (8). Experiments were carried out in the following solvents: ○ — toluene; □ — dibutyl ether; ♦ — methyl isobutyl ketone; ← — isooctane.
When the rates are plotted as a function of the $X_{DCA}$ in the organic phase, substantial variation between the different solvents is observed (Figure 2A). However, when the same rate data are plotted against $a_{DCA}$-values, the shape of the curves become similar (Figure 2B). The data for isooctane are, in contrast to the other solvents, adequately described by a Michaelis Menten equation. In toluene, dibutylether, and methylisobutylketone, however, a maximum rate is observed at $a_{DCA}$-values of approximately 0.5. Therefore, it was decided to use specificity constants for DCA ($k_{sp} = V_0/K_{m,DCA}$) that are obtained from rate data up to an $a_{DCA}$ of 0.3 mol/mol. As shown in Table 4, the $k_{sp}^{X}$-values (based on molar fractions) in toluene and isooctane varied a factor 20 but the corresponding $k_{sp}$-values (based on thermodynamic activities) ranged from $8 \times 10^3$ to $14 \times 10^3$ μmol.min/mg PcL, less than a factor two.

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 3.** Panel A: Thermodynamic activities (left axis, closed symbols) and activity coefficients (right axis, open symbols) of DCA (circles) and DOH (squares) for varying $X_{DCA}^{sp}$-values in a DCA/DOH-aqueous (4/96 v/v) biphasic system. Panel B: Initial rates of DCA hydrolysis by PcL at varying $X_{DCA}^{sp}$-values in the DCA/DOH-aqueous biphasic system (Panel B).

**Initial rates of DCA hydrolysis in biphasic systems containing DCA and DOH**

It has already been observed in conversion experiments that the reaction rates decrease strongly after 5% conversion.²⁸ It was investigated, therefore, whether addition of DOH had inhibitory effects on the initial rates of DCA hydrolysis. The $γ^0$-values of DCA and DOH were determined for the actual concentrations at which the kinetic experiments were carried out (Figure 3 A). As shown in Figure 3B, Eqn. (8) adequately describes the rate data of these experiments, and the intrinsic parameters derived were $β_1 = 104$ μmol/min.mg; $β_2 = 0.16$ mol/mol; $β_3 = 0.027$ mol/mol; $β_4 = 0.35$ mol/mol. The $k_{sp}$-value of $4 \times 10^3$ μmol/min/mg PcL, calculated with parameters obtained from this experiment via $k_{sp} = \frac{β_3}{β_4}$.
(\(V_{\text{max}} = \beta_1/\beta_3\); \(K_m = \beta_2/\beta_3\)), is in reasonable agreement with the \(k_{sp}\)-values obtained from the data presented in Figure 2 (Table 4). Therefore, the inhibitory effect of decanol is simply caused by the reversibility of the ping pong mechanism and not by a specific effect on the enzyme.

**Table 4.** Specificity constants of PcL for DCA hydrolysis in emulsions of various solvents. Specificity constants (\(k_{sp} = V_i/K_{m,i:DCA}\)) are given based on molar fractions (\(k_i^{X}\)) or thermodynamic activities (\(k_{sp}\)). They were calculated from the kinetic parameters obtained by fitting Eqn. (8) to the experimental data (for \(a_{\text{DOH}} = 0\) and \(a_{\text{DCA}} < 0.3\)). The individual parameter values from which the \(k_{sp}\)-values were calculated (\(k_{sp} = \beta_1/\beta_3\)) are given in brackets.

<table>
<thead>
<tr>
<th>Organic solvent</th>
<th>(k_i^{X}) (\mu\text{mol/min.mg})</th>
<th>(k_{sp} = (\beta_1/\beta_3)) (\mu\text{mol/min.mg})</th>
</tr>
</thead>
<tbody>
<tr>
<td>methylisobutylketone</td>
<td>(4.1 \times 10^3)</td>
<td>(4.0 \times 10^3) (919/0.23)</td>
</tr>
<tr>
<td>toluene</td>
<td>(1.5 \times 10^3)</td>
<td>(8.0 \times 10^3) (718/0.09)</td>
</tr>
<tr>
<td>dibutylether</td>
<td>(7.2 \times 10^3)</td>
<td>(7.4 \times 10^3) (670/0.09)</td>
</tr>
<tr>
<td>isoctane</td>
<td>(30 \times 10^3)</td>
<td>(14 \times 10^3) (530/0.038)</td>
</tr>
</tbody>
</table>

**Initial rates of DCA hydrolysis in biphasic systems containing DCA, DOH and Isooctane**

Since the experimental data in isooctane are adequately described with Eqn. (8), this solvent was chosen for a further investigation of the kinetics. At \(a_{\text{DCA}}\)-values of 0.9, 0.3, 0.2, and 0.1, three series of rate measurements (\(a_{\text{DCA}} = 0.9\) in duplo) were performed for each value (Figure 4A). Each series of measurements was carried out with a freshly prepared PcL solution. The standard deviation of the measurements within a series appeared to be 2%. The standard deviation of the hydrolysis rate at \(a_{\text{DCA}} = 1\) from the different PcL solutions was 6% (as determined for 9 solutions, \(r_{\text{DCA}} = 612 \pm 35\mu\text{mol/min/mg PcL}\)). Since the variation for the various PcL solutions was higher than the experimental error for the rate measurements, all rate data were normalized on the rates at \(a_{\text{DCA}} = 1\) (i.e. at \(a_{\text{DCA}}=1\), \(r_{\text{DCA}} = 1\)) to compensate for variation in the activity of PcL.

The data at various \(a_{\text{DCA}}\) and \(a_{\text{DOH}}\) values (Figure 4A) were simultaneously fitted with Eqn. (8), the parameter values are given in Table 5. To evaluate the validity of the ping-pong model, the linearity of the curve connecting data points of the reciprocal, relative rate versus \(a_{\text{DOH}}\) was checked (Eqn. 10). The plots show that straight lines can be drawn through the experimental data (Figure 4B). Data at high \(a_{\text{DOH}}\)-values were excluded because the rates were found to be very low and large errors are introduced by taking their reciprocal. The slope of the individual series at \(a_{\text{DCA}} = 0.1\) deviates from the slope obtained with the parameters of the joint fit (Figure 4B), which is probably due to inaccuracies in the low-rate measurements.
However, the linearity of the slopes determined at the other $a_{DCA}$-values appears to be convincing (Figure 4C). The intercept determined from the individual reciprocal curves is in good agreement with the intercept simulated with the parameters obtained from the joint fitting (Figure 4D).

**Figure 4.** Initial rates of DCA hydrolysis by PCL in an isooctane-buffer biphasic system. The normalized rates ($r_n$) are plotted as a function of $a_{DCH}$ for various $a_{DCA}$-values (Panel A). The experimental data were simulated with Eqn. (8) for $a_{DCA}$-values of: 0.1 (circles, – – – – – – ); 0.2 (triangles, – – – – – – ); 0.3 (squares, – – – – – – ); 0.9 (diamonds, – – – – – – ). Open, closed and large symbols indicate duplo measurements. Panel B shows the reciprocal values of the normalized rates ($1/r_n$). The lines were simulated with Eqn. (10), the symbols and line styles are as in panel A. Panel C shows the slopes of the $1/r_n$ versus $a_{DCH}$ curves at constant $a_{DCA}$-values. The slopes (●) were determined from the experimental data presented in panel B, the curve being simulated with Eqn. (11). Panel D shows the intercepts of the $1/r_n$ versus $a_{DCH}$ curves at constant $a_{DCA}$. The intercepts (●) are determined from the experimental data in panel B, the curve being simulated with Eqn. (12). For all simulations in panel B, C, and D, the parameter values from fitting of Eqn. (8) to the experimental data in panel A were used (Table 4).
Figure 5. Initial normalized rates ($r_n$) of PdL for DCA hydrolysis in MIBK-buffer biphasic systems. Panel A shows plots of $r_n$ as a function of $a_{DOH}$ at various $a_{DCA}$ values. The $a_{DCA}$ values applied are: 0.1 (○--○); 0.3 (●--●); and 0.9 (Δ--Δ--Δ). Panel B shows plots of $r_n$ as a function of $a_{DCA}$ at various $a_{DOH}$ values. The $a_{DOH}$ values applied were: 0 (+---+); 0.05 (•--•--•--•); 0.1 (○--○); and 0.2 (●--●--●--●). The data were simulated with Eqn. (8) and the parameters derived from this are presented in Table 4.

Initial rates of DCA hydrolysis in biphasic systems containing DCA, DOH and MIBK

Since MIBK is frequently used for practical purposes, this solvent deserves further attention. It is clear from Figure 2 that Eqn. (8) does not adequately describe the experimental data for the full range of $a_{DCA}$-values. However, in the presence of varying amounts of DOH and $a_{DCA}$ values of 0.1; 0.3; and 0.9, the experimental data could be fitted with Eqn. (8), except the data at $a_{DOH} = 0$ (Figure 5A). On the other hand, it is clear that the fit is rather coincidental (Figure 5B), due to the choice of the $a_{DCA}$-values at which the measurements were taken. This view is confirmed by the large standard deviations, which were found for the parameter estimates (Table 5).

Discussion

After correcting for substrate-solvent interactions by using thermodynamic activities instead of concentrations, the values for the equilibrium ($K_{eq}$) in different media should be identical. Theoretical considerations indicate that enzyme kinetic parameters based on thermodynamic activities are also identical in different solvents, provided that binding of substrate and catalysis are not affected by the medium. Use of thermodynamic activities
has been applied on enzyme kinetics in aqueous and organic monophasic media. Here it is checked whether this approach is able to predict kinetic behavior of lipase in biphasic systems (adsorbed to emulsion droplets) with different organic solvents. It appears that correction for solvent-substrate interaction leads to a substantial decrease of the kinetic differences observed between the biphasic systems with different organic solvents. This follows from a comparison of the curves in Figure 2 and from that of corrected and uncorrected parameter values (Table 4). However, small differences in kinetics remain for the different solvents. Possible causes could be: non-equilibrium between the two phases; incorrect estimates of $\gamma$-values; unrealistic premises made in the theoretical considerations for the correction method (equal binding of the solvents to the active site, substrate completely shielded from the solvent in the substrate-enzyme complex, equal activity coefficients of the enzyme species in the catalytic cycle, no effect of the solvents on the catalytic mechanism of the enzyme). The likeness of these possibilities is discussed below.

Table 5. Parameter values and standard deviations (in brackets), obtained from fitting Eqn. (8) to the experimental rate data in isooctane and methylisobutylketone (MIBK).

<table>
<thead>
<tr>
<th>Lumped parameter</th>
<th>Solvent</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isooctane</td>
<td>MIBK</td>
</tr>
<tr>
<td>$\beta_i (\mu\text{mol/min/mg})$</td>
<td>0.31 (± 0.09)</td>
<td>0.2 (± 0.1)</td>
</tr>
<tr>
<td>$\beta_i (\text{mol/mol})$</td>
<td>0.28 (± 0.09)</td>
<td>0.2 (± 0.1)</td>
</tr>
<tr>
<td>$\beta_i (\text{mol/mol})$</td>
<td>0.03 (± 0.01)</td>
<td>0.02 (±0.01)</td>
</tr>
<tr>
<td>$\beta_i (\text{mol/mol})$</td>
<td>0.7 (± 0.3)</td>
<td>0.7 (± 0.6)</td>
</tr>
</tbody>
</table>

Since increasing the stirrer speed had no effect on the reaction rate, it can be concluded that no diffusion limitation existed and that the phases are in equilibrium during the conversions. To correct for medium-substrate interactions, the $\gamma^\text{N}$-values of the components are determined for the bulk (organic and aqueous) phases. However, DOH it is (partly) located at the surface, as concluded from the decrease of the surface tension upon DOH addition. The $\gamma$-value for DOH at the interface will be different from its values in the bulk phases. However, for the amounts DOH used in the experiments, the surface area of the aqueous-organic interface is relatively small (as estimated from the surface area of decanol). Therefore, the $a_{\text{DOH}}$-values in the system can be calculated adequately by using the bulk $\gamma_{\text{DOH}}$-values.

As shown in Table 1, the $\gamma$-values derived from solubility and partition experiments ($\gamma^{\text{exp}}$) can deviate substantially from those determined with the Unifac method ($\gamma^\text{N}$). For the
values in water, this is not unexpected since the Unifac method is known to give inaccurate values in this medium. Since the variation in concentration of DCA and DOH in the aqueous phase is low, their \( \gamma \)-values will be virtually constant. Therefore, the experimental \( \gamma \)-values in water are derived from solubility and partition experiments.

To determine the \( \gamma \)-values of DCA and DOH in the organic phase (in view of the concentrations applied, these will not be constant), the Unifac method was used. The reliability of these \( \gamma^\prime \)-values can only be estimated from a comparison with their \( \gamma^\text{exp} \)-values (which are based on experimental data). The difference between these values can be a factor 3, as shown for \( \gamma^\prime_{\text{DOH}} \) in isoctane. Similarly, for HCA in isoctane the \( \gamma^\prime_{\text{HCA}} \)-value appeared to deviate from the \( \gamma^\text{exp}_{\text{HCA}} \)-value by a factor 3. In contrast to this, for HCA in MIBK and DOH the \( \gamma^\prime_{\text{HCA}} \)-values agreed well with the \( \gamma^\text{exp}_{\text{HCA}} \)-values (the \( \gamma^\text{exp}_{\text{HCA}} \)-values derived from solubility experiments are similar to those determined from partition experiments, suggesting that they are reliable).

It appears that \( K^\text{obs.} \)-values (measured at equal pH-values) vary with a factor 6, but that the corresponding \( K^\text{th.} \)-values are rather similar (Table 2). The latter implicates that the applied \( \gamma \)-values are reliable. After correction for substrate-solvent interactions the kinetic parameter values also become similar (Table 4), although the variation is larger in this case. In view of the estimated errors, the variation in the kinetic parameters could originate from the error in the \( \gamma \)-values and no other causes.

Experimental data obtained for the biphasic system with isoctane as well as the inhibition of DOH could be well fitted with Eqn. 8. This suggests that the kinetic mechanism adopted is correct and the use of activity coefficients is justified. However, the fit was poor for the other solvents. A maximum for the initial rate of DCA hydrolysis was observed around a \( a_{\text{DCA}} \)-values of 0.5 (Figure 2). Such a maximum has also been observed by others. The maximum is not caused by variation in amount of lipase adsorbed to the interface since PcL in the DCA/PcL system remains fully adsorbed to the interface under the applied conditions. Furthermore, the maximum is not caused by an effect of the surface tension on the activity of PcL, as observed for hydrolysis of triglycerides, since no correlation with the surface tension of the various solvents was observed (Table 6).

