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# Pyruvate Metabolism in *Saccharomyces cerevisiae*

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In yeasts, pyruvate is located at a major junction of assimilatory and dissimilatory reactions as well as at the branch-point between respiratory dissimilation of sugars and alcoholic fermentation. This review deals with the enzymology, physiological function and regulation of three key reactions occurring at the pyruvate branch-point in the yeast *Saccharomyces cerevisiae*: (i) the direct oxidative decarboxylation of pyruvate to acetyl-CoA, catalysed by the pyruvate dehydrogenase complex, (ii) decarboxylation of pyruvate to acetaldehyde, catalysed by pyruvate decarboxylase, and (iii) the anaplerotic carboxylation of pyruvate to oxaloacetate, catalysed by pyruvate carboxylase. Special attention is devoted to physiological studies on *S. cerevisiae* strains in which structural genes encoding these key enzymes have been inactivated by gene disruption.

KEY WORDS — Yeast; glycolysis; TCA cycle; sugar metabolism; metabolic engineering; pyruvate decarboxylase; pyruvate carboxylase; pyruvate dehydrogenase complex; alcoholic fermentation; Crabtree effect

CONTENTS



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## INTRODUCTION

Pyruvic acid (from Greek, πυρ=fire; Latin, *uva*=grape; German Brenztraubensäure) derives its name from the fact that it is formed upon heating of tartaric acid, a major organic acid in wine, as first demonstrated by Erlenmeyer.[39] As

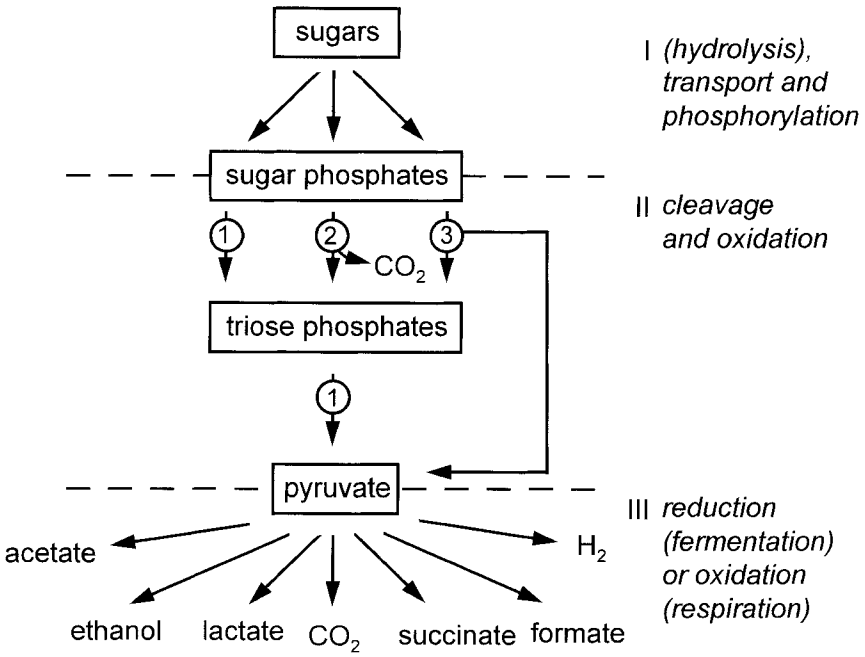


Figure 1. Schematic representation of the diversity of sugar dissimilation by microorganisms. Multiple routes exist for transport and sugar phosphorylation (I), conversion of sugar phosphates to pyruvate (II) and conversion of pyruvate to end products of dissimilation (III). Sugars may be transported by facilitated diffusion, active transport or group translocation; oligosaccharides can either be transported and then hydrolysed intracellularly or, alternatively be hydrolysed extracellularly and then transported as monosaccharides. Cleavage and oxidation of sugar phosphates may occur via the Embden-Meyerhof pathway (1), the hexose-monophosphate pathway (2) and the Entner-Doudoroff pathway (3). Under some conditions, triose phosphates may also be converted to pyruvate via the methylglyoxal by-pass. Virtually all possible combinations of the variants of I, II and III are encountered in nature.

illustrated by its central position on many metabolic pathway maps, pyruvate is located right at the heart of heterotrophic carbon metabolism. Neuberg first pointed to the importance of pyruvate as an intermediate in the fermentative metabolism of sugars by yeasts.[95] Kluyver, who was later to become a pioneer in the taxonomy, biochemistry and physiology of yeasts, was not immediately convinced. One of the (obligatory) statements accompanying his PhD thesis read: 'Pyruvate is not, as postulated by Neuberg, an intermediate in the alcoholic fermentation'.[71] This youthful transgression did not keep Kluyver from becoming a full professor in our department (1922–1956). We hope that readers will extend a similar clemency to the inevitable errors and omissions in this review.

*Modes of carbohydrate metabolism in yeasts and other microorganisms*

Microorganisms use different routes for the metabolism of sugars. As illustrated in Figure 1, this metabolic diversity is apparent at three levels: (i) transport and, in the case of oligosaccharides, hydrolysis; (ii) conversion of sugar phosphates to pyruvate by cleavage and oxidation; and (iii) further metabolism of pyruvate. Figure 1 represents a simplified scheme to which many exceptions exist. For example, some bacteria (including various pseudomonads) first carry out one or more oxidation steps outside the cell membrane, after which transport of the resulting organic acid, phosphorylation and cleavage occur.[82]

A common motif in virtually all sugar-metabolizing microorganisms is that the lower part

of the Embden-Meyerhof pathway is involved in the conversion of triose phosphates to pyruvate. In some cases, this reaction sequence can be by-passed by a route involving methylglyoxal and D-lactate as intermediates.[27] The methylglyoxal by-pass, which is not coupled to substrate-level phosphorylation, probably functions mainly during 'overflow metabolism'.[139]

The diversity of microbial sugar metabolism is especially evident in the further metabolism of pyruvate (Figure 1). During fermentative growth, pyruvate may be converted into a multitude of compounds, including molecular hydrogen, carbon dioxide and many organic metabolites. Alternatively, respiratory dissimilation of pyruvate via the tricarboxylic acid (TCA) cycle leads to its complete oxidation to carbon dioxide and water.

In view of the staggering diversity of sugar metabolism in the microbial world, a surprising unity exists among yeasts. Of the ca. 700 yeast species that are currently recognized, all strains investigated seem to predominantly use the Embden-Meyerhof pathway for conversion of hexose phosphates to pyruvate. In order not to make yeasts appear overtly boring in this respect, it should be mentioned that important differences occur in the initial steps leading from extracellular sugar to intracellular hexose phosphates (Figure 2).[4] Furthermore, depending on growth conditions and yeast species, the hexose monophosphate pathway may make an important contribution to sugar metabolism.[20,21,24]

The unity in the carbohydrate metabolism of yeasts becomes most evident from their fermentative sugar metabolism. Whenever yeast species exhibit a fermentative sugar metabolism, ethanol and carbon dioxide are the predominant fermentation products.[33] The glycerol that is also frequently found does not primarily result from sugar dissimilation. Rather, glycerol formation enables the reoxidation of NADH that is generated during the conversion of sugar into biomass.[102,146,147] Under aerobic conditions, this assimilatory NADH can easily be disposed of by respiration. When, under conditions of extreme oxygen limitation or anaerobiosis, this becomes impossible, glycerol formation acts as an essential redox valve.[158]

In addition to ethanol and glycerol, fermenting yeast cultures often excrete small amounts of other fermentation products, in particular organic acids (e.g. acetate and succinate[102,151]). Under some conditions, D-lactate is produced in small amounts by cultures of *Saccharomyces cerevisiae* and *Candida utilis* (Luttik *et al.*, unpublished),[66] suggesting involvement of the methylglyoxal by-pass, albeit at very low rates. Other metabolites excreted by yeasts include higher alcohols, esters and aldehydes. Although specific rates of formation of these compounds are often orders of magnitude lower than the rates of ethanol formation, some play an important role as (off-)flavours in alcoholic beverages.

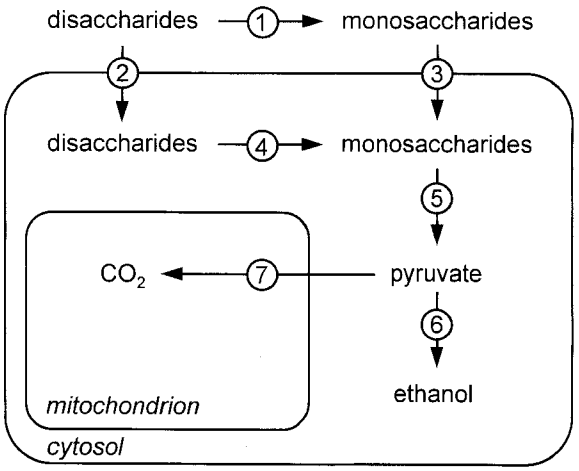


Figure 2. Schematic representation of carbohydrate dissimilation by yeasts. Hydrolysis of disaccharides may occur by extracellular enzymes with a low pH optimum (1) or intracellularly (4) by enzymes with a near-neutral pH optimum. Disaccharide transport (2) appears to occur exclusively via proton symport, whereas monosaccharide transport (3) may involve either facilitated diffusion or proton symport, depending on yeast species and environmental conditions. In the case of hexoses, the Embden-Meyerhof pathway is the main route of sugar dissimilation (5) in yeasts. Pyruvate, the end-product of glycolysis, is either converted to ethanol and carbon dioxide (6) or respired to carbon dioxide and water (7) in the mitochondria.

*Regulation of sugar metabolism in yeasts*

The range of sugars that support growth differs greatly among yeast species. Nevertheless, all wild-type yeast strains that have so far been tested can utilize glucose as a carbon source.[5] However, not all yeast strains are able to ferment glucose to ethanol. At the time of writing this review, the total number of strains deposited in the collection of the Centraalbureau voor Schimmelcultures was 4738. Of these, 4180 strains had been tested for their fermentative capacity, using the standard taxonomic test method (i.e. measuring gas

Table 1. Regulation of fermentative sugar metabolism in yeasts.