Since theoretical considerations indicated that the kinetics would be identical in all solvents, the cause for the maximum must be found in unrealistic premises. It has been reported that organic solvents bind to the active site. If applicable to the DCA/PcL systems, this would mean that inhibition should be taken into account. It has also been suggested that bound substrate may be partially exposed to the bulk solvent, or that solvents affect catalysis by changing the polarity of the active site. Whether these effects also occur in the system studied here, is presently unknown. In order to study this, comparisons are required between kinetic parameter values from which the contribution of substrate-solvent interactions have been removed. As shown here, the method proposed is able to do this for
biphasic systems. In addition to this, predictions can be made for kinetic behavior from a single set of kinetic parameters and $\gamma$-values.

Table 6. Kinetic parameter values of PCL in various organic solvents and physico-chemical properties of these solvents used in the biphasic systems. The kinetic parameters concern the maximal reaction rates relative to the rate at $a_{\text{ref}} = 1$ ($r_{\text{max}}/r_{\text{ref}}$), and the $a_{\text{ref}}$-value ($a_{\text{max}}$) at which the maximal rate is obtained from (Fig 2). Solvent physico-chemical properties at 25 °C are: $\Gamma$, surface tension to air; $\eta$, dynamic viscosity; $\mu$, dipolar moment; $\varepsilon$, dielectric constant; log P, octanol/water partitioning coefficient; $\delta$, Hildebrand's parameter. Definition of the superscripts: $^a$, value at 20°C; $^b$, value at 20°C obtained from linear interpolation between reported values at other temperatures.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>kinetic parameter</th>
<th>Solvent physico-chemical property</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_{\text{max}}/r_{\text{ref}}$</td>
<td>$a_{\text{max}}$ (mol/mol)</td>
</tr>
<tr>
<td>MIBK</td>
<td>1.30</td>
<td>0.55</td>
</tr>
<tr>
<td>Dibutylether</td>
<td>1.19</td>
<td>0.55</td>
</tr>
<tr>
<td>Toluene</td>
<td>1.12</td>
<td>0.46</td>
</tr>
<tr>
<td>Isooctane</td>
<td>0.88</td>
<td>0.5</td>
</tr>
</tbody>
</table>

References


Chapter 5

The catalytic performance of pig pancreas lipase in enantioselective transesterification in organic solvents

J. Bert A. van Tol, Diana E. Kraayveld, Jaap A. Jongejan, and Johannis A. Duine
Abstract

Transesterification of vinyl acetate with racemic glycidol (\(R,S-2,3\)-epoxy-1-propanol) by pig pancreas lipase (PPL) was studied in hexane, diisopropylether, tetrachloromethane, and 2-butaneone. Correction for substrate-solvent interactions was carried out by using thermodynamic activities of the substrates in the equations. Data of initial rate measurements could be fitted with a pingpong bibi model, taking competitive inhibition by glycidol into account. Although plotting of the rates against thermodynamic activities resulted in similar curves for the various solvents, significant variation of the intrinsic parameters still remained. Similarity of the kinetic parameter values increased, however, when competitive inhibition by the solvents was taken into account, suggesting that simple interaction of the solvents with the active site occurs rather than exertion of specific effects on the catalytic properties of the enzyme. It appeared that the enantiomeric ratio, \(E\), and the selectivity factor, \(\alpha\) (the choice between vinyl acetate and (S)-glycidyl acetate), of PPL remained constant during the conversion in all solvents tested. Since \(E\) is a sensitive indicator for changes in the catalytic properties of an enzyme, this also confirms that the solvents have no specific effect on PPL. Seen in the light of reports concluding the opposite, the validity of generalization is discussed.

Introduction

In the past few years, organic solvents have been explored as medium for enzyme-catalyzed conversions.\(^1\) Information has been provided with respect to the stability of the protein,\(^2\) and the effect of water\(^3\) and solvent\(^4\) on the catalytic performance (rate and selectivity). However, no general theory on the catalytic performance of enzymes in organic solvents exists, explaining the deviating behaviour as compared to water.

Recently we showed for lipase-catalyzed hydrolysis of esters in different solvents that many deviations in performance can be ascribed to substrate-solvent interactions, the effects of these hidden in the values of the ordinary kinetic parameters.\(^5\) Theoretical considerations demonstrated that correction for this is possible by using thermodynamic activities \((a)\) in the equations instead of concentrations.\(^5,6,7\) Correction for the substrate-solvent interaction provides the so-called intrinsic affinity parameters \((K_{m}^{\text{int}}, K_{p}^{\text{int}})\), i.e. parameters which should be solvent-independent unless specific effects of solvent on the enzyme are at hand.\(^5,6\)

Although the correction method showed the intrinsic kinetic parameters in the various solvents to become similar, in some of them significant deviations still remained.\(^5,7\(^b,c\) In order to prove or disprove specific solvent effects as a cause, an experimental set up was chosen in which (small) changes in water activity (the water content mostly having a dramatic effect on activity\(^4\)) or an error in the estimation of the activity coefficient \((\gamma)\) could be rejected as
variables responsible for deviations. Transesterification, neither requiring nor producing water, under well defined conditions with respect to water activity seemed the method of choice for this.

It has been reported that also enantioselectivity is affected by organic solvents. Since substrate-solvent interactions are by definition equal for the enantiomers (in a non-chiral solvent), enantioselectivity does not require correction for substrate-solvent interactions, bringing along with it the impossibility of introducing errors due to incorrect γ-estimates. Since discrimination between the enantiomers requires only a few kilocalories per mole, this implicates that subtle changes in an enzyme caused by the solvent can be detected by the ratio of the specificity constants for the enantiomers, the so called enantiomeric ratio (E).

In a number of reports, the effect of the solvent on E has been determined via initial rate measurements. In others the effect was determined from conversion experiments in which the conversion had already taken place for at least 50%. However, as shown by us for R,S-glycidyl (2,3-epoxy-1-propyl) butyrate hydrolysis by pig pancreas lipase (PPL), the E-value determined from a single enantiomeric excess (ee) and degree of conversion (η) measurement, can differ dramatically from the E-value as defined above. The reason for this is the ping-pong mechanism of the hydrolase, providing the opportunity to transfer the butyrate moiety either to water or to the R-glycidol produced in the course of the reaction. The preference of the enzyme exhibited for two nucleophiles is expressed by the selectivity factor (α). Thus, ignorance about this phenomenon (observed as a plateau-effect in plots of ee versus η) could also explain the reported effects of solvent on enantioselectivity. Since values of the selectivity factor (here the choice between vinyl acetate and S-glycidyl acetate, see below) for transesterification in organic solvents are unknown, not only initial rate measurements but also progression studies were performed. To avoid disturbing equilibrium effects in the latter, transesterification was performed with vinyl acetate, the vinyl alcohol produced tautomerizing to acetaldehyde, making the reaction practically irreversible.

Materials and Methods

Chemicals

All chemicals were of the highest purity available, the solvents were of pro analysis grade. Porcine pancreas lipase (PPL, type II) was purchased from Sigma (USA), R,S-glycidol and vinyl acetate from Janssen Chimica (Belgium), and vinyl propionate from Pfaltz and Bauer Inc (USA). 4Å zeolite molecular sieve was from Union Carbide Int. Co., (USA). Before use, it was dried overnight at 400 °C in an oven. R,S-Glycidyl acetate was synthesized by refluxing 1 mol glycidol and 1.5 moles acetic acid chloride with a catalytic amount of pyridine. The glycidyl acetate had a purity (after distilling twice) of 98%, as judged from GC analysis, using a HP-1 column (Hewlett Packard, 1=30m, i.d. =0.53 mm).
GC analyses

The concentration and enantiomeric composition of glycidol and glycidyl acetate were determined with a Hewlett Packard HP-5890 series II GC, equipped with flame ionisation detection, HP Chemstation integration software, and a trifluoroacetyl derivatized γ-cyclodextrine column (γ-TFA, l = 25 m, i.d. = 0.23 mm, Astec Inc., USA). To quantify the compounds, internal standard (2-octanone) was added to the reaction mixture. The temperature trajectory was: initial temperature of 80°C for 8 min, linear temperature increase at a rate of 10°C/min to 95°C, and 95 °C for 6 min (allowing both glycidol and glycidyl acetate to be determined in one run).\(^{14}\)

Thermodynamic activities and activity coefficients

The thermodynamic activity coefficient of a compound A (γ\(_A\)) in a given solvent is defined as γ\(_A\) = \(a_A / X_A\), in which \(a_A\) is the thermodynamic activity and \(X_A\) is the molar fraction of the compound. Since several reference states can be chosen, it should be stressed that the activities here are defined in the sense of Raoults's law, i.e. the activity coefficient of a compound in its pure form is 1. The activity coefficients of vinyl acetate were estimated by using the Unifac method.\(^{15}\) Since no data are available in data bases to calculate the activity coefficients of glycidol and glycidyl acetate, these had to be determined experimentally. When the solvent and racemic glycidol (acetate) are mutually poorly soluble, its activity coefficient can be determined from the solubility via:

\[
γ_{A}^{\text{sol}} = \frac{1}{X_{A}^{\text{sol}}} \quad \text{(1)}
\]

In the case that the glycidol (acetate) is miscible with the organic solvent, its activity coefficient can be estimated from the partition coefficient (\(P\)) over the organic solvent and a second solvent (e.g. water) for which the activity coefficient is known, since:

\[
P_{2/3} \equiv \frac{X_{A}^{s2}}{X_{A}^{s1}} = \frac{γ_{A}^{s1}}{γ_{A}^{s2}} \quad \Leftrightarrow \quad γ_{A}^{s1} = γ_{A}^{s2} \cdot P^{1/3} \quad \text{(2)}
\]

Solubility and partitioning parameters

Since glycidol is poorly soluble in hexane, tetrachloromethane and diisopropylether, and glycidyl acetate is poorly soluble in hexane, their activity coefficients were determined from the solubility in these solvents according to eq 1. Small amounts (1-5 µl) of the solute were added to 4 ml hexane under stirring until saturation was reached, as judged from clouding. The solubilities of glycidol and glycidyl acetate in hexane were 19 µl/4 ml and 179
μl/4 ml, respectively. The solubilities of glycidol in diisopropylether and tetrachloromethane were 97 μl/ml and 35 μl/ml, respectively. The corresponding activity coefficients are given in Table 1. Thermodynamic activities of glycidyl acetate in diisopropylether, tetrachloromethane and 2-butanone, and of glycidol in 2-butanone were determined from partitioning in an aqueous two phase systems, as follows: 4 ml solvent was mixed with 50μl glycidyl acetate or 50 μl glycidol, and 20 μl nonane was added as internal standard. 2 ml of this mixture was mixed with 2 ml 10 mM potassium phosphate buffer, pH 7.8. The concentration of glycidol/glycidyl acetate was measured (with GC) in the organic solvent layer before and after mixing (and settling of the layers) with the buffer.

Table 1. Activity coefficients of the reactants for the concentration ranges used. The values were obtained: 1 from Dechema database for activity coefficients; 15 or experimentally via b: solubility determination (eq 1) and c: partitioning determination (eq 2).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>γ_{glycidol}</th>
<th>γ_{glycidyl acetate}</th>
<th>γ_{vinyl acetate}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>54b</td>
<td>17b</td>
<td>5.8a</td>
</tr>
<tr>
<td>Diisopropylether</td>
<td>5.7h</td>
<td>3.0c</td>
<td>1.9a</td>
</tr>
<tr>
<td>Tetrachloromethane</td>
<td>17e/20b</td>
<td>2.3c</td>
<td>1.9a</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>0.9c</td>
<td>1c</td>
<td>1.1a</td>
</tr>
</tbody>
</table>

Screening of various racemic alcohols

Since hydrolases account for the majority of the reported kinetic resolutions, 16 the screening was focused on hydrolases. A number of racemic alcohols were studied for their suitability in transesterification. The conversions were performed in a mixture of 15 ml dried 2-butanone (see below), 1 ml vinyl acetate and 5 ml 2-butanone containing 500 mg PPL. The conversion rate was estimated from the degree of conversion (ξ) reached in a given time period. ξ was determined from the ee, and ee, value according to ξ=ee,/(ee,+ee,). Since no deviations from the model derived for a minimal kinetic scheme was observed under the applied conditions, the E-values were determined according to Chen and coworkers 9 from multiple samples taken during conversion.

Equilibration of PPL

2-Butanone, used because of its relatively high capacity to dissolve water, was treated prior to use with 4 Å zeolite, resulting in a residual water concentration of 0.04% w/w. Equilibration of the enzyme was performed by suspending 700 mg PPL in 20 ml of the treated 2-butanone. The cooled suspension was sonified for 1 min and subsequently shaken
in a stoppered centrifuge tube for 16 hours on an orbital shaker at 250 rpm. The enzyme suspension was then centrifuged for 15 min at 27,000 g and the supernatant decanted. The water content of the sedimented enzyme preparation was 0.1 % w/w. The sediment was resuspended in 20 ml treated 2-butanolone, and specified volumes of this "PPL-suspension" were administered to the reaction mixtures. For incubation experiments to detect irreversible inhibition, 150 and 250 µl racemic glycidol was added to 1.5 ml "PPL suspension", and the mixture was shaken for 30 min.

The water content of organic solvents and enzyme suspension was determined according to the optimized coulometric Karl-Fischer titration method. To eliminate interference by ketones, Baker Reaquant solvent and titrant (Baker Chemicals) were used. The accuracy of the measurements was 3% and the relative standard deviation is also 3%, as was determined with the dihydrate salt of sodium tartrate as a reference compound.

Initial rate measurements

150 ml of the organic solvent was mixed with vinyl acetate and 50 µl 2-octanone, which served as the internal standard. The mixture was incubated with 25 g of 4Å zeolite for 16 hours under shaking. After removal of the zeolite, 20 ml of this mixture was pipetted in the reaction vial, racemic glycidol was added and the reaction was started by addition of 0.5 ml of the "PPL-suspension". Final concentrations of vinyl acetate and glycidol are indicated in the text. The reactions were performed in magnetically stirred 50 ml vials with air tight caps at 298 K. To follow the reaction, samples of 0.5 ml were withdrawn from the reaction mixture, the PPL was removed by centrifugation, and the R- and S-glycidyl acetate concentration in the supernatant was analyzed with GC. In the case of tetrachloromethane, the sample was filtrated over glass wool prior to analysis. The reaction rate was determined from the slope of the linear line connecting 4 R- and S-glycidyl acetate concentrations determined every 10 minutes.

Conversion experiments

The conversion experiments were performed as indicated for the initial rate measurements, except that they were carried out under a continuous flow of solvent-saturated nitrogen (5 ml/min) to remove the formed acetaldehyde. The reaction was started by the addition of 1 ml PPL suspension (prepared as described above, except that the sedimented enzyme was resuspended in 3 ml solvent). Final concentrations of the compounds are indicated in the text.

Simulations and fitting

Kinetic parameters of initial rate experiments (eqs 4 and 6) were estimated by non-linear regression. Standard deviations of the parameters were estimated from the diagonal
elements of the covariance matrix, and they are given in brackets.

Simulated curves for the data of the conversion experiments were obtained after numerical integration by a fourth order Runge Kutta procedure of the differential equation 8, yielding S- versus R-glycidol concentration data. These were transformed into curves relating ee$_s$ to $\xi$ values. The curves were fitted simultaneously by orthogonal (allowing error in both ee$_s$ and $\xi$ values) non-linear regression to experimental points at various concentrations of substrates (glycidol and vinyl acetate) and product (glycidyl acetate). An absolute standard deviation of 0.01 in both $\xi$ and ee was used, which was estimated by averaging the deviations of all data points to the fitted curves. The errors in the experimental $\xi$ and ee values are correlated since they were both estimated from the same GC measurement. However, this correlation does not exceed 0.5, even for high $\xi$ and ee values. Furthermore, it can be neglected since it has virtually no influence on the parameter estimations. Standard deviations of the parameter ($E$ and $\alpha$) values were also estimated from the diagonal elements of the covariance matrix.

Since the activity coefficients will vary with a changing medium composition, an error is introduced by using constant values in a conversion experiment. However, since the coefficients were determined under conditions similar to those used in the kinetic experiments and a relatively narrow range of concentrations was applied in the experiments, the values (Table 1) can be regarded as constant. Therefore, the kinetic parameters based on molar fractions can be recalculated into parameters based on thermodynamic activities. The parameters derived from eqs 4, 6 and 8 (in which thermodynamic activities are used) are called intrinsic parameters since they are corrected for solvent effects. They are related to the uncorrected parameters based on molar fractions (indicated with the superscript X) according to:

$$K_i^X = \frac{K_i}{\gamma}, \quad V_i^X/K_m^X = \gamma \cdot V_i/K_m, \quad \alpha^X = \frac{\gamma}{\gamma_Q} \cdot \alpha$$  \hspace{1cm} (3)$$

For a non-chiral solvent, the activity coefficients of the enantiomers are equal, so that no correction of $E$ is required. In contrast to this, the selectivity factor $\alpha$ has to be corrected for substrate-solvent interaction since the parameter is related to different substrates with different activity coefficients (eq 3).
Theory

Initial rate kinetics

PPL is a serine hydrolase, a class of enzymes to which a ping-pong BiBi mechanism applies. Therefore, the kinetic resolution of glycidol via transesterification with vinyl acetate can be classified as a pingpong bibi B-Q resolution, as depicted in Scheme 1.

Scheme 1. The reaction scheme of PPL for transesterification of \( R,S \)-glycidol with vinyl acetate according to a ping-pong bibi mechanism and \( R,S \)-glycidol as competitive inhibitor. Abbreviations in the scheme and their symbols in the equations in brackets are: VA: vinyl acetate (A); VOH: vinyl alcohol (P); Gly\(^R\): \( R \)-glycidol (B); Gly\(^S\): \( S \)-glycidol (Q).

Based on theoretical considerations, it has been shown that enzymatic reaction rates are related to the thermodynamic activity of the substrates instead of their concentration (or molar fraction). Thus, the expression for the initial rates of the conversion of the glycidol enantiomers contains the thermodynamic activities of vinyl acetate \( (a_A) \) and \( R \)- and \( S \)-glycidol \( (a_B^R \) and \( a_B^S \), respectively). Under the conditions of the initial rate experiments, product activities are still zero and \( a_B^R = a_B^S \). The rate equations were derived with the King-Altman method using the steady state assumption for all enzyme intermediates. Previous results on transesterification of tributyrine with hexanol indicated inhibition effects of hexanol on PPL. Therefore, rate equations for competitive and uncompetitive inhibition by glycidol were derived. Competitive substrate inhibition of the enzyme by the alcohol is indicated in Scheme 1 and the corresponding initial rate equation is given by:

\[
r_B^R = - \frac{a_A \cdot a_B^R}{V_i^R/(1 + \frac{K_{m,B}^R}{K_{i,m,B}})} + (1 + E) \cdot \frac{a_A}{V_i^R/K_{m,A}} \cdot \frac{a_B^R}{V_i^R/K_{m,B}}
\]

(4)
in which the enantiomeric ratio \((E)\) for such a mechanism is defined as:\textsuperscript{12}

\[
E = \frac{V_i^S / K_{m,B}^S}{V_i^R / K_{m,B}^R}
\]  

(5)

Since the initial rate experiments were performed with the racemic glycidol, an inhibition constant \((K_i)\) is used combining the effects of both enantiomers \((1/K_i = 1/K_i^R + 1/K_i^S)\). The terms \(V_i^R/K_{m,A}^R\) and \(V_i^R/K_{m,B}^R\) are the specificity constants for vinyl acetate and \(R\)-glycidol, respectively. The terms with the microscopic rate constants of the lumped parameters are given in Table 2.

When the alcohol acts as an uncompetitive inhibitor, it may bind to the three enzyme intermediates proposed in Scheme 1, EVAc, EGly\textsuperscript{R}Ac, and EGly\textsuperscript{S}Ac. The initial rate equation derived for this is as follows:

\[
V_i^R = \frac{a_A \cdot a_B^R}{\left(\frac{\kappa_i^R}{k_4^R} + \frac{E \kappa_i^S}{k_4^S} + (1 + E) \cdot \frac{\kappa_i^S}{k_2^S} \cdot a_A \cdot a_B^R + (1 + E) \cdot \frac{a_B^R}{V_i^R/K_{m,A}^R} + \frac{a_A}{V_i^R/K_{m,B}^R}\right)}
\]  

(6)

in which:

\[
\kappa_i^R = \frac{a_{B}^R}{K_{i,EVAc}^R} + \frac{a_{B}^S}{K_{i,EGly^RAc}^S} \land \kappa_i^S = \frac{a_{B}^R}{K_{i,EGly^SAc}^R} + \frac{a_{B}^S}{K_{i,EGly^SAc}^S} \land \kappa_i^R = \frac{1}{K_{i,EVAc}^R} + \frac{1}{K_{i,EGly^RAc}^S} + \frac{1}{K_{i,EGly^SAc}^R}
\]  

(7)

The rate of the \(S\)-enantiomer for both inhibition cases can be obtained by multiplying the numerator of eqs 4 and 6 by \(E\). Therefore, the \(E\)-value can be estimated from a single measurement by determining the ratio of the initial rates on the \(S\) and \(R\)-enantiomer. The total reaction rate for racemic glycidol is obtained by summatng the rates of the two enantiomers.

**Conversion kinetics**

Since the vinyl alcohol formed during the tranesterification reaction tautomerizes to acetaldehyde \((K_{eq} \text{ of acetaldehyde/vinyl alcohol in water is approximately } 10^5)\textsuperscript{22} and the acetaldehyde is removed by continuously refreshing the head space of the reaction vessel with solvent saturated nitrogen) the reaction is regarded to go to completion. Evidence that this also applies to experiments in organic solvents has been reported.\textsuperscript{23} Taking the formation of glycidyl acetate \((Q)\),\textsuperscript{12} the ratio of the conversion rates of the enantiomers (obtained from eq 4 or 6) is given by:
\[
\frac{r^R_B}{r^S_B} = \left( \frac{dc^R_B}{dc^S_B} \right) = \frac{(E.\alpha a^R_A + a^S_Q).a^R_B - a^S_Q.a^S_B}{(\alpha a^R_A + a^S_Q).a^R_B - a^S_Q.a^R_B} \tag{8}
\]

in which \( \alpha \) represents the selectivity factor, expressing the preference of the free enzyme for the forward reaction as compared to the reverse reaction, defined as follows:

\[
\alpha = \frac{V^R_k/K^R_{m.A}}{V^-k/K^R_{m.Q}} \tag{9}
\]

**Table 2.** Definition of the lumped parameters (according to Cornish-Bowden\(^{21}\) and Straathof et al.\(^{12}\)).

<table>
<thead>
<tr>
<th>Lumped parameter</th>
<th>Elementary rate constants</th>
<th>Dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_1 )</td>
<td>( k_2^R k_4^R/(k_2 + k_4) )</td>
<td>( \mu\text{mol/min.g} )</td>
</tr>
<tr>
<td>( V_4 )</td>
<td>( k_3 k_4^R/(k_1 + k_3) )</td>
<td>( \mu\text{mol/min.g} )</td>
</tr>
<tr>
<td>( K_{m.A} )</td>
<td>( (k_1 + k_2)k_4/[k_1(k_2 + k_4)] )</td>
<td>( \text{mol/mol} )</td>
</tr>
<tr>
<td>( K_{m.B} )</td>
<td>( k_2 k_3 + k_4)/[k_3(k_2 + k_4)] )</td>
<td>( \text{mol/mol} )</td>
</tr>
<tr>
<td>( K_i )</td>
<td>( k^R_i/k^S_i + k^S_i/k^S_i )</td>
<td>( \text{mol/mol} )</td>
</tr>
<tr>
<td>( K_{\text{tolv}} )</td>
<td>( k_{i_{\text{tolv}}}/k_{i_{\text{tolv}}} )</td>
<td>( \text{mol/mol} )</td>
</tr>
<tr>
<td>( E )</td>
<td>( k_2^S k_2^S(k_3^R + k_4^R)/[k_3^R k_4^R(k_3^S + k_4^S)] )</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>( k_1 k_2 (k_3^S + k_4^S)/[(k_1 + k_2)(k_3^R k_4^R)] )</td>
<td>-</td>
</tr>
</tbody>
</table>

**Results**

**The experimental design**

For unambiguous interpretation of the resolution curves and accurate determination of \( E \) and \( \alpha \), the transesterification reaction must fulfill a number of requirements. To avoid side reactions (hydrolysis of esters), the reaction must be carried out at low water activity \( (a_w) \). Various hydrolases, such as *Pseudomonas cepacia* lipase,\(^{31}\) show catalytic activity only at \( a_w \geq 0.2 \), and are thus unsuited. PPL is catalytically active at low \( a_w \)-values\(^{38} \) (the specific activity of PPL per surface area is 146, 86 and 53 \( \mu\text{mol/min/m}^2 \) at water activities of 0.01, 0.05, and 0.15, respectively\(^{58} \)). To avoid plateaus (caused by equilibrium phenomena), the reaction must be forced to completion. \( E \) and \( \alpha \) values should be below 20, otherwise the mechanism-inherent intricacies are not detectable. To find a reaction which meets the requirements, transesterification of several racemic alcohols with PPL was investigated. It
appeared (Table 3) that the systems \( R,S-2\)-methyl-1-propanol/vinyl acetate, \( R,S\)-glycidol/proponyl acetate, and \( R,S\)-glycidol/vinyl acetate were suited. In view of our experience with glycidol and glycicyl esters,\(^{11}\) the latter system was chosen.

**Table 3.** Screening of racemic alcohols for their suitability in transesterification. The \( E \)-values for the various alcohol/acyl donor combinations were derived from eight measurements taken at different \( \xi \)-values. No deviation from minimal (Michaelis Menten) kinetics was observed for the concentrations indicated, so \( E \) could be determined according to the equations given by Chen et al.\(^9\) The superscript \( VP \) indicates that vinyl propionate was used as acyl donor. No transesterification was observed in the absence of PPL or in the presence of bovine serum albumin. Since \( \varepsilon \), and thus \( \xi \), cannot be determined accurately at high \( E \)-values, no estimation for the rate of 2-octanol is given.

<table>
<thead>
<tr>
<th>Racemic alcohol</th>
<th>Amount of alcohol (µl)</th>
<th>Amount of vinyl acetate (ml)</th>
<th>Initial rate (µmol/min/g PPL)</th>
<th>( E )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Butanol</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>2-Pentanol</td>
<td>100</td>
<td>1</td>
<td>0.1</td>
<td>7</td>
</tr>
<tr>
<td>2-Hexanol</td>
<td>100</td>
<td>1</td>
<td>2.5</td>
<td>68</td>
</tr>
<tr>
<td>2-Methyl-1-pentanol</td>
<td>50</td>
<td>2</td>
<td>5.2</td>
<td>6</td>
</tr>
<tr>
<td>2-Octanol</td>
<td>100</td>
<td>1</td>
<td>N.D.</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Glycidol</td>
<td>100</td>
<td>1</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Glycidol</td>
<td>100</td>
<td>0.5(^{VP})</td>
<td>3.1</td>
<td>3</td>
</tr>
</tbody>
</table>

**Initial rate measurements**

The reaction rate increased with increasing racemic glycidol concentrations, although at a certain concentration inhibition occurred (Figure 1, panel A). This resulted in a maximum, the height and concentration at which these were attained (Table 4) varying with the solvent used. To determine the contribution of glycidol - solvent interaction to this, the rates were also plotted as a function of the thermodynamic activity of \( R \)- and \( S \)-glycidol in these solvents. As appears from Figure 1, panel B, this leads to profiles being more or less similar. The variation in glycidol concentration at which the maximum is attained decreases from a 22-fold variation (molar fractions) to a 2.5 fold variation (thermodynamic activity) (Table 4). Since the \( E \)-value (as determined from the ratios of \( S \) and \( R \)-glycidol conversion rates in the various solvents, Figure 1) in the range of glycidol concentrations applied did not vary, the high glycidol concentrations did not change the catalytic properties connected with enantioselectivity. It was also confirmed that no irreversible inhibition took place since incubating PPL with high concentrations of glycidol (1 and 2 M) for 30 min did not affect its
catalytic activity. Therefore, it was attempted to see whether the decrease in activity at rising glycidol concentrations could be ascribed to competitive or uncompetitive substrate inhibition by glycidol.

Table 4. Molar fractions ($X_{\text{max}}$) and thermodynamic activities ($a_{\text{max}}$) at which maximal initial rates ($r_{\text{max}}$) were observed in the curves presented in Figure 1 (panel A and B, respectively)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$r_{\text{max}}$ $\mu$mol/min/g PPL</th>
<th>$X_{\text{max}}$ mol/mol</th>
<th>$a_{\text{max}}$ mol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>33</td>
<td>$0.46\times10^{-3}$</td>
<td>0.025</td>
</tr>
<tr>
<td>Diisopropylether</td>
<td>195</td>
<td>$2.5\times10^{-3}$</td>
<td>0.015</td>
</tr>
<tr>
<td>Tetrachloromethane</td>
<td>20</td>
<td>$1.0\times10^{-3}$</td>
<td>0.020</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>120</td>
<td>$10\times10^{-3}$</td>
<td>0.010</td>
</tr>
</tbody>
</table>

On applying simulations to the experimental data according to eqs 4 and 6, it appeared that competitive substrate inhibition (eq 4) gave the best fit (Figure 1, panel A and B) (the fits for uncompetitive inhibition are not shown). The intrinsic kinetic parameters (using thermodynamic activities in the equation) derived from this are presented in Table 5. From these data it appears that: the $E$-values in the different solvents are constant; the $K_{(\text{glycidol})}$-value does not vary with the solvent, except in the case of hexane, where it is rather high; specificity constant ratios for vinyl acetate and $R$-glycidol vary with the solvents in a different way (a factor 3 and a factor 50, respectively).

In view of the large variation in the specificity constant for vinyl acetate, this suggested that the solvents might be competitive inhibitors for the enzyme. Since the solvents are in large excess as compared to the solute, their thermodynamic activity can be regarded as being 1.