| Regulatory phenomenon | Definition and environmental conditions | Proposed mechanism | References |
|---|---|---|---|
| Crabtree effect (long-term) | Aerobic alcoholic fermentation at high growth rates, irrespective of the mode of cultivation (growth under sugar limitation or growth with excess sugar) | Insufficient capacity of respiratory routes of pyruvate dissimilation | 107,109,114 |
| Crabtree effect (short-term) | Instantaneous aerobic alcoholic fermentation after transition of sugar-limited cultures to sugar excess | Respiratory metabolism becomes saturated, causing overflow at the level of pyruvate | 114,140,144 |
| Pasteur effect | Suppression of alcoholic fermentation in the presence of oxygen. Observed in all facultatively fermentative yeasts that do not exhibit a Crabtree effect. In *S. cerevisiae* only evident at low glycolytic fluxes (e.g. in slowly growing cells) | The affinity ($V_{max}/K_m$) of the respiratory system for pyruvate, acetaldehyde and/or NADH is higher than that of the fermentative route | 78 |
| Kluyver effect | Absence of alcoholic fermentation during oxygen-limited growth on a sugar (often a disaccharide), even though glucose is readily fermented. Widespread among yeasts, does not occur in *S. cerevisiae* | Control of the synthesis and/or activity of the sugar carrier. When the effect is observed for disaccharides, these are hydrolysed intracellularly | 4,66,133,158 |
| Custers effect | Oxygen requirement for alcoholic fermentation, evident upon transfer from oxygen-limited to anaerobic conditions. Observed with glucose (e.g. in *Brettanomyces* spp.) as well as with other sugars (e.g. xylose in *Candida utilis*) | Redox imbalance, either due to an inability to form glycerol or other reduced metabolites (*Brettanomyces*), or to different cofactor specificity of reduction and oxidation reactions (xylose fermentation) | 22,23,33,125 |

production in Durham tubes). According to this test, 1555 strains (or 37% of the total) were unable to perform alcoholic fermentation.

As the Durham test is rather insensitive, and therefore prone to false-negative results,[34] the true percentage of non-fermentative yeasts is probably lower than the taxonomic tests suggest. Since the group of strictly-fermentative yeasts (i.e. yeasts that are unable to respire sugars) is also relatively small, the large majority of yeast strains can either respire sugars or ferment them to ethanol and carbon dioxide. All these facultatively fermentative yeasts exhibit alcoholic fermentation under oxygen-limited growth conditions.[33,34] This is where uniformity ends, as in many yeasts the oxygen concentration is not the sole factor determining the contribution of respiration and fermentation to the overall rate of sugar metabolism.

The diversity among facultatively fermentative yeasts with respect to the regulation of alcoholic fermentation is evident from phenomena indicated by such terms as 'Pasteur effect', 'Crabtree effect', 'Kluyver effect' and 'Custers effect'. All these 'effects' represent regulatory mechanisms that affect the balance between fermentation and respiration. Since it would be beyond the scope of this review to discuss these phenomena in detail, only a brief summary of the phenomenology of these effects and their proposed mechanistic explanations are given in Table 1. The Crabtree effect (occurrence of alcoholic fermentation under aerobic conditions) is one of the most important metabolic phenomena in biomass-directed industrial applications of *S. cerevisiae* and, as will be discussed below, a major incentive for studying pyruvate metabolism in this yeast.

## S. cerevisiae: *laboratory model and cell factory*

It is not necessary to reiterate the etymology of the word 'enzyme' to illustrate the unique role of *S. cerevisiae* in the development of biochemistry and physiology. While continuing to be an important model organism for studies in these fields (as will hopefully be illustrated in this review), *S. cerevisiae* has also become a key model organism for studying eukaryotic genetics and cell biology. This role will only become more important now that this yeast has become the first eukaryote to have its complete genome sequenced. The functional analysis of the many open reading frames to which no discrete physiological function has yet been attributed, is one of the major challenges in yeast biology.[99]

Developments with respect to the industrial applications of *S. cerevisiae* are in many aspects similar to those in fundamental research. Areas of yeast biology that have already existed for decades or even centuries, including the production of alcoholic beverages and bakers' yeast, continue to merit research. This is partly due to the need for improved process control, which necessitates a quantitative description of the metabolic fluxes within the cells. Furthermore, in spite of the long history of *S. cerevisiae* as an industrial micro-organism, some inherent problems are still not completely understood or solved. This is perhaps best illustrated by the classical bakers' yeast production process.

During the production of bakers' yeast, the costs of the carbohydrate feedstock are a major factor in the overall economy of the process. Thus, a high biomass yield on the sugar feedstock (usually molasses) is a major optimization criterion. This implies that fermentative sugar metabolism (which leads to a much lower biomass yield than respiratory metabolism; for a review see ref. 148) should be avoided during the production phase. On the other hand, commercial bakers' yeast should have a high fermentative capacity in the dough application. During the industrial production of bakers' yeast, the strong inclination of *S. cerevisiae* to perform alcoholic fermentation is largely overcome by careful manipulation of the rate of sugar supply and by controlling other environmental conditions.[12] So far, it has not been possible to use metabolic engineering to control the tendency of *S. cerevisiae* towards aerobic fermentation, while, at the same time, maintaining a high fermentative capacity in the dough environment.

While classical applications of *S. cerevisiae* continue to present challenges, novel processes are rapidly gaining ground. The most important example of this is the use of *S. cerevisiae* as a host for the expression of heterologous proteins.[52,117] In addition to problems dealing with expression of heterologous genes and the excretion and modification of the products, a number of physiological properties of *S. cerevisiae* need to be taken into consideration for this application. To facilitate downstream processing, high product concentrations are desirable. Therefore, large-scale heterologous protein production is performed at high biomass densities. At high biomass densities, even low specific rates of by-product formation will rapidly lead to the build-up of toxic metabolite levels, with detrimental effects on productivity.[51,148]

Minimization of by-product formation is not the only way in which physiological research may contribute to heterologous protein production. At present, only a small fraction of the carbon and nitrogen substrates fed to heterologous-protein-producing cultures is converted into the product of interest. Future research will therefore also have to address the question how the fluxes leading to heterologous protein can be maximized while at the same time keeping the yeast cell viable under the conditions used for industrial production.