Figure 1 (next page). The transesterification rates of $R,S$-glycidol plotted as a function of the molar fraction (panel A) and thermodynamic activity (panel B) of $R$-glycidol for four different solvents. The curves represent best fits of eq 4 (based on molar fractions or thermodynamic activity, respectively) to the experimental data in each solvent. The corresponding parameter values are given in Table 5. The curves simulated according to the model for competitive inhibition of the solvents are shown in panel C. The parameter values (Table 7) were obtained from the fit of eq 10 to the combined data in the four solvents. The open and closed symbols represent the $R$ and $S$-enantiomer, respectively. The thermodynamic activities (molar fraction) of vinylacetate are in: hexane, $\Delta$ 0.17 (0.044), $\bigcirc$ 0.32 (0.083), $\square$ 0.60 (0.15); diisopropylether, $\Delta$ 0.083 (0.044), $\bigcirc$ 0.15 (0.083), $\square$ 0.26 (0.15); tetra, $\Delta$ 0.11 (0.052), $\bigcirc$ 0.17 (0.089), $\square$ 0.31 (0.152); 2-butanone: $\Delta$ 0.04 (0.043), $\bigcirc$ 0.08 (0.083), $\square$ 0.14 (0.14).
Enantioselectivity of PPL in organic solvents

PANEL A
HEXANE

PANEL B
DIISOPROPYLETHER

PANEL C
TETRACHLORO-METHANE

2-BUTANONE

\[ X_{\text{R-Glycidol}} \quad a_{\text{R-Glycidol}} \quad a_{\text{R-Glycidol}} \]
Table 5. Intrinsic kinetic parameters determined from the data presented in Figure 1, panel B with eq 4. The parameters-values are given for the specificity constants for vinyl acetate (V_{i}^{A}/K_{m,A}^{E}) and R-glycidol (V_{i}^{R}/K_{m,B}^{E}), the enantiomeric ratio E (note: V_{i}^{A}/K_{m,A}^{E} = E \cdot V_{i}^{R}/K_{m,B}^{E}), the inhibition constant for glycidol (K_{i}), and a lumped parameter V_{i}^{R}/(1 + K_{m,B}^{E}/K_{m,B}^{S}).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>V_{i}^{A}/K_{m,A}^{E}</th>
<th>V_{i}^{R}/K_{m,B}^{E}</th>
<th>E</th>
<th>K_{i}</th>
<th>V_{i}^{R}/(1 + K_{m,B}^{E}/K_{m,B}^{S})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol/min.g</td>
<td>mol/min.g</td>
<td></td>
<td>mol/mol</td>
<td>mol/min.g</td>
</tr>
<tr>
<td>Hexane</td>
<td>7.4(±0.5)×10^{-3}</td>
<td>3.8(±0.6)×10^{-3}</td>
<td>5.5(±1.0)</td>
<td>8.6(±2.2)×10^{-3}</td>
<td>3.0(±1.6)×10^{-4}</td>
</tr>
<tr>
<td>Diisopropylether</td>
<td>3.7(±0.5)×10^{-3}</td>
<td>1.3(±0.2)×10^{-2}</td>
<td>4.6(±0.2)</td>
<td>1.1(±0.2)×10^{-3}</td>
<td>2.5(±0.7)×10^{-4}</td>
</tr>
<tr>
<td>Tetrachloromethane</td>
<td>1.1(±0.1)×10^{-4}</td>
<td>4.1(±0.3)×10^{-3}</td>
<td>4.6(±0.5)</td>
<td>1.1(±0.3)×10^{-1}</td>
<td>1.1(±1.1)×10^{3}</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>1.5(±0.4)×10^{-3}</td>
<td>1.1(±0.3)×10^{-2}</td>
<td>5.4(±0.8)</td>
<td>1.3(±0.6)×10^{-2}</td>
<td>1.1(±5.8)×10^{-3}</td>
</tr>
</tbody>
</table>

Eq 10 describes the inhibition of the various solvents, as compared to the situation in diisopropylether (showing the highest specificity constants for glycidol and vinyl acetate). In this equation, $K_{i}^{s}$ is the inhibition constant of the solvents relative to that in diisopropylether ($K_{i}^{solv}/K_{i}^{dipe}$). The combined experimental data from the four solvents were fitted with eq 10, as shown in Figure 1, panel C, resulting in the parameters given in Table 6. When comparing the $K_{i}^{solv}/K_{i}^{dipe}$-values derived from this, it is clear that strong inhibition by the solvent prevents a strong mitigating effect of glycidol on the reaction rate (as expected from the mechanism proposed). Thus, taking solvent inhibition into account, it appears that the behaviour of PPL in transesterification is similar in the various solvents used here. However, as shown in Figure 1, panel C, the fit is still not perfect in the case of hexane and trichloromethane.

$$
\begin{align*}
\tau_{i}^{R} & = \frac{a_{A} \cdot a_{B}^{R}}{V_{i}^{R}/(1 + K_{m,B}^{E}/K_{m,B}^{S})} + \frac{a_{A} \cdot a_{B}^{R}}{(1 + E) \cdot (1 + \frac{a_{B}^{R}}{K_{i}^{solv}} + \frac{1}{K_{i}^{dipe}}) \cdot \frac{a_{B}^{R}}{V_{i}^{R}/K_{m,A}^{E}} + \frac{a_{A}^{solv}}{V_{i}^{R}/K_{m,B}^{E}}} \\
& = \frac{a_{A} \cdot a_{B}^{R}}{V_{i}^{R}/(1 + K_{m,B}^{E}/K_{m,B}^{S})}
\end{align*}
$$

(10)

Table 6. Intrinsic inhibition constants of the solvents relative to that in diisopropylether ($K_{i}^{solv}/K_{i}^{dipe}$), determined from the experimental data shown in Figure 1, panel C, with eq 10. It was assumed that in the different solvents only the competitive inhibition varied. The parameters that are considered to be equal in each solvent are: $V_{i}^{A}/K_{m,A}^{E} = 3.0(±0.3)×10^{-3} \text{ mol/min.g}$, $V_{i}^{R}/K_{m,B}^{E} = 1.2(±0.1)×10^{-2} \text{ mol/min.g}$, $E = 4.8(±0.2)$, $K_{i} = 1.0(±0.2)×10^{2} \text{ mol/mol}$, $V_{i}^{R}/(1 + K_{m,B}^{E}/K_{m,B}^{S}) = 3.4(±1.2)×10^{4} \text{ mol/min.g}$. 

Diisopropylether | 1 |
Hexane | 0.017 (±0.0002) |
Tetrachloromethane | 0.015 (±0.0002) |
2-Butanone | 0.9 (±0.1) |
Conversion Experiments

The same solvents were applied in conversion experiments as those used in the initial rate experiments, except hexane since the solubility of glycidol in this is too low to measure a reliable progression curve. At $\xi > 0.98$ the ee$_5$-values tended to decrease, suggesting that the reaction is not completely irreversible, despite the tautomerisation of vinyl alcohol and removal of acetaldehyde. However, since at these high $\xi$-values the determination of ee$_5$ is cumbersome (due to the low glycidyl acetate concentration), these data are not accurate so that they were not included in the fitting routines. Data from 6 conversion experiments in each solvent were fitted with eq 8, and some are presented in Figure 2. As shown in Table 7, the intrinsic $E$ and $\alpha$-values are similar in the solvents (95% confidence intervals). It also appears that the $E$-values determined from the conversion experiments (via eq 8) are similar to those determined by initial rate measurements (via eq 4). Apparently, $\alpha$ is neither affected by solvent inhibition. This can be expected from a competitive inhibition mechanism, lowering the specificity constants of the substrate A and the product Q to the same extent.

![Graphs showing ee$_5$ vs $\xi$ for different solvents](image)

**Figure 2.** Enantiomeric excess values of the remaining glycidol (ee$_5$) and the degree of conversion for the transesterification of R,S-glycidol with vinyl acetate in three different solvents. Molar fractions of respectively R and S-glycidol, R and S-glycidyl acetate, and vinyl acetate in: diisopropyl ether: $\nabla$ 0.00093, 0.0216, 0.0842, ■ 0.00095, 0.011, 0.0886, ○ 0.00195, 0, 0.0878, ▲ 0.00098, 0, 0.1262; tetrachloromethane $\nabla$ 0.0008, 0.127, 0.0839, ■ 0.0008, 0.00644, 0.085○ 0.00164, 0, 0.043+0.00082, 0, 0.1573; 2-butanone $\nabla$ 0.00316, 0.02, 0.078, ■ 0.0032, 0.0102, 0.0793, ○ 0.00335, 0, 0.0658, ▲ 0.0017, 0, 0.066. The curves are simulated using eq 7, the parameter values are given in Table 7, and the molar fractions indicated above.
Table 7. Kinetic parameters derived from progression curves shown in Figure 2 with eq 8. Values for the intrinsic selectivity factor, $\alpha_i$, were derived from the parameter estimates based on molar fractions, according to eq 3 and the $\gamma$-values given in Table 1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$E$</th>
<th>$\alpha^i$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diisopropylether</td>
<td>5.4 (± 0.2)</td>
<td>0.14 (± 0.01)</td>
<td>0.22</td>
</tr>
<tr>
<td>Tetrachloromethane</td>
<td>5.5 (± 0.3)</td>
<td>0.16 (± 0.02)</td>
<td>0.19</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>5.6 (± 0.3)</td>
<td>0.32 (± 0.04)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

**Discussion**

In principle, organic solvents may affect the stability as well as the activity of an enzyme. In the case of PPL, its stability has been proven to be excellent in these media, even at elevated temperatures. Thus, to provide the enzyme with a standardized amount of water, which is a crucial factor for activity, the long equilibration times necessary for this were allowed. The extent of hydration is maintained during the reaction since transesterification is a water-neutral reaction. The use of vinyl acetate combined with removal of the acetaldehyde formed, forced the reaction to go to completion. In view of the reproducible results obtained, the experimental approach chosen here appeared to work well in practice.

Since kinetic parameters derived in the ordinary way contain contributions from substrate-solvent interactions, it was attempted to carry out correction according to the procedure already so successfully applied. Unfortunately, the $\gamma$-values required for this could not be obtained from data bases for the compounds in the reactions studied here. Therefore, they were experimentally determined. As shown in Figure 1, the phenomenon of diverging trends in plots of rates versus concentrations, disappeared in plots of rates versus thermodynamic activities. However, equalities were less pronounced from the comparison of the kinetic parameters, the intrinsic values still showing significant deviations, despite the fact that competitive inhibition by glycidol was taken into account. The inequalities can be explained from a number of reasons: unreliable estimates of the $\gamma$-values; experimental errors and the covariance which exists between the parameters; different catalytically available areas of the suspended enzyme particles in the various solvents, leading to variation in $k_{cat}$; specific effects of certain solvents on the catalytic performance of the enzyme, e.g. by affecting the conformer structure, by binding to free or intermediate enzyme forms or changing the dielectric constant of the active site. The probability of these possibilities as explanation for the deviations is discussed below.

Several reports indicate that binding occurs of organic solvent molecules to enzymes, including their active sites. However, *a priori* this does not mean that fundamental changes
in the mechanism of action are introduced by this. In the simplest way of interference, binding could just occur to the active site of free or intermediate enzyme forms without affecting the overall or active site conformations, leading to competitive or uncompetitive inhibition, respectively. Competitive inhibition by the solvent should be independent of the type of reaction (hydrolysis, (trans)esterification) and of the nature of the substrates. Thus, for all types of reactions the same inhibition constants should be found. It has been found indeed that the solvents used in this study showed similar inhibition effects on the transesterification of tributyrine with hexanol catalyzed by PPL.\textsuperscript{5b} However, the results cannot directly be compared in a quantitative way since in the latter study not all the kinetic parameters were determined. Although the simulation based on the equation for competitive inhibition gave the best fit of the experimental data, this was not satisfactory in all cases (hexane and tetrachloromethane). Accordingly, the results of this approach cannot exclude the possibility that other effects are exerted by organic solvents on enzyme properties in some cases.

Just as for glycidyl ester hydrolysis,\textsuperscript{11} PPL has a preference for the $R$-glycidol enantiomer in transesterification (note that according to the Prelog priority rules, $R$-glycidol has the same configuration as $S$-glycidyl ester). A relatively low $E$-value was observed in the transesterification, similar to the low enantioselectivity reported in transesterification of vinyl propionate and valerate.\textsuperscript{13} In accordance with theoretical considerations,\textsuperscript{12} the $E$-value of esterification of $R,S$-glycidol with butyric acid (about 6, data not shown) is similar to that for hydrolysis of $R,S$-glycidyl butyrate (6.0) carried out in organic solvents.\textsuperscript{25} This value is much lower than for $R,S$-glycidyl butyrate hydrolysis in a two phase system (about 20) but very similar to that of hydrolysis in water (about 9).\textsuperscript{11} This might indicate that the PPL conformer catalyzing transesterification in organic solvents has more similarity to that catalyzing hydrolysis in a monophasic (aqueous) than in a biphasic system. Apparently, the catalytic properties of PPL dissolved in water are maintained in the (commercial) powder preparation and no change occurs when the powder is suspended in organic solvents, as is observed in emulsions by interfacial activation. Whether this situation also applies to other lipases is presently unknown. If so, induction of the interfacial conformation in preparations could be a target to improve the suitability of lipases for application in organic solvents.

In analogy with hydrolysis of $R,S$-glycidyl butyrate under certain conditions,\textsuperscript{11} a plateau value was observed in plots of $e_\alpha$ versus $\xi$ in $R,S$-glycidol transesterification. Since in ester hydrolysis this is due to the choice the enzyme has between glycidol and water, it was investigated whether in transesterification this could be due to the choice the free enzyme has between vinyl acetate and $S$-glycidyl acetate formed during the course of the reaction. From the adequate simulation obtained by fitting the experimental data with the equation taking this into account (eq 8), it is clear that disturbing back reactions also occur in organic solvents whereas the $E$ and intrinsic $\alpha$-values calculated are very similar for the diverse organic solvents. Consequently, since theoretical considerations predict that the parameter $E$ is a very
sensitive indicator for changes in the transition states of kinetic resolutions, the organic solvents studied here do not affect PPL in a specific way. Apparently, the competitive inhibition exerted by certain solvents does not lead to specific changes.

The plateau-effect shows that high enantiomeric purity cannot be achieved in batch conversions with substantial initial concentrations of the racemic substrate, a serious drawback in applications. Since the intrinsic $\alpha$ and $E$ do not vary, to diminish the effect, a possibility left is to use a solvent with a low apparent $\alpha$-value (i.e. the $\gamma$-value of vinyl acetate should be high as compared to that of $S$-glycidyl acetate in that solvent). Otherwise, since the intrinsic $\alpha$-value is strikingly low (0.5, indicating that the preference for glycidyl acetate is two times higher than that for vinyl acetate), a vinyl alcohol ester could be used for which the enzyme has a higher preference than for vinyl acetate.

It has been stated for a number of cases that the enantioselectivity of an enzyme in organic solvent is different from that in water. However, in view of the conclusions reached here for PPL, could this be due to unreliable $E$-determinations or unawareness of the plateau effect? In most enzyme-catalyzed kinetic resolutions reported today, $ee$ and $\xi$ are determined just for one single point in the conversion. These data are used to calculate $E$ via relations derived by Chen and coworkers, which is not allowed in view of the significant aberration introduced when the contribution of $\alpha$ is neglected (as indicated in eq 8). In this connection, it should also be stressed that large errors may occur in $E$ depending on its value and method of measurement (perhaps not surprising, therefore, standard deviations for the $E$ values are scarcely given in publications). Thus, in cases where evidence is abstracted from fluctuation of the $E$-value, one should pay attention to the possibility of more trivial causes for this. At first sight, suspicion seems not justified in cases where the $E$-value is derived from the kinetic parameters determined with initial rate measurements or where the preference of the enzyme switches to that for the opposite enantiomer. However, caution should still be exercised when $E$ is derived from initial rates of the separate enantiomers at one single concentration of the second substrate, since the variations can be due to effects of the solvent on the second substrate, leading to varying kinetic parameter ($K_m$ or $K_r$, based on concentrations) values and consequently to varying $E$-values.

In is concluded that: 1. The enantioselectivity of PPL for the transesterification of glycidol is not affected by the substrates and solvents used here. 2. In cases where the preference of the enzyme switches, the properties of the enzyme as such or the enzyme-substrate complex are modified by the solvents. In view of the pitfalls observed here, the reported cases with a changing $E$-value have to be reevaluated using the appropriate methods before conclusions can be made with respect to effects of organic solvents on the enzymes.
Reference


(b) Dechema Database-PC (1991) DDBST Gmbh, Dortmund, Germany.


Chapter 6

Towards intrinsic enzyme substrate affinities by using thermodynamical activities to correct ordinary kinetic parameters for substrate-water interactions

J. Bert A. van Tol, Rob M. M. Stevens, Jaap A. Jongejan, and Johannis A. Duine
Abstract

On considering binding of water-dissolved substrate to an enzyme as a thermodynamic cycle, a number of elementary steps can be discerned of which the variability in Gibbs energy as a function of the substrate nature can be examined. It is argued that only substrate dehydration and binding of dehydrated substrate to active site are relevant in this. Thus, to reveal the specificity of an enzyme exclusively based on differences in binding energy, corrections have to be made for substrate-water interactions. It appears that the correction can be achieved by replacing substrate concentration with activity or activity coefficient ($\gamma$) times concentration. It is shown how to obtain $\gamma$-values and correct the kinetic parameters of Candida rugosa lipase and pig liver esterase for esters, and of horse liver alcohol dehydrogenase for alcohol and aldehydes. Since the method provides quantitative data, better insight can now be obtained into the factors governing enzyme-substrate binding, i.e. in the accessibility (dimensions) of the substrate binding pocket and its real (solvent independent) preference of an enzyme for a certain type of substrate. The possibility to relate experimental kinetic data, obtained in aqueous media, to binding energies, from molecular modelling studies carried out in vacuum, are discussed.

Introduction

To understand the catalytic performance of an enzyme, ideally what one would like to compare are the equilibrium binding constants of the substrates for the substrate pocket as well as the actual conversion rates. This requires, however, detailed kinetic studies and only in a few cases the rate constants for binding and dissociation of substrate and enzyme are known. Therefore, mostly substrate specificity is related to the kinetic parameters of the substrates, that is by comparing the Michaelis constants ($K_m$, either the genuine or the apparent ones), the specificity constants ($k_{sp} = k_{cat}/K_m$) or, when these parameters are not known, by comparing relative rates determined at fixed concentrations. Since the kinetic parameters are lumped parameters, comprising several other microscopic rate constants, the impression obtained is frequently indicated as "the affinity of the enzyme for the substrate". Despite these shortcomings, the substrate specificity of an enzyme determined in this way is an important feature to know as it may address the physiological role, the dimensions of the substrate pocket, the way in which the substrate is positioned with respect to the catalytic residues in the active site, and the catalytic process as such.

Some enzymes are very restrictive, catalyzing the conversion of one sole compound, some are promiscuous, in accepting a large number of substrates of a homologous series, others are in between. For the latter two cases, the constraints which determine specificity can
be probed by determining the kinetic parameters of the active compounds. However, the character of the compounds may change enormously, e.g. by going from methanol to octanol in probing an alcohol oxidoreductase. Contributions to understanding enzyme mechanisms mainly originate from results of in vitro studies conducted in buffered aqueous solutions. Since mostly dilute reactants are involved, in deriving thermodynamic and kinetic equations, concentrations instead of thermodynamic activities are commonly used. However, is this allowed for comparative purposes if it is realized that hydration energies may differ significantly in a homologous series of substrates? Taking a closer look at the binding process, a number of indicatives relevant to this question can be mentioned: hydrophobic substrates will be expelled from water into the (frequently hydrophobic) binding pocket where if structural water is present in this (seen in several 3-dimensional structures of crystallized enzymes), it may become replaced by substrate; reversibly, a more hydrophilic substrate will experience the opposite effect (of course, similar considerations can be held for the product). Thus, when substrates with different character are to be compared, it is intuitively obvious that the common ways of obtaining substrate specificities will not take these phenomena into account. For such purposes it is necessary to know the "solvent-independent availability" of the substrate for the enzyme, that is the "intrinsic substrate specificity values". The intrinsic parameters are suited for use in molecular modelling studies for which the computations are usually carried out in vacuum.

As will be shown here, intrinsic binding and specificity parameters can be derived from the "ordinary parameters" by correcting for the effect of water on the substrates in the overall process. The correction can be achieved by introducing thermodynamic activities for the substrates in the kinetic equations. The ways to obtain the activity coefficients of the substrates, are indicated. We applied this to: esterase from pig liver (PLE) and lipase from Candida rugosa (CCL), for which our own kinetic data were taken; NAD-dependent alcohol dehydrogenase from horse liver (ADH), for which data on the specificity constants as well as rate constants for binding of alcohols (forward reaction) and for aldehydes (reverse reaction) were available from the literature.¹

Theory

Binding of hydrated substrate (A(w₁)) to the hydrated active site of an enzyme (E(w₁)) can be theoretically split up² in elementary steps in which complete dehydration of substrate and active site take place, in which the dehydrated species form a Michaelis complex (EA) and in which this complex becomes subsequently hydrated (EA(wₑ)) (Scheme 1).
To determine how binding is affected by the nature of the substrate, the variability of each step should be considered. For this, a number of (reasonable) assumptions (see below) can be made: the substrate becomes fully dehydrated and shielded from the bulk water by the enzyme in the EA complex; the extent of dehydration of the active site (w) does not depend on the nature of the substrate; apart from the active site, the hydration of the enzyme species (E and EA) hardly varies. Under these assumptions only $\Delta G_{s,A}$ and $\Delta G_b^g$ (the superscript g refers to the pure gas phase as reference state, *vide infra*), that is the Gibbs energies of dehydration of substrate and binding of dehydrated substrate to the active site, respectively, vary with the substrate. Thus, to compare different substrates, the difference of overall Gibbs binding energies, $\Delta \Delta G_b^{eq}$, can be expressed as:

$$\Delta \Delta G_b^{eq} = -\Delta G_{s,A} + \Delta G_b^g$$  \hspace{1cm} (I)

A number of arguments support the assumptions on invariable steps in Scheme 1. From substrate specificity determinations on chymotrypsin with dioxane as cosolvent, Bell et al.\(^3\) concluded that the enzyme species had similar extents of hydration for varying ratios of cosolvent. Additional support comes from the results with horse liver alcohol dehydrogenase (ADH) presented here (see the Discussion section). Studies on thermolysin inhibited with a number of homologous phosphonamide and phosphonate esters, showed that the extent of hydration of the active site did not vary in the series.\(^2\) Finally, in many 3-dimensional structures of crystallized enzyme-substrate (analogue) complexes, the substrates appear to be well shielded from the bulk water surrounding the enzyme.

The assumption that only $\Delta \Delta G_b^g$ and $\Delta \Delta G_{s,A}$ are relevant in comparing binding of different substrates, means that in order to reveal the intrinsic affinity differences of the enzyme for the substrates, corrections for substrate-water interactions should be made in the overall binding process. An absolute correction is provided by the Gibbs energy of solvation for bringing the substrate from the pure gas phase to the water dissolved state.\(^4\) This Gibbs energy of solvation ($\Delta G_{s,A}$) is related to the thermodynamic activity of the substrate (also with the gas phase as a reference state), as follows (for definition of $a_k^g$ see below):

$$\Delta G_{s,A} = R.T. \ln a_k^g$$  \hspace{1cm} (2)
In the definition of the thermodynamic equilibrium for a reaction, the equilibrium constant is related to the thermodynamic activities of the participating species. Thus, for a substrate-enzyme complex, this constant (indicated here as the intrinsic dissociation constant, \( K_{d}^{\text{int}} \), as it concerns the dehydrated species) can be presented as follows:

\[
K_{d}^{\text{int}} = \frac{a_{E}^{E} \cdot a_{A}^{E}}{a_{EA}^{E}}
\]

(3)

Since \( a_{A}^{E} = \gamma_{A}^{E} \cdot c_{A} \) (\( \gamma_{A}^{E} \) is the activity coefficient and \( c_{A} \) the concentration of A in water) and it was assumed that the activity coefficients for the different enzyme species are equal (\( \gamma_{E}^{E} = \gamma_{EA}^{E} \)), Eqn. (3) can be converted into:

\[
K_{d}^{\text{int}} = \frac{\gamma_{A}^{E} \cdot c_{A} \cdot c_{E}}{c_{EA}} = \gamma_{A}^{E} \cdot K_{d}
\]

(4)

Thus, the experimentally obtained dissociation constant, \( K_{d} \), which is based on concentrations, is related to the intrinsic dissociation constant via \( \gamma_{A}^{E} \). It is also related to the Gibbs energy of binding, \( \Delta G_{b}^{\text{int}} \), of dehydrated substrate to dehydrated active site, regarded here as the "intrinsic" Gibbs energy of binding, \( \Delta G_{b}^{\text{int}} \), as follows:

\[
\Delta G_{b}^{\text{int}} = R \cdot T \cdot \ln K_{d}^{\text{int}} = R \cdot T \cdot \ln \gamma_{A}^{E} \cdot K_{d}
\]

(5)

Based on the transition state theory and assuming that equality of the activity coefficients of the enzyme species also applies to those of the transition states, it can be shown that the rate of binding of substrate A to the enzyme depends on the thermodynamic activity of A.\(^{5,6}\) Thus, the binding (\( r_{1} \)) and dissociation (\( r_{-1} \)) rate of A to and from the enzyme are given by:

\[
r_{1} = k_{1}^{\text{int}} \cdot c_{E} \cdot a_{A} \quad \text{and} \quad r_{-1} = k_{-1}^{\text{int}} \cdot c_{EA}
\]

(6)

in which \( k_{1}^{\text{int}} \) and \( k_{-1}^{\text{int}} \) are the intrinsic binding and dissociation rate constants, respectively. It can be shown in a similar way that the rates of all elementary steps in the catalytic cycle are related to the thermodynamic activity of the substrates and products as indicated in Eqn. (6). Applying this to the minimal enzymatic scheme, the reaction rate and the intrinsic kinetic parameters are related to each other in the following way:

\[
r_{A} = \frac{k_{\text{cat}} \cdot c_{E} \cdot a_{A}}{K_{m}^{\text{int}} + a_{A}}
\]

(7)
In analogy, for more complex reaction mechanisms equations can be derived in a similar way by replacing concentrations in the rate equations by the thermodynamic activities of the reactants. Thus, Eqn. (7) can be rearranged to:

\[
\gamma_A = -\frac{k_{\text{cat}} \cdot c_{E_a} \cdot c_A}{K_m^{\text{int}} \cdot \gamma_A^S + c_A}
\]  

(8)

Since most kinetic experiments are carried out in a limited concentration range, the activity coefficient will approximately remain constant. As appears from Eqn. (8), correction for the hydration effect only affects \( K_m \) and the following relationships apply:

\[
K_m^{\text{int}} = \gamma_A^S \cdot K_m \quad (9A)
\]

\[
k_{\text{sp}}^{\text{int}} = \frac{k_{\text{cat}}}{K_m^{\text{int}}} = \frac{k_{\text{cat}}}{\gamma_A^S \cdot K_m^S} = \gamma_A^S \quad (9B)
\]

It should be noted that the intrinsic parameters obtained are in fact the parameters that would have been found if the enzyme acted in the gas phase, with catalytic properties identical to that in the aqueous phase.

When two substrates are involved in the enzymatic conversion and only apparent kinetic parameter values (\( V_{\text{max}}^{\text{app}} \) and \( K_{m}^{\text{app}} \)) are available, caution should be exercised when converting the apparent parameters into intrinsic ones. Thus, for a mechanism in which a compulsory ordered complex is involved (the generally accepted mechanism for NAD-dependent alcohol dehydrogenase), the effect of the second substrate (NAD\(^+\) or NADH) has to be taken into account, as follows from the equations\(^7\) for the forward reaction (also applicable to the reverse reaction when \( c_{\text{NAD}^+} \) is replaced by \( c_{\text{NADH}} \)):

\[
V_{\text{max}}^{\text{app}} = \frac{c_{\text{NAD}^+}}{K_{m,\text{NAD}^+} + c_{\text{NAD}^+}} \cdot V_{\text{max}} \quad \text{and} \quad K_{m}^{\text{app}} = \frac{K_{d,\text{NAD}^+} c_{\text{NAD}^+}}{K_{m,\text{NAD}^+} + c_{\text{NAD}^+}} \cdot K_m \quad \Rightarrow
\]

\[
k_{\text{sp}}^{\text{app}} = \frac{V_{\text{max}}^{\text{app}}}{K_{m}^{\text{app}}} = \frac{c_{\text{NAD}^+}}{K_{d,\text{NAD}^+} + c_{\text{NAD}^+}} \cdot k_{\text{sp}}
\]

(10)

However, it is also clear from this that \( k_{\text{sp}}^{\text{app}} \) becomes equal to \( k_{\text{sp}} \) when \( c_{\text{NAD}^+} \) values far exceed those of \( K_{d,\text{NAD}^+} \) (\( K_{d,\text{NAD}^+} \) represents the ratio of the dissociation to the association rate constant for enzyme and NAD\(^+\)). The kinetic constants of ADH derived from initial rate measurements, in the example presented here, were determined at a concentration of 2 mM NAD\(^+\) or 0.1 mM NADH. Since \( K_{d,\text{NAD}^+} \) is 0.5 mM and \( K_{d,\text{NADH}} \) is 0.5 \( \mu \text{M} \), according to
Eqn. (10), \( k_{sp} \approx k_{sp}^{app} \).