## *Aim and scope of this review*

Further optimization of *S. cerevisiae* strains as dedicated 'cell factories' will to a large extent depend on the rerouting of metabolism by metabolic pathway engineering. Rational strategies for modifying the distribution of fluxes at key branch-points in the metabolic network depend on detailed knowledge of the physiological role of the different branches and of the regulatory mechanisms that operate in wild-type cells.

The aim of this paper is to provide an overview of the literature on the junction in the *S. cerevisiae* metabolic network where fermentative and respiratory sugar metabolism diverge: the pyruvate branch-point. We have focussed on the biochemistry and physiology of pyruvate metabolism and, in particular, on the use of molecular genetic techniques to study the role and regulation of key enzymes active at this branch-point. Regulation of enzyme synthesis is discussed in terms of enzyme activity and (in some cases) mRNA levels, but we

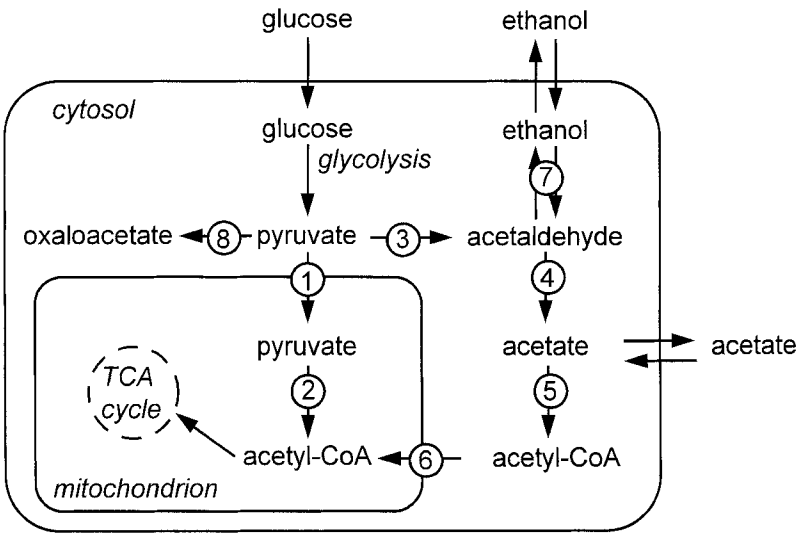


Figure 3. Key enzymic reactions at the pyruvate branch-point in *S. cerevisiae*. Numbered reactions are catalysed by the following enzymes: 1, mitochondrial pyruvate carrier; 2, pyruvate dehydrogenase complex; 3, pyruvate decarboxylase; 4, acetaldehyde dehydrogenase; 5, acetyl-CoA synthetase; 6, carnitine shuttle; 7, alcohol dehydrogenase; 8, pyruvate carboxylase. In addition to a cytosolic acetaldehyde dehydrogenase, *S. cerevisiae* also contains a mitochondrial isoenzyme.[63] The pyruvate dehydrogenase by-pass consists of enzymes 3, 4 and 5. Formation of acetyl-CoA from acetate requires two ATP equivalents, since acetyl-CoA synthetase hydrolyses ATP to AMP and pyrophosphate.

have chosen not to discuss signal transduction mechanisms.

A further restriction is that we will focus on three major conversions of pyruvate that occur during growth on sugars: oxidative decarboxylation to acetyl-CoA, decarboxylation to acetaldehyde and carboxylation to oxaloacetate.

This paper deals with a single yeast species, *S. cerevisiae*. Although 'yeast' and '*S. cerevisiae*' are often used as synonyms in the literature, it should be realized that in many physiological aspects, *S. cerevisiae* is an exceptional yeast. For example, *S. cerevisiae* is one of very few yeasts capable of growth under strictly anaerobic conditions.[150] The regulation of pyruvate metabolism in *S. cerevisiae*, with its strong tendency towards alcoholic fermentation, is also clearly different from other yeasts used as laboratory model organisms and/or industrial microorganisms (e.g. *C. utilis, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia stipitis, Yarrowia lipolytica*, etc.). Therefore, the data discussed in this paper cannot necessarily be extrapolated to other yeast species.

## MAJOR ROUTES OF PYRUVATE METABOLISM: ENZYMES AND GENES

The three major metabolic pathways in *S. cerevisiae* that originate from pyruvate are schematically shown in Figure 3. Of these pathways, only the conversion of pyruvate into ethanol has an exclusively dissimilatory function: ATP production from substrate-level phosphorylation reactions in glycolysis can only occur when the NADH produced in this pathway is reoxidized. Acetaldehyde, the electron acceptor used for NADH reoxidation during fermentative growth, is formed from pyruvate by pyruvate decarboxylase (EC 4.1.1.1).