Ping-pong reactions (the mechanism involved in hydrolysis by lipases and esterases) do not suffer from such complications, as follows from the equations:  

\[
V_{\text{max}}^{\text{app}} = \frac{c_B}{K_{m,B} + c_B} V_{\text{max}}^{\text{app}} \quad \text{and} \quad K_{m}^{\text{app}} = \frac{c_B}{K_{m,B} + c_B} K_m
\]  

(11)

in which B is the second substrate, water. From this it appears that:

\[
\frac{k_{sp}^{\text{app}}}{k_{sp}} = \frac{V_{\text{max}}^{\text{app}}}{K_{m}^{\text{app}}} = \frac{V_{\text{max}}}{K_m} = k_{sp}
\]  

(12)

Thus, the intrinsic specificity constants of the enzymes considered here can be obtained from the apparent specificity constants via Eqn. (9B).

**Activity coefficients**

To calculate the Gibbs hydration energy of the substrates, their pure gas phase was chosen as reference state (molar fraction \( X^g = 1 \), \( P^0 = 1 \) bar). A gas phase is considered to behave ideal at low pressure, since the molecules have negligible interactions with each other under this condition. In this case the fugacity of substrate A equals the pressure of A (\( P_A \)). It is assumed that the substrates studied here behave ideally since their saturated vapour pressures are low. Under these (ideal) conditions, the thermodynamic potential of A in the gas phase at saturated vapour pressure at room temperature (\( \mu_{A,298}^{\text{sat,g}} \)) is related to the chemical potential at reference pressure (\( \mu_{A,298}^{0,g} \)) by:

\[
\mu_{A,298}^{\text{sat,g}} = \mu_{A,298}^{0,g} + R.T. \ln \left( \frac{P_{A,298}}{P^0} \right)
\]  

(13)

When gas and liquid phase are in equilibrium, \( \mu_{A,298}^{\text{sat,g}} \) equals the chemical potential of A in the pure liquid phase (\( \mu_{A,298}^{\text{sat,l}} \)). Since at low pressures the molar volume of liquids is independent of the pressure, the chemical potential of A in the liquid phase is independent of the pressure, as follows:

\[
\mu_{A,298}^{\text{sat,g}} = \mu_{A,298}^{\text{sat,l}} = \mu_{A,298}^{0,l}
\]  

(14)

in which \( \mu_{A,298}^{0,l} \) is the chemical potential of A in the pure liquid phase at reference temperature and pressure. The chemical potential of A in an aqueous solution (\( \mu_{A,2098}^{aq} \)) is related to \( \mu_{A,298}^{0,l} \) by:
\[ \mu_{A,298}^{aq} = \mu_{A,298}^{0,i} + R.T. \ln (a_A^i) \]  \hspace{1cm} (15)

The activity of A in the aqueous solution \((a_A^i)\) can be expressed as the product of the molar fraction of A \((X_A)\) and the activity coefficient \(\gamma_A^{X,i}\) (it should be noted that the standard state in Eqn. (15) is the pure liquid A, and that in the pure liquid, \(\gamma_A^{X,i} = 1\)). On combining Eqns. (13), (14) and (15), Eqn. (16) is obtained:

\[ \mu_{A,298}^{aq} = \mu_{A,298}^{0,g} + R.T. \ln \gamma_A^g c_A \] \hspace{1cm} (16)

in which the activity coefficient in dilute aqueous solutions with the pure gas as reference state \((\gamma_A^g)\) is given by:

\[ \gamma_A^g = \frac{P^A}{P^0} \cdot \frac{\gamma_A^{X,i}}{55.6} \] \hspace{1cm} (17)

Thus, \(\gamma_A^g\) depends on the reference pressure \(P^0\). From Eqns. (4), (9) and (17) it is clear that the "absolute" intrinsic parameters also depend on \(P^0\). This complication is avoided in the comparative studies presented here, since \(P^0\) cancels in the ratios of the kinetic parameters and activity coefficients that are used to indicate the relative variation of the kinetic parameters as a function of substrate variation.

Materials and Methods

Estimation of \(\gamma^g\)

Substrates for which saturated vapour pressure \((P^{sat})\) relations were available\(^8\) are compiled in Table 1 and the calculated values are indicated. Substrates for which no relations were available are compiled in Table 2. In that case, \(P^{sat}\)-values were obtained with the Riedel-Planck-Miller method\(^9\) which requires knowledge about the boiling point \((T_b)\) and critical properties \((T_c\) and \(P_c\)). The critical properties were determined by the Ambrose method\(^10,11\) using the molecular masses and \(T_b\)-values. When the actual \(T_b\)-values are not known they were estimated from \(T_b\)-values of isomeric compounds\(^12,13\).

For alcohols, aldehydes and ethyl acetate, \(\gamma^{X,i}\)-values could be calculated from non-random two-liquids equation (NRTL) parameters obtained from vapour-liquid equilibria\(^14\) and liquid-liquid equilibria\(^15\) experiments. Data for ethanol and acetaldehyde were only available for high temperatures. To obtain the data for 298 K, linear extrapolation of \(\ln \gamma^{X,i}(T)\) versus
1/T was performed. For the other esters, $\gamma^{X,1}$-values were calculated from solubilities found in the literature\textsuperscript{12} (ethyl acetate, ethyl butyrate, and propyl butyrate) or by experimental determination in 10 mM potassium buffer, pH 7.8 (hexanal, $R,S$-ethyl 2-chloropropionate). In the case of hexyl butyrate, a correlation method was used to estimate $\gamma^{X,1}$: extrapolation of log $\gamma^{\text{sat}}$ ($\gamma^{\text{sat}} = 1/$solubility) of homologous esters versus log $\gamma^{\text{calc}}$ ($\gamma^{\text{calc}}$ calculated according to the Unifac procedure based on liquid-liquid parameters\textsuperscript{15}) yielded a value of $1.2 \times 10^4$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$P^{\text{sat}}$ (bar)</th>
<th>$\gamma^{X,1}$</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.078</td>
<td>8.3</td>
<td>0.012</td>
</tr>
<tr>
<td>Propanol</td>
<td>0.027</td>
<td>29</td>
<td>0.014</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.0090</td>
<td>80</td>
<td>0.013</td>
</tr>
<tr>
<td>Hexanol</td>
<td>0.001</td>
<td>960</td>
<td>0.018</td>
</tr>
<tr>
<td>Ethanal</td>
<td>1.21</td>
<td>4.6</td>
<td>0.10</td>
</tr>
<tr>
<td>Propanal</td>
<td>0.42</td>
<td>45</td>
<td>0.34</td>
</tr>
<tr>
<td>Butanal</td>
<td>0.15</td>
<td>70</td>
<td>0.18</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.13</td>
<td>70</td>
<td>0.16</td>
</tr>
<tr>
<td>Ethyl butyrate</td>
<td>0.019</td>
<td>871</td>
<td>0.31</td>
</tr>
<tr>
<td>Propyl butyrate</td>
<td>0.0070</td>
<td>3600</td>
<td>0.45</td>
</tr>
</tbody>
</table>

$\gamma$-values as presented in Tables 1 and 2 were calculated with Eqn. (17), using the $P^{\text{sat}}$ and $\gamma^{X,1}$ (calculated for a composition of $X_A = 0.001$) values compiled there. It should be stressed again that the $\gamma$-values are not absolute (since they depend on $P^0$). As is shown in Table 3 for ethyl acetate, the $\gamma^{X,1}$-value is affected by the concentration (molar fraction) of the substrate in the buffer. Thus, since considerable variation in substrate concentrations occurred in experiments with esterase and lipase (represented in Tables 4 and 5), $\gamma^{X,1}$-values corresponding with the applied concentration were used. Due to the high affinities of ADH for its substrates (e.g. $K_d = 2.7 \times 10^{-4}$ and $5.0 \times 10^{-6}$ for ethanol and ethanolal, respectively), these experiments could be carried out in a concentration range where the value of the activity coefficient remains practically constant.

The accuracy of $P^{\text{sat}}$ and $\gamma^{X,1}$-values achieved depends on which method was used for
their estimation. An indication for this can only be obtained from literature examples in which the measured \( P^a \) and \( \gamma^{X,1} \)-values were compared with the calculated ones. The accuracy of \( P^a \) values determined according to Reid et al.\(^8\) appears to be better than 2 %. For the Ambrose method, having an average error of 4.3 K in \( T_c \) for 400 tested compounds, the accuracy decreases for compounds with increasing molecular weight.\(^8\) It is estimated that the activity coefficients of alcohols and aldehydes obtained from NRTL parameters will be accurate within 10 % of their actual value.\(^14,15\) The temperature correction used assumes that the excess enthalpy and entropy are approximately independent of the temperature in the range of 35 K. The \( \gamma^{X,1} \)-values of the alkyl esters determined from the solubility of the esters, will be accurate within 10 % of the actual value. Since the potassium phosphate buffer concentrations applied in the experiments were low (10 mM for the experiments with hydrolases, 33 mM for those with ADH\(^1\)), no significant deviations originating from salt effects on the calculated vapour pressures and activity coefficients of the substrates are expected to occur. Deviations due to the fact that the experiments were conducted at a pH higher than 7 (the reference pH) can be excluded because the substrates are not ionized.

Table 2. Thermodynamic parameters of substrates for which no direct relations exist to calculate \( P^a \). Estimation of \( P^a \) occurred as indicated in the Materials and Methods section. The superscripts have the following meaning: \(^a\), boiling points from literature\(^11,12\); \(^b\), boiling points estimated from values determined for isomers;\(^11,12\); \(^c\), \( \gamma^{X,1} \) values calculated from experimentally determined solubilities;\(^4\), \( \gamma^{X,1} \) values determined from estimated solubilities (from \( \gamma^{alc} \) correlations).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( T_b ) (K)</th>
<th>( T_c ) (K)</th>
<th>( P_c ) (bar)</th>
<th>( P^{a,s} ) (bar)</th>
<th>( \gamma^{X,1} \times 10^5 )</th>
<th>( \gamma^e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanal</td>
<td>404(^a)</td>
<td>592</td>
<td>35.1</td>
<td>0.0115</td>
<td>0.98(^c)</td>
<td>0.20</td>
</tr>
<tr>
<td>Hexyl butyrate</td>
<td>473(^b)</td>
<td>641</td>
<td>21.3</td>
<td>0.00235</td>
<td>12(^d)</td>
<td>0.51</td>
</tr>
<tr>
<td>R,S-Ethyl 2-chloropropionate</td>
<td>421(^a)</td>
<td>618</td>
<td>33.2</td>
<td>0.0061</td>
<td>2.8(^c)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**Determination of kinetic parameters**

Initial reaction rates of ester hydrolysis by esterase and lipase were obtained by monitoring the acid production by alkali (10 mM NaOH) administration in a pH-stat apparatus (Metrohm: dosimat 665, impulsomat 614, pH-meter 691). The reactions were carried out in a thermostatted vessel (50 ml) at 298 K in 10 mM potassium phosphate buffer, pH 7.8. Concentrations of ester (below the solubility value) and enzyme used are given in the text.\(^9\) Kinetic parameters of the hydrolases for the esters were obtained from a direct fitting of the data for reaction rates at varying thermodynamic activity of the substrates (Eqn. 5). It is assumed, therefore, that no error occurs from variation of the \( \gamma^e \)-value within the
concentration range used in the experiments (Table 3). The standard deviations of $V_{\text{max}}$ and $K_m$, determined from the covariance matrix, are given in brackets.

<table>
<thead>
<tr>
<th>$c_{\text{ethyl acetate}}$ (M)</th>
<th>$X_{\text{ethyl acetate}}$ (mol/mol)</th>
<th>$\gamma^{X_{\text{ethyl acetate}}}_{\text{ethyl acetate}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>0.002</td>
<td>70</td>
</tr>
<tr>
<td>0.22</td>
<td>0.004</td>
<td>68</td>
</tr>
<tr>
<td>0.44</td>
<td>0.008</td>
<td>63</td>
</tr>
<tr>
<td>0.67</td>
<td>0.012</td>
<td>59</td>
</tr>
<tr>
<td>0.89</td>
<td>0.016</td>
<td>56</td>
</tr>
</tbody>
</table>

Table 3. Variation of $\gamma^{X_{\text{ethyl acetate}}}_{\text{ethyl acetate}}$ with increasing ethyl acetate concentrations. $\gamma^{X_{\text{ethyl acetate}}}_{\text{ethyl acetate}}$-Values were calculated from liquid-liquid equilibria NRTL parameters.\(^{15}\)

Materials

Porcine liver esterase (E.C. 3.1.1.1) and Candida rugosa lipase (E.C. 3.1.1.3) were purchased from Sigma Chemical Company. Propyl butyrate and hexyl butyrate were prepared by refluxing propanol and butyric acid in the presence of sulphuric acid as catalyst. The esters had a purity of 99%, as judged from GC. All other compounds were from the highest purity available.

Results

Pig liver esterase (PLE) and Candida rugosa lipase (CCL)

Experiments on enzymatic hydrolysis were carried out with a series of homologous esters which were available to us, which showed good activity, and for which reliable $\gamma^\#$-values could be obtained. In comparing the $k_{sp}$-values of PLE (Table 4), it appears that esters with a large acid or alcohol moiety are better substrates. However, such a trend is not indicated by the $k_{sp}^{\text{ox}}$-values. To reveal the difference between the two parameters, the relative specificity constants were plotted against the relative energy of hydration, as expressed by $\ln \gamma^{\#}_{\text{ethyl acetate}}$ (see Eqn. (2)), with ethyl acetate as a reference compound (Fig. 1). It can be concluded from this that PLE prefers esters with increasing hydration energy but that this preference is virtually absent when correction is applied for the substrate-water interactions. Since the correction concerns the $K_m$-values and the $V_{\text{max}}$-values are more or less similar (Table 4), it follows that the intrinsic affinity of PLE for esters of the size as used here does not vary so much. Apparently, the preference deduced from the $k_{sp}$-values is in fact caused
Table 4. Kinetic parameters for hydrolysis of esters by pig liver esterase. Hydrolysis occurred with PLE (0.2 mg/ml) and ester concentrations in the range indicated (below the solubility concentration). Ordinary kinetic parameters were obtained by fitting the Michaelis Menten equation to the data from rate measurements. $k_{\text{eq}}$-values were calculated from the kinetic parameters obtained by fitting equation 7 to the data, using $\gamma^e$-values to calculate $a^e$-values corresponding with the concentrations applied.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration range applied (mM)</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ ($\mu$mol/min/µl PLE)</th>
<th>$k_{\text{sp}}$ ($\times 10^3$ l/min/µl)</th>
<th>$k_{\text{int}}$ ($\times 10^3$ mol/min/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>0.07 - 830</td>
<td>0.8 (0.4)</td>
<td>1.2 (0.1)</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>Ethyl butyrate</td>
<td>0.1 - 63</td>
<td>0.24 (0.08)</td>
<td>3.1 (0.2)</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td>Propyl butyrate</td>
<td>0.02 - 17</td>
<td>0.35 (0.09)</td>
<td>3.3 (0.2)</td>
<td>9.4</td>
<td>22</td>
</tr>
<tr>
<td>Hexyl butyrate</td>
<td>0.01 - 2.7</td>
<td>0.10 (0.03)</td>
<td>3.2 (0.2)</td>
<td>32</td>
<td>63</td>
</tr>
<tr>
<td>$R,S$-ethyl 2-chloropropionate</td>
<td>0.02 - 20</td>
<td>1.3 (0.3)</td>
<td>3.4 (0.3)</td>
<td>2.6</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Figure 1. Specificity of pig liver esterase for esters with varying hydration energies. To construct the lines, data for $\gamma^e$-values from Tables 1 and 2 and $k_{\text{sp}}$ and $k_{\text{int}}$-values from Table 4 were used. Open symbols represent the uncorrected data, closed symbols the corrected ones. The data for $R,S$-ethyl 2-chloropropionate (indicated with the triangles) are not connected with the line since this compound does not fit in the homologous series.

Figure 2. Specificity of Candida cylindracea lipase for esters with varying hydration energies. To construct the lines, $\gamma^e$-values were used from Tables 1 and 2, and $k_{\text{sp}}$ and $k_{\text{int}}$-values from Table 5. The meaning of the symbols is indicated in the legend of Fig. 1.
by the expelling effect of water, as compared to the gas phase, on the large esters, pushing them into the active site of the enzyme.

To probe the effect of correction on the behaviour of CCL, the same substrates were used as for PLE. Table 5 shows significant differences for the specificity constants and far less for the intrinsic ones. As appears from Fig. 2, similar trends as in the case of PLE exist.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration range (mM)</th>
<th>( k_u ) ( \times 10^4 ) l/min.mg</th>
<th>( k_{u *} ) ( \times 10^2 ) mol/min.mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>4.0 - 830</td>
<td>0.13</td>
<td>1.1</td>
</tr>
<tr>
<td>Ethyl butyrate</td>
<td>3.6 - 61</td>
<td>0.048</td>
<td>0.15</td>
</tr>
<tr>
<td>Propyl butyrate</td>
<td>0.7 - 17</td>
<td>1.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Hexyl butyrate</td>
<td>0.02 - 2.7</td>
<td>5.0</td>
<td>9.9</td>
</tr>
<tr>
<td>R,S-ethyl 2-chloro-propionate</td>
<td>0.7 - 20</td>
<td>1.5</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Apparently, the size of the esters used here was not large enough to probe the dimensions of the active sites. On the other hand, PLE has been examined with racemic esters\(^{17,18}\) for that purpose. From the enantioselectivity observed, the active site map was derived. Since enantiomers are hydrated to the same extent, the enantioselectivity does not require correction, implicating that the reported model may represent the actual geometry of the active site.

**Horse liver alcohol dehydrogenase (ADH)**

Ethanol is a good substrate for many NAD-dependent alcohol dehydrogenases. However, with a few exceptions the enzymes have broad specificities, acting on primary, secondary and cyclic alcohols (steroids) as well as aldehydes and ketones. To answer the question which compound is the normal, physiological substrate, usually a variety of substrates are chosen for a survey and the kinetics studied. From this comparative work it has been deduced that an inverse relationship exists between \( K_m \) and the chain length of aliphatic alcohols and aldehydes or the size of the ring of cyclic alcohols and ketones. Since linear relationships have been obtained when the free energy of binding (derived from \( K_m \)-values) is plotted against the number of carbon atoms in linear alcohols\(^{19}\) or the ring size of cyclic alcohols\(^{20}\) or by plotting the logarithm of \( V_{max}/K_m \) against the hydrophobicity (log \( P \)-value\(^{21} \)) or the number of \( CH_2 \)-units\(^{22} \), it is generally accepted that hydrophobic interactions play an
important role in substrate binding to these enzymes. For the trends observed, however, how much is due to the expelling effect of the aqueous solvent on the hydrophobic substrate and how much to the intrinsic affinity of the pocket?

Figure 3. Specificity of horse liver alcohol dehydrogenase for alcohols and aldehydes of varying size. The values of the kinetic parameters used were derived from steady state kinetics by Sekhar and Pllap. Panel A presents the uncorrected and panel B the corrected data for alcohols (□) and aldehydes (●). γ" values can be found in Tables 1 and 2.

Table 6. Specificity and dissociation constants of horse liver alcohol dehydrogenase for alcohols and aldehydes. The specificity constants used were derived from initial rate kinetics and the dissociation constants (K_{4,3} and K_{4,6}) from stopped-flow kinetics. Dissociation constants used were those derived for the ordered BiBi mechanism with isomerization of the enzyme-NAD" complex (see Table IV of reference 1).

<table>
<thead>
<tr>
<th>Substrate couple</th>
<th>k_{sp} (*10^6 l/mol.s)</th>
<th>k_{sp}^{int} (*10^6 mol/mol.s)</th>
<th>K_{4,3}/K_{4,6} (*10^3 M^{-1})</th>
<th>K_{4,3}^{int}/K_{4,6}^{int} (*10^3 mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol/Ethanol</td>
<td>0.044 /0.19</td>
<td>3.7 /1.9</td>
<td>2.3 /0.70</td>
<td>2.8 /2.8</td>
</tr>
<tr>
<td>Propanol/Propanal</td>
<td>0.24 /0.85</td>
<td>17 /2.5</td>
<td>0.34 /0.030</td>
<td>0.48 /0.75</td>
</tr>
<tr>
<td>Butanol/Butanal</td>
<td>0.61 /9.7</td>
<td>47 /54</td>
<td>0.087 /0.006</td>
<td>0.11 /0.11</td>
</tr>
<tr>
<td>Hexanol/Hexanal</td>
<td>2.4 /14</td>
<td>130 /70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When comparing the relative k_{sp}'s (Table 6. Fig. 3A), it is clear that aldehydes are better substrates than alcohols (of the same size) and an increase in the chain length gives a better substrate, especially in the case of aldehydes. However, when comparing k_{sp}^{int}'s (Table
6, Fig. 3B), it appears that the effect of the chain length is mitigated and that the difference between an alcohol and its corresponding aldehyde nearly vanishes. Thus, the forward and the reverse reaction are in fact equally effective for a given substrate/product couple. Since \( k_{\text{cat}} \)-values for alcohols and aldehydes are comparable,\(^1\) it appears that ADH has similar "intrinsic affinities" for an alcohol/aldehyde couple. Finally, since it appears that the better performance of the more hydrophobic substrates is largely due to the expelling effect of water on these compounds, the contribution of hydrophobic amino acid residues in substrate binding may be less than previously thought.

According to the theory developed here, the effect of water on the kinetic parameters concerns the interaction with the substrate, provided that the assumptions made for the other steps in binding are correct. The validity of these assumptions can be verified by comparing the trend in \( k_{\text{int}} \)-values with that of \( K_d \)-values since deviations would indicate additional effects of water in the catalytic cycle. Therefore, it was interesting to compare \( K_d \) with \( K_{\text{int}} \) values. Kinetic studies on the steps in the catalytic cycle of ADH performed by Sekhar and Plapp\(^1\) gave data which could be fitted with two different models, yielding different kinetic parameter values. However, the relative \( K_d \)-values derived for the two models appeared to be similar. The values chosen here concern the parameters in the ordered BiBi mechanism with isomerisation of the enzyme-NAD\(^+\) complex, because their accuracy is higher than those for the other mechanism. To indicate which \( k \)-values should be considered in the forward as well as in the reverse reaction, part of the catalytic cycle is presented (according to Sekhar and Plapp\(^1\)) (Scheme 2):

\[
\begin{align*}
E-\text{NAD}^+ & \rightleftharpoons E-\text{NAD}^+-\text{RCH}_2\text{OH} & \ldots \rightleftharpoons & E-\text{NADH-RCOH} & = & E-\text{NADH} & \quad \text{Scheme 2} \\
k_3 \cdot a_{\text{RCH}_2\text{OH}} & \quad k_6 \\
k_3 & \quad k_6 \cdot a_{\text{RCOH}}
\end{align*}
\]

From Table 6 and Fig. 4A, it appears that \( K_d \)-values (\( k_3/k_3 \) and \( k_6/k_6 \) ratios for alcohols and aldehydes, respectively) decrease with increasing chain length and that the values for aldehydes are much lower than for the corresponding alcohols. However, the comparison of \( K_{\text{int}} \)-values reveals (Table 6 and Fig. 4B) that intrinsic binding values of an alcohol/aldehyde couple are very similar and the same trend exists as in the comparison of the \( k_{\text{int}} \)-s. Since \( k_{\text{sp}} \)-s were derived from steady state kinetics and \( K_d \)-s from pre-steady state kinetics, the similar trends strongly support the view that activity coefficients of the enzyme conformers are similar, implicating that the assumptions made for the here presented method are valid for this enzyme, and probably also for others.
Figure 4. Relative dissociation constants of horse liver alcohol dehydrogenase for alcohols and aldehydes of varying size. The data used were obtained with stopped flow kinetics by Sekhar and Plapp.\textsuperscript{1} Panel A presents the uncorrected and panel B the corrected data for alcohols (□) and aldehydes (●).

Discussion

In evaluating the reliability of the correction method, two pertinent questions have to be answered: are the assumptions made in the theoretical framework justified and do the equations in which \( \gamma \) is introduced, really provide intrinsic kinetic parameters and the binding energy of the substrate to active site (Eqn. (5))? With respect to the first question, the examples from the literature (already mentioned) and the consistent trends observed here for the intrinsic parameters of ADH, support the view that the \( \gamma \)-values of the enzyme species are similar. With respect to the second question, the binding energies calculated according to Eqn. (5) (Table 7), using the \( \gamma \)-values shown in Tables 1 and 2, are in the range of those determined for n-alkylamines and trypsin\textsuperscript{23} and the transfer of hydrocarbons from a non-polar solvent to water\textsuperscript{24}. However, before concluding that the method provides the genuine intrinsic binding energies, a more close inspection is required.

The binding energies calculated consist of enthalpic and entropic contributions, and if the correction method is inadequate, of substrate hydration. Entropic contributions can be neglected if entropy loss upon binding does not vary between the different substrates, a premise which will usually not be met.\textsuperscript{2,25,26} To examine this, the contribution of a CH\textsubscript{2}-unit in the ADH example will be considered. Assuming that fixation of alcohol and corresponding aldehyde take place in a similar way in the binding pocket of ADH, the differences observed
for the couples will be from enthalpic origin (however, since in the ternary complexes NAD$^+$ or NADH participate, respectively, this may be an oversimplification). From Table 7, it appears that the $\Delta G_b^{\text{int}}$-values for corresponding alcohol/aldehyde couples are very similar. This suggests that the contribution of a CH$_2$-group to substrate binding is the same for the alcohols as well as for the aldehydes. In deriving the contribution of a CH$_2$-group in binding in the series of alcohols and aldehydes, $\Delta AG$ and $\Delta AG^{\text{int}}$-values should be taken. Surprisingly (Table 7), all these values are similar (about 4.2 kJ/mol), suggesting that the CH$_2$-group in the substrate does not interact with water, which is in contradiction with the fact that the $\Delta G$ and $\Delta G^{\text{int}}$-values vary strongly. Therefore, the suggestion derived from the similarity cannot be correct. Perhaps it originates from cancelling of the hydration energy against the entropic contribution.

**Table 7.** Contribution of the substrate CH$_2$ unit in binding to ADH. Binding energies were calculated from the dissociation constants given in Table 6, according to Eqn. (5).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_d$ (mol/l)</th>
<th>$\Delta G_b$ (kJ/mol)</th>
<th>$\Delta G_b^{\text{int}}$ (kJ/mol CH$_2$)</th>
<th>$K_{d}^{\text{int}}$ (mol/mol)</th>
<th>$\Delta G_b^{\text{int}}$ (kJ/mol)</th>
<th>$\Delta G_b^{\text{int}}$ (kJ/mol CH$_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>2.3*10$^{-3}$</td>
<td>-15.1</td>
<td></td>
<td>2.8*10$^{-5}$</td>
<td>-26.0</td>
<td></td>
</tr>
<tr>
<td>Propanol</td>
<td>3.4*10$^{-4}$</td>
<td>-19.7</td>
<td></td>
<td>4.8*10$^{-6}$</td>
<td>-31.7</td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>8.7*10$^{-5}$</td>
<td>-23.1</td>
<td></td>
<td>1.1*10$^{-6}$</td>
<td>-34.4</td>
<td></td>
</tr>
<tr>
<td>Ethanal</td>
<td>7.0*10$^{-4}$</td>
<td>-18.0</td>
<td></td>
<td>2.8*10$^{-5}$</td>
<td>-26.0</td>
<td></td>
</tr>
<tr>
<td>Propanal</td>
<td>3.0*10$^{-5}$</td>
<td>-26.0</td>
<td></td>
<td>7.5*10$^{-6}$</td>
<td>-29.4</td>
<td></td>
</tr>
<tr>
<td>Butanal</td>
<td>6.1*10$^{-6}$</td>
<td>-30.2</td>
<td></td>
<td>1.1*10$^{-6}$</td>
<td>-34.4</td>
<td></td>
</tr>
</tbody>
</table>

To examine the role of entropic contributions further, the equation for the difference in binding enthalpies between substrates A1 and A2 is presented (derived from $\Delta G = \Delta H - T \cdot \Delta S$ and Eqn. (5)):

$$\Delta \Delta H_b^{\text{int}} = T \cdot \Delta \Delta S_b^{\text{int}} + R \cdot T \cdot \ln \frac{\gamma_{A2}^{\text{g}} \cdot K_{d,A2}^{\text{int}}}{\gamma_{A1}^{\text{g}} \cdot K_{d,A1}^{\text{int}}}$$  \hspace{1cm} (18)

in which $\Delta \Delta H_b^{\text{int}}$ and $\Delta \Delta S_b^{\text{int}}$ are the enthalpy and entropy differences, respectively, for binding of two substrates. In case one wishes to take the entropy changes between different substrates into account, corrections of the kinetic parameters in Eqns. (4) and (9) should be performed with a coefficient $\gamma'$ which equals $\gamma_{A}^{\text{g}} \cdot \exp(\frac{-\Delta S}{R})$. Although the differences in entropy changes ($\Delta \Delta S = \Delta S_{A2} - \Delta S_{EA2} - \Delta S_{A1} + \Delta S_{EA1}$) can in principle be obtained from $K_d^{\text{int}}$-values at varying
temperature, these data are not available for ADH. Another approach has been developed by Williams et al. for making an estimate for the entropic contribution in bimolecular association of antibiotics. However, the structural information required for this, is lacking for our cases. Therefore, to estimate the importance of entropic contributions, the following cases were considered:

1. \[ \Delta S_{A1} - \Delta S_{EA1} = \Delta S_{A2} - \Delta S_{EA2}, \text{ i.e. the entropy change upon binding is equal for the different substrates. With this assumption, the calculated data represent the binding enthalpies.} \]

2. \[ \Delta S_{EA1} = \Delta S_{EA2}, \text{ i.e. the entropy of the various enzyme-substrate complexes is equal (this includes the special case} \Delta S_{E} = \Delta S_{EA}, \text{ in which binding of the substrate does not influence the entropy of the enzyme).} \]

3. \[ \Delta S_{EA1} \neq \Delta S_{EA2}, \text{ i.e. the entropy of the different enzyme-substrate complexes varies.} \]

Although case 3 is probably the correct option, the entropic contribution cannot be calculated with the thermodynamic methods presented here. Therefore, the limiting values of the entropy changes, represented by options 1 and 2, should be considered. If it is assumed that the entropy of the enzyme does not change upon substrate binding (\( \Delta S_{E} = \Delta S_{EA} \), a realistic assumption since in binding the structural water in the active site is replaced by substrate), the entropic contribution only concerns that of the absolute entropy of the substrate in the gas phase. This can be calculated with the method of Benson and the results are presented in Table 8. From this it appears that the contribution of the \( CH_2 \) group in the series of alcohols and aldehydes is equal, and the value is similar to that obtained for the affinity of Ile-tRNA synthetase for the 3-methyl group of L-isoleucine (15 kJ/mol). Thus to describe the active site of ADH with respect to affinity, the enthalpy differences given in Table 8 seem a reliable estimate of their maximal values.