The energetically more favourable, respiratory dissimilation of carbohydrates requires coupling of glycolysis to the TCA cycle. Acetyl-CoA, the fuel of the cycle, can be synthesized from pyruvate by a direct oxidative decarboxylation, catalysed by the pyruvate–dehydrogenase complex.[60,75,76] In contrast to the enzymes of glycolysis, which are all located in the cytosol, the pyruvate–dehydrogenase complex is located in the

Table 2. Structural genes of enzymes involved in pyruvate metabolism by *S. cerevisiae*, predicted size of the encoded peptides and chromosomal localization. Nomenclature of genes is according to the Yeast Protein Database.

| Enzyme | Structural gene | Peptide size (kDa) | Chromosome | Reference |
|---|---|---|---|---|
| *Pyruvate dehydrogenase* heteromultimer, 8–9 MD | | | | |
| E1α subunit | *PDA1* | 45 | V | 7,136 |
| E1β subunit | *PDB1* | 36 | II | 90 |
| E2 subunit | *LAT1* | 49 | XIV | 98 |
| E3 subunit | *LPD1* | 52 | VI | 118,120 |
| Protein X | *PDX1* | 42 | VII | 8 |
| *Pyruvate decarboxylase* tetramer, 248 kDa | | | | |
| | *PDC1* | 62 | XII | 69,128 |
| | *PDC5* | 62 | XII | 54,131 |
| | *PDC6* | 62 | VII | 55 |
| *Pyruvate carboxylase* tetramer, 520 kDa | | | | |
| | *PYC1* | 130 | VII | 92 |
| | *PYC2* | 130 | II | 137,153 |

mitochondrial matrix.[60] Therefore, pyruvate oxidation by the complex requires transport of the substrate across the mitochondrial membranes.

In addition to the pyruvate dehydrogenase reaction, conversion of pyruvate to acetyl-CoA can occur via an indirect route (Figure 3). This 'pyruvate dehydrogenase by-pass' involves the enzymes pyruvate decarboxylase (which is also a key enzyme in alcoholic fermentation), acetaldehyde dehydrogenase and acetyl-CoA synthetase.[60] The by-pass route is the source of the acetate that accumulates in sugar-grown *S. cerevisiae* cultures under some growth conditions (e.g. during transfer from glucose limitation to glucose excess.[144]

Conversion of pyruvate into acetyl-CoA is not only a dissimilatory reaction: acetyl-CoA, as well as some TCA-cycle intermediates synthesized from it, is an essential biosynthetic building block. This assimilatory function of the TCA cycle is, in principle, incompatible with its role in dissimilation, since complete dissimilation of acetyl-CoA requires that oxaloacetate be regenerated at each turn of the cycle. During growth of *S. cerevisiae* on carbohydrates, the withdrawal of TCA-cycle intermediates for biosynthesis is compensated for by the third major pathway originating from pyruvate, i.e. the carboxylation of pyruvate to oxaloacetate. This vital assimilatory reaction is catalysed by pyruvate carboxylase (EC 6.4.1.1).

Some relevant enzymological and regulatory characteristics of the three enzymes that compete for the common intermediate pyruvate, as well as some characteristics of their structural genes, are discussed below. In addition, the scarce information on transport of pyruvate into *S. cerevisiae* mitochondria is summarized.

### *The pyruvate dehydrogenase complex*

As in other organisms, the *S. cerevisiae* pyruvate dehydrogenase complex is a large multicomponent enzyme complex ($M_r = 8–9 \times 10^6$).[75,76] It belongs to the family of α-oxoacid-dehydrogenase complexes, which catalyse the lipoamide-mediated oxidative decarboxylation of α-oxoacids.[112,161] These complexes consist of three major catalytic components called E1, E2 and E3.[161] In contrast to the E1 and E2 subunits, which are complex-specific, the E3 subunit of the pyruvate dehydrogenase complex is also part of 2-oxoglutarate dehydrogenase and branched-chain 2-oxoacid dehydrogenase, the other α-oxoacid-dehydrogenase complexes in *S. cerevisiae*,[29,30] and of glycine decarboxylase.[134] A fourth component, X, does not appear to have a catalytic function but is probably involved in assembly of the complex.[80] The structural genes encoding the subunits of the *S. cerevisiae* pyruvate dehydrogenase complex have all been cloned (Table 2).

Conversion of pyruvate into acetyl-CoA is catalysed by the concerted action of the catalytic subunits.[112,113] In the first step, pyruvate is covalently linked to thiamine pyrophosphate (TPP), the cofactor of the E1 subunit (pyruvate

dehydrogenase, EC 1.2.4.1). This reaction yields 2-α-hydroxy-ethyl-TPP ('active aldehyde').[61] In *S. cerevisiae*, as in most other organisms, the E1 moiety consists of two subunits, E1α and E1β.[76] The α-hydroxy group of active aldehyde is oxidized and the resulting acetyl moiety is coupled to the lipoamide cofactor of the E2 subunit (dihydrolipoamide acetyl transferase, EC 2.3.1.12), which subsequently transfers it to coenzyme A. During the oxidation of active aldehyde, an E2 lipoamide group is reduced to dihydrolipoamide. Its reoxidation is catalysed by the E3 subunit (dihydrolipoamide dehydrogenase, EC 1.6.4.3) that uses $NAD^+$ as the electron acceptor. Thus, the overall reaction catalysed by the complex is:

$$\text{pyruvate} + \text{coenzyme A} + NAD^+ \rightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

Kinetic analysis with the purified pyruvate dehydrogenase complex from *S. cerevisiae* yielded $K_m$ values for pyruvate, $NAD^+$ and coenzyme A of 625 μM, 23 μM and 18 μM, respectively.[75] These $K_m$ values were measured at pH 8·1; the $K_m$ for pyruvate decreases to ca. 200 μM when the pH is lowered to 6·5.[67,75] Cooperativity with respect to pyruvate, as occurs with prokaryotic pyruvate dehydrogenase complexes, was not observed with the *S. cerevisiae* complex.[67,75] The products NADH and acetyl-CoA exhibited competitive inhibition with respect to $NAD^+$ and coenzyme A ($K_i$=23 μM and $K_i$=18 μM, respectively).

Activity of the pyruvate dehydrogenase complex is difficult to assay in crude cell extracts due to proteolytic degradation and interference by other enzymes, in particular pyruvate decarboxylase.[75,136] However, evidence from measurements on crude mitochondrial fractions indicates that pyruvate dehydrogenase activity is expressed under conditions which do not require its dissimilatory function. For example, activity has been measured in cells from anaerobic cultures and in cultures grown aerobically on ethanol.[155] This may be indicative of a role of the pyruvate dehydrogenase complex in mitochondrial assimilatory processes requiring active aldehyde or acetyl-CoA. An observation that may be relevant to this hypothesis is that, with the exception of *LAT1*, all genes encoding subunits of the complex contain putative GCN4 boxes.[155] Gcn4p is a general regulator of amino acid metabolism.[53] Although it is as yet unclear to what extent these sequences are functional, their presence is consistent with a role of the pyruvate dehydrogenase complex in amino acid synthesis. This is further supported by a partial leucine requirement of mutants lacking pyruvate dehydrogenase activity.[154]

Transcriptional regulation has been studied for two of the structural genes encoding subunits of the complex. Levels of the *PDA1* transcript, which encodes the E1α subunit, were essentially constant under all conditions tested, including aerobic growth on ethanol and anaerobic growth in glucose-limited chemostat cultures.[155] In fact, due to its constant level and stability, it has been proposed that the *PDA1* transcript is a suitable loading standard for quantitative mRNA assays.[157] In contrast, transcription of the *LPD1* gene (encoding the E3 subunit, which is also part of the two other α-oxoacid-dehydrogenase complexes) is subject to glucose catabolite repression, although significant transcription levels are still observed in the presence of glucose.[120] Full derepression of *LPD1* requires a HAP2/3/4 binding site in its promoter.[17]

The possible involvement of phosphorylation of the pyruvate dehydrogenase complex in the regulation of its activity will be discussed in a separate paragraph.

### *Pyruvate decarboxylase*

Pyruvate decarboxylase (EC 4.1.1.1) catalyses the TPP- and magnesium-dependent decarboxylation of pyruvate to acetaldehyde and carbon dioxide.[47,84,96] The native *S. cerevisiae* enzyme, which occurs in the cytosol,[143] is a tetramer, composed of four identical or highly related subunits of ca. 62 kDa.[46,77]

Pyruvate decarboxylase exhibits cooperativity with respect to pyruvate, an effect which is enhanced by phosphate.[14,62] At the same time, phosphate is a competitive inhibitor of the enzyme: the $K_m$ for pyruvate (1–3 mM in the absence of phosphate) increases about fourfold in the presence of 25 mM phosphate.[14,143] Normal intracellular concentrations of phosphate in *S. cerevisiae* (5–15 mM[58,143]) are higher than the $K_i$ for phosphate (ca. 1 mM), indicating that phosphate is a physiologically relevant effector of *in vivo* pyruvate decarboxylase activity.

Studies on pyruvate decarboxylase are complicated by the presence of three structural genes (Table 2) that each potentially encode an active enzyme. The *PDC1* gene was isolated by complementation of mutants with low pyruvate decarboxylase levels.[69,128] The mutants used for these

complementation studies were obtained by mutagenesis with ethyl methane sulfonate.[127] Surprisingly, a null mutation of the *PDC1* gene resulted in a strain with a much higher pyruvate decarboxylase activity than that of the previously isolated point mutants.[123] This activity was subsequently shown to be encoded by a second, highly homologous structural gene called *PDC5*.[54,131] In glucose-grown shake-flask cultures, *PDC5* was expressed to a much higher level in the *pdc1* deletion mutant than in strains containing point-mutation alleles of *PDC1*. This strongly suggests that expression of *PDC* genes is subject to autoregulation.[54]

Inactivation of both *PDC1* and *PDC5* yields strains which, during growth in complex medium with glucose, do not express detectable levels of pyruvate decarboxylase. A third *PDC* gene, *PDC6*, was isolated by low-stringency hybridization of a genomic library with a *PDC1* probe.[55] Although the *PDC6* sequence had a high similarity with *PDC1* and *PDC5*, its disruption did not cause significant changes of pyruvate decarboxylase activity.[40,55] However, a number of spontaneous revertants of *pdc1 pdc5* double mutants have been isolated in which a recombination event had caused a fusion of the *PDC6* open reading frame with the *PDC1* promoter.[56] So far, it is unclear whether growth conditions exist under which the native *PDC6* gene contributes significantly to pyruvate decarboxylase activity in wild-type *S. cerevisiae*.

Full expression of *PDC1* and *PDC5* requires the presence of a functional *PDC2* gene, which encodes a positive transcription regulator.[57] The role of two other genes that are required for optimal expression of pyruvate decarboxylase genes, *PDC3* and *PDC4*[160] (Seehaus, cited in ref. 57) remains unclear.

Regulation of pyruvate decarboxylase expression in *S. cerevisiae* has been studied almost exclusively in shake-flask cultures. In such experiments, a strong increase of pyruvate decarboxylase activity is invariably observed when cultures are switched from a non-fermentable carbon source (e.g. ethanol) to a glucose-containing medium (see e.g. refs 15,16,40,87). In mutants affected in the expression of various glycolytic enzymes, induction of pyruvate decarboxylase is correlated with the levels of metabolites originating from the lower part of glycolysis.[15,16] While these experiments demonstrate that the activity of pyruvate decarboxylase in *S. cerevisiae* varies as a function of growth conditions, shake-flask experiments do not allow discrimination between the effects of individual growth parameters. Changes of the carbon source will have a profound impact on many growth parameters, including pH, growth rate, viability, metabolite concentrations and dissolved-oxygen concentration.

In particular, the dissolved-oxygen concentration has been shown to have a strong effect on the regulation of pyruvate decarboxylase in yeast species.[41,135,145,158] This parameter cannot be adequately controlled in batch cultures which, especially during rapid growth on sugars, become oxygen limited.[34] In aerobic, carbon-limited chemostat cultures of *S. cerevisiae* grown on ethanol or glucose at a dilution rate of 0·10 $h^{-1}$, no significant differences in pyruvate decarboxylase activities were observed.[110] In aerobic, sugar-limited cultures, an increase of the pyruvate decarboxylase activity occurs above the critical growth rate at which alcoholic fermentation is triggered.[88,109] So far, no experiments have been performed to study the differential expression of the three structural *PDC* genes under carefully controlled growth conditions (e.g. as a function of the oxygen supply in cultures grown at a fixed rate or as a function of growth rate in aerobic and anaerobic cultures).

### *Pyruvate carboxylase*

In *S. cerevisiae*, the anaplerotic synthesis of oxaloacetate from pyruvate is catalysed by pyruvate carboxylase (EC 6.4.1.1).[42,86] The enzyme catalyses the magnesium- and ATP-dependent carboxylation of pyruvate to oxaloacetate:[121]

$$\text{pyruvate} + HCO_3^- + \text{ATP} \rightarrow \text{oxaloacetate} + \text{ADP} + P_i$$

In contrast to many higher organisms, in which pyruvate carboxylase is a mitochondrial enzyme, its location in *S. cerevisiae* is exclusively cytosolic.[49,116,143,153]

The native *S. cerevisiae* enzyme is a tetramer consisting of identical or highly related 130 kDa subunits.[3,26,115] Each subunit contains a covalently linked biotin cofactor that is attached to the inactive apoenzyme by a specific, ATP-dependent holoenzyme synthetase.[138]

The $K_m$ values of the *S. cerevisiae* pyruvate carboxylase for its substrates pyruvate, bicarbonate and ATP have been estimated at 0·8 mM, 2·7 mM and 0·24 mM, respectively.[121] In addition to the concentrations of its substrates, activity of

pyruvate carboxylase can be modulated by a number of other metabolites, including acetyl-CoA, palmitoyl-CoA and aspartate. These metabolites not only affect the maximum specific activity of pyruvate carboxylase, but also the affinity for its three substrates.[6,130]

In contrast to pyruvate carboxylase from a variety of other sources, the yeast enzyme exhibits activity in the absence of acetyl-CoA. This basal activity is dependent on the presence of potassium or other monovalent cations[93]. At saturating concentrations of acetyl-CoA ($K_a$=6·6 μM;[93]), maximum specific activities are three- to four-fold higher than in its absence[93,121]. The positive effect of acetyl-CoA, the fuel of the TCA cycle, is in line with the anaplerotic role of pyruvate carboxylase: accumulation of acetyl-CoA will increase replenishment of the acceptor molecule oxaloacetate. The activation by acetyl-CoA has been reported to be competitively inhibited by NADH which, however, did not affect the basal acetyl-CoA-independent activity[25].

In addition to acetyl-CoA, long-chain acyl-CoA esters are potent activators of the enzyme. In fact, the $K_a$ for palmitoyl-CoA (0·04 μM) is two orders of magnitude lower than that for acetyl-CoA.[93] The maximum activity of *S. cerevisiae* pyruvate carboxylase also depends on the relative concentrations of adenine nucleotides: activity increases with increasing adenylate energy charge.[89]

Aspartate is a non-competitive inhibitor of the enzyme ($K_i$=1·9 mM), whereas the product oxaloacetate exhibits competitive inhibition with respect to pyruvate ($K_i$=0·22 mM). Inhibition by aspartate, a biosynthetic building block directly derived from oxaloacetate, appears to be a specific feed-back control mechanism since most other dicarboxylic acids do not cause substantial inhibition.[104] Inhibition by α-oxoglutarate[83] is unlikely to be relevant under physiological conditions in view of its high $K_i$ (ca. 18 mM).

In contrast to the extensive regulation of pyruvate carboxylase at the enzyme activity level, synthesis of the enzyme seems to be largely constitutive. In aerobic batch and chemostat cultures grown on a number of substrates, the pyruvate carboxylase activity in cell extracts varied by no more than two-fold.[36,48,65,103] In comparison with aerobic, glucose-limited chemostat cultures grown at the same dilution rate, activities in anaerobic cultures were about two-fold higher.[48,103] Addition of aspartate to growth media led to a decrease of pyruvate carboxylase activity in dialysed cell extracts of ca. 50%.[48] However, even in aspartate-containing media, carbon dioxide fixation via pyruvate carboxylase continued.[103]

*S. cerevisiae* contains two structural genes for pyruvate carboxylase (Table 2), each encoding an apoenzyme that can be activated by binding of biotin. The *PYC1* gene was cloned using an oligonucleotide probe based on a biotin-attachment consensus sequence.[92] The highly homologous *PYC2* gene was subsequently isolated by hybridization of genomic libraries with a *PYC1* probe.[137,153] Kinetic studies with the two isoenzymes, partially purified from disruption mutants, yielded similar $K_m$ values for pyruvate and ATP, while aspartate inhibition kinetics were also the same.[137]

Both *PYC* genes are transcribed in wild-type *S. cerevisiae* grown on glucose or ethanol in shake-flask cultures, although transcript levels were dependent on the growth phase.[18] Peculiarly, levels of the *PYC1* transcript were higher in ethanol-grown cultures than in cultures grown on glucose. This is unexpected, since the glyoxylate cycle is generally assumed to account for replenishment of oxaloacetate during growth on $C_2$-compounds. However, since transcript levels were related to total RNA content of the samples, the observed difference might, at least in part, be caused by different rRNA contents of glucose- and ethanol-grown cells.

### *Transport of pyruvate into the mitochondrion*

As discussed above, the *S. cerevisiae* pyruvate dehydrogenase complex is located inside the mitochondria. The flux through this enzyme might therefore not only be affected by synthesis of its subunits and by the intramitochondrial concentrations of its substrates and effectors, but also by regulation of pyruvate transport into the mitochondrial matrix. The kinetics of pyruvate transport into the mitochondria are also likely to have an impact on the competition of mitochondrial pyruvate oxidation with the cytosolic enzymes pyruvate decarboxylase and pyruvate carboxylase.[59] Of the key reactions at the pyruvate branch-point in *S. cerevisiae*, pyruvate transport into the mitochondria is by far the least studied and characterized.

Being a small molecule, pyruvate can readily cross the outer mitochondrial membrane via pores.[9,31] Free diffusion of the non-dissociated acid across the phospholipid bilayer may contrib-

ute to transport across the inner mitochondrial membrane.[2] Involvement of a transporter was demonstrated by the observation that ΔpH-dependent uptake of pyruvate by *S. cerevisiae* mitochondria is competitively inhibited by α-cyano-3-hydroxycinnamate,[19] a well-known inhibitor of mammalian mitochondrial pyruvate transporters.

Two peptides of 26 and 50 kDa, isolated by affinity-chromatography on immobilized 4-hydroxy-cyanocinnamate, catalysed pyruvate transport upon reconstitution in proteoliposomes.[94] In addition to pyruvate/pyruvate exchange, the reconstituted proteoliposomes also catalysed exchange of acetoacetate and branched-chain oxoacids. When exchange with acetoacetate was measured, the $K_m$ for pyruvate was 0·8 mM. This is close to the $K_m$ for pyruvate oxidation by intact mitochondria (0·3 mM)[143] and the $K_m$ of the pyruvate dehydrogenase complex (0·2–0·6 mM),[75] suggesting that the overall $K_m$ of mitochondria for pyruvate is of the order of 0·2 to 1 mM.

Although the systematic sequencing of the yeast genome has yielded a number of sequences that encode putative mitochondrial transporters, none of these has been linked conclusively to a pyruvate carrier. It therefore remains unclear which genes are involved in the uptake of pyruvate into the mitochondrial compartment of *S. cerevisiae* and how this important process is regulated at the level of carrier synthesis and/or transport activity.

## GENE DISRUPTIONS

Inactivation of structural and regulatory genes by disruption or replacement, using the one-step gene disruption procedure[100,119] has become an indispensable tool for physiological studies. By comparing null mutants with the isogenic wild type, important information can be obtained about the physiological role of the gene involved. Many marker genes that are available to disrupt or replace *S. cerevisiae* genes complement auxotrophic requirements and can therefore only be applied in auxotrophic laboratory strains. As will be briefly discussed below, such strains are poorly suited for quantitative studies on intermediary carbon metabolism.

The substrates (normally amino acids or bases) for which a yeast is auxotrophic have to be added to its growth medium. In a physiological sense, this is not equivalent to the situation in the complemented disruptant, which is able to synthesize the amino acid or base itself. For example, the kinetics of amino-acid uptake from the medium may be different from those of intracellular amino-acid synthesis. This will directly affect the specific growth rate and intracellular metabolite pools and thus have regulatory effects that extend beyond amino acid metabolism. Furthermore, although amino-acid synthesis is not a major energy-requiring step in the synthesis of yeast biomass,[149] small effects on growth energetics cannot be excluded. Also energy requirements for active uptake of amino acids may affect bioenergetics.

Synthesis of amino acids is an integral part of intermediary carbon metabolism. Effects of gene disruption may therefore easily be obscured when amino acids have to be added to the growth medium. An example of this will be given below when the disruption of the *PDA1* gene, encoding the E1α subunit of the pyruvate dehydrogenase complex,[154] is discussed.

A problem that is not inherent to the use of auxotrophic markers, but nevertheless worth mentioning, is that the concentrations of amino acids or nucleotides in growth media for auxotrophic strains used in the literature are often too low. For example, L-leucine is often added at a fixed concentration of 20 mg $l^{-1}$ to media containing 10 g $l^{-1}$ or more of glucose. As will be illustrated by the following calculation, this is inadequate. The protein content of *S. cerevisiae* is about 45% and the biomass yield on glucose in (respirofermentative) batch cultures is about 0·4 g g glucose$^{-1}$ at the time of ethanol exhaustion. Approximately 10% of *S. cerevisiae* protein consists of leucine.[101] This means that, to achieve a biomass concentration of 4 g $l^{-1}$, at least 160 mg $l^{-1}$ leucine should be added to the medium of a leucine auxotroph. At lower leucine concentrations, growth will be limited by the amino acid long before the carbon source is exhausted.

The disadvantages of auxotrophic markers do not hold for dominant selectable marker genes (encoding, for example, antibiotic resistance), which can be used in prototrophic *S. cerevisiae