<table>
<thead>
<tr>
<th>Substrate couple</th>
<th>( \Delta G^e_{p} ) (kJ/mol)</th>
<th>( T \Delta S^e_{p,abs} ) (kJ/mol)</th>
<th>( \Delta H^e_{p} ) (kJ/mol)</th>
<th>( \Delta \Delta H^e_{p} ) (kJ/mol CH(_2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol/Ethanol</td>
<td>-26 / -26</td>
<td>-84 / -79</td>
<td>-110 / -105</td>
<td>16 / -15</td>
</tr>
<tr>
<td>Propanol/Propanol</td>
<td>-31 / -29</td>
<td>-96 / -91</td>
<td>-126 / -120</td>
<td>-16 / -15</td>
</tr>
<tr>
<td>Butanol/Butanol</td>
<td>-34 / -34</td>
<td>-108 / -102</td>
<td>-142 / -137</td>
<td>-16 / -17</td>
</tr>
</tbody>
</table>

X-ray diffraction studies of a crystallized enzyme provide insight into the geometry of the binding pocket and the residues surrounding the substrate. Energies of binding for a series of homologous substrates can, therefore, also be obtained with the free energy
perturbation method. However, the outcome of the computations is strongly affected by the choice of the parameter set. Furthermore, the perturbation method appears applicable only to the cases where high resolution X-ray structures are available and where the substrates/ligands have a high degree of homology. Kinetical studies with a series of substrates provide data reflecting the ability of the binding pocket to accommodate, bind and convert the substrate under actual operation conditions. Since the correction method can transform these data into reliable, solvent-independent intrinsic parameters, a second approach is now available. It will be interesting, therefore, to test the perturbation method directly with the here presented method, which is essentially based on experimentally determined parameters.

In taking the pure gas phase of the substrates as a reference state, the correction applied to the kinetic parameters compensates for all interactions of substrate with water (by introducing other reference states (and thus other types of $\gamma$) such as the compound dissolved in hexane, correction for a specific interaction like van der Waal’s interaction or hydrogen bonding$^{33}$ can be obtained). Use of the partition coefficient of a compound in water/octanol ($\log P$ value),$^{34}$ is not recommended to reveal the contributions of hydrogen bonding to the total binding energy, since the compound will form hydrogen bonds in both phases of the reference system, resulting in only partial corrections.$^{35}$

The series of substrates used here concern uncharged molecules of esters, alcohols and aldehydes. Activity coefficients for charged substrates can also be obtained, i.e. $\gamma^{	ext{K.L.}}$-values.$^{36}$ Unfortunately, since $P^\text{w}^{36}$ values of these compounds are not available, $\gamma^\text{K.L.}$-values cannot be derived so that the correction is only partial. Accepting this limitation, it means that the scope of application of the method presented here is very large. The enzyme-substrate combinations studied here did not reveal the dimensions of the active sites, as judged from the fact that the largest substrate applied still showed reasonable activity. Thus, the results address the aspect of "affinity", not of "accessibility". The trends revealed show that the preference for hydrophobic substrates, as expressed by the intrinsic parameters, is much less than that derived from ordinary parameters as compared with that for the (hydrophilic) reference substrate. Intuitively, this can be explained from the expelling effect of water on these compounds, pushing them into the active site of the enzyme. A similar explanation has already been given for a reversed case, the inability of sugar converting enzymes to release their product when acting in an organic solvent.$^{37}$

It could be reasoned that ordinary kinetic parameters give a better insight into the physiological role of an enzyme than the intrinsic ones since cellular reactions proceed in water so that the effect of water on the substrate forms part of the overall behaviour of the enzyme. However, it should be realized that the prevailing conditions at the site where the enzyme is located in vivo, can be completely different from those in the test tube so that the "solvent effect" on the kinetics and thus the indications for the physiological role will also be
different. Therefore, as soon as reliable estimates can be made of the activity coefficient in
the cytosol, membrane, etc., intrinsic kinetic parameters will be indispensable for estimating
real in vivo behaviour. Similarly, to enable comparison of the action of an enzyme in water
and in organic solvents, intrinsic parameters should be derived since the large differences
between the effect of water and organic solvents on the substrate could be responsible for the
different performances reported in the literature. This has indeed been found by us (results
to be published elsewhere) in a number of cases, the outcome having strong implications for
the presently accepted view that organic solvents modify the properties of an enzyme.

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References

1 Sekhar, V. C. and Plapp, B. V (1990) Rate constants for a mechanism including intermediates in the
interconversion of tertiary complexes by horse liver alcohol dehydrogenase, Biochemistry 29, pp
4289-4295.
bonding group in an enzyme inhibitor, Science, 235, pp 569-571.
5 Tol, J. B. A. van, Jongejan, J. A., Duine, J. A. (1992) Relation of enzymatic reaction rate and
hydrophobicity of the solvent, in Biocatalysis in non-conventional media (J. Tramper, M.H.
biphasic aqueous - organic systems, in Biocatalysis in non-conventional media (J. Tramper, M.H.
London.
McGraw-Hill Inc, N.Y.
Fundam., 2, pp 78-79.
11 Ambrose, D. (1979) Correlation and estimation of vapour-liquid properties. II. Critical pressures and
volumes of organic compounds, National Physical Laboratory, Teddington, NPL Rep. Chem., 98.
methods of purification (Weissberger, A., ed.) Vol 2, Wiley, J. and Sons, N.Y.


Summary

Since enzymes can catalyze reactions with a high selectivity and under mild conditions, they are applied in the production of fine chemicals, such as pharmaceuticals and food additives. The rate and the selectivity of the reactions depend on the characteristics of the enzyme and the medium in which the reaction is carried out. In this thesis the effects of the kinetic mechanism and the medium on the rate and selectivity of enzyme catalyzed reactions are described. Since lipases have large potentials for production of fine-chemicals, the experiments were performed with this group of enzymes.

To study the effect of the medium on the enzyme, the contribution of substrate-medium interactions should be taken into account. Correction for substrate-solvent interactions in the overall kinetics can be carried out by substituting thermodynamic activities for substrate concentrations in the ordinary rate equations. Theoretical considerations indicate that the kinetics in different media are identical, provided that a number of premises are valid.

Experiments to verify these premises were carried out with lipase-catalyzed hydrolysis and ester formation in monophasic as well as biphasic systems. It appeared that the often reported, sigmoid curves obtained when plotting enzyme activity against the hydrophobicity (log P values) of the solvent, are due to the varying expelling/attracting effect of the solvent on the substrate. Furthermore, the differences in the values of the kinetic parameters became smaller. Although errors in the thermodynamic activity values and rate measurements can contribute, specific medium effects on the enzymes are the main cause for these differences remaining after correction for substrate-medium interactions. First attempts to explain the differences by assuming solvent binding to the active site, extending the rate equations with competitive inhibition by the solvents, leads to further similarity of the kinetic parameters.

The use of thermodynamic activities of substrates in the kinetic equations is also required to determine the contribution of substrate-solvent interaction to enzyme specificity. Quite different patterns were obtained for the corrected and ordinary kinetic parameters in plots of a series of homologous substrates. The method to correct for substrate-solvent interactions could be used to combine computations for substrate-enzyme binding, carried out in vacuum, with experimental kinetic data, obtained from experiments in aqueous environments.

The commonly used equation to describe enantioselective conversions, derived for Michaelis Menten kinetics, is often not correct for reactions with multiple substrates or products. For lipase catalyzed resolutions a plateau occurs in plots of the optical purity of the remaining substrate versus the degree of conversion. This plateau is, as a consequence of the ping-pong mechanism of lipase, caused by the occurrence of a reverse reaction with the
product enantiomer. An extended equation, taking the latter into account, is able to predict the kinetic resolutions with lipase in monophasic as well as biphasic systems for hydrolysis and transesterification. In view of common practice to make predictions from one analysis during the conversion and using the wrong equation, many statements in the literature about kinetic resolutions, especially those made for the effect caused by the media or by modifying agents, seem not tenable.

Based on thermodynamic activities of the substrates and the correct kinetic model, it appeared that the substrate specificity and enantioselectivity (as judged from the intrinsic selectivity factor and enantiomeric ratio, respectively) of porcine pancreas lipase (PPL) remained constant in different organic solvents. This suggests that the remaining dissimilarities in rate, observed after correction for substrate-solvent interactions, are due to enzyme modifications affecting conversion rate of the substrates to the same extent. However, since examples exist in the literature in which solvents cause reversal in e.g. enantiomer preference, it appears that organic solvents can affect certain enzyme/substrate combinations and that the situation for PPL is not general. Thus, further studies on the kinetics in various media are required to obtain insight in the effects of the solvent on rate and (enantio)selectivity. From the work presented here, it will be clear that proper kinetic mechanisms and correction for substrate-solvent interactions should be applied to reveal these effects, and to derive reliable models that predict enzyme behaviour under preparative conditions.
Samenvatting

Enzymen zijn in staat om onder milde omstandigheden en met een hoge selectiviteit reacties te katalyseren. Deze eigenschappen zijn noodzakelijk voor de produktie van een aantal fijnchemiciën, zoals geneesmiddelen en levensmiddel-additieven. In toenemende mate wordt dan ook onderzoek gedaan naar de toepassing van enzymen in de produktie van deze fijnchemiciën. Het blijkt dat de snelheid en (inent)selectiviteit van enzymreacties afhangen van het type enzym en van het medium waarin de reacties plaatsvinden. In dit proefschrift worden de invloeden van het medium en het kinetische mechanisme op de snelheid en (inent)selectiviteit van enzymreacties beschreven op basis van thermodynamische en kinetische modellen. Omdat lipasen toegepast kunnen worden voor de produktie van fijnchemiciën zijn de experimenten uitgevoerd met deze groep enzymen.

Om de katalytische activiteit van enzymen in verschillende media te kunnen vergelijken, moet gecorrigeerd worden voor de invloed van het medium op de substraten. Deze correctie kan uitgevoerd worden door in plaats van concentraties de thermodynamische activiteit van de substraten te gebruiken. Uit theoretische beschouwingen blijkt dat de katalytische activiteit van enzymen in verschillende media gelijk is indien: (1) de media gelijke interactie met de active site van het enzym hebben, (2) de activiteitsoëfficiënten van de enzym-intermediairen in de katalytische cyclus dezelfde waarde hebben, (3) het medium geen effect heeft op het katalytisch mechanisme van het enzym.

De invloed van media op enzymen is experimenteel bepaald voor lipases in verschillende mono- en bifasische systemen. Het blijkt dat de zogenaamde log P-afhankelijkheid van enzymen wordt veroorzaakt door verschil in solvatatie van de substraten en dat de verschillen tussen de kinetische parameters op basis van thermodynamische activiteiten sterk vermindert zijn. Fouten in de bepaling van de activiteitsoëfficiënten en de reactiesnelheden dragen bij aan de verschillen tussen de kinetische parameters die resteren na correctie voor substraat-medium interacties. Echter, de verschillen worden voornamelijk veroorzaakt door een effect van het medium op het lipase. Dit effect kan bestaan uit een verschillende mate van binding van de media aan de active site. Wanneer dit verschil verdisconteerd wordt door competitieve inhibtie door de media in de kinetische vergelijkingen op te nemen, nemen de verschillen in de kinetische parameters verder af.

Het gebruik van thermodynamische activiteiten in de kinetische vergelijkingen is ook vereist wanneer de bijdrage van substraat-medium interacties aan enzymspecificiteit wordt bestudeerd. Aangezien verschillende substraten een verschillende mate van solvatatie hebben, draagt solvatatie bij tot de enzymspecificiteit. De beschreven methode om voor substraat-medium interacties te corrigeren, kan gebruikt worden om computerberekeningen aan
substraat-enzym complexen, die in vacuüm uitgevoerd zijn, te koppelen aan kinetische experimenten, die in waterige oplosmiddelen uitgevoerd zijn.

De enantioselectieve omzettingen van racemische substraten door lipase worden niet adequaat beschreven door vergelijkingen die afgeleid zijn voor Michaelis Menten type enzymen. Zo leidt een verhoging van de omzettingsgraad bij hoge produkt concentraties niet tot een stijging van optische zuiverheid van het overblijvende substraat. Dit effect wordt waargenomen als een plateau in grafieken van de optische zuiverheid tegen de omzettingsgraad. Het plateau is een gevolg van het ping-pong mechanisme van lipase, waardoor er een terugreactie kan optreden van het enantiomere produkt. De mate waarin dit fenomeen optreedt wordt gekwantificeerd door de selectiviteitsfactor. De vergelijking voor kinetische resolutie waar de selectiviteitsfactor was ingebracht, voldoet voor hydrolyse en transesterificatie in zowel mono- als bifasische systemen. Met deze kennis kan aangegeven worden hoe de plateau-effecten in de praktijk vermeden kunnen worden.

Op basis van thermodynamische activiteiten van de substraten en het correcte enzymatische model, blijkt dat de enantiomere ratio en de selectiviteitsfactor van lipase voor glycidol (2,3-epoxypropanol) onafhankelijk van het oplosmiddel zijn. De effecten van het oplosmiddel op het enzym zijn voor de twee enantiomeren dus even sterk. Aangezien in de literatuur ook omkeren van de enantioselectiviteit in verschillende media vermeld wordt, hangt het effect van het medium op het enzym dus ook af van het substraat/enzym koppel. Uit de resultaten die in dit proefschrift vermeld zijn, is het duidelijk dat de geschikte kinetische mechanismen en correctie voor substraat-oplosmiddel interactie nodig zijn om deze effecten te kunnen onderzoeken en om adequate beschrijvingen te geven van de reacties onder preparatieve condities.
List of Publications


Chapters 2, 3, 4, and 5, and parts of chapter 6 are submitted for publication.
CURRICULUM VITAE


Aansluitend verrichte hij een promotieonderzoek bij de vakgroep Microbiologie en Enzymologie van de TU Delft met Hans Duine als promotor. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Tijdens zijn promotieperiode heeft hij als inval-docent aan de Haagse Laboratorium Hogeschool gewerkt. Tevens was hij als penningmeester betrokken bij de organisatie van een studiereis naar India.1

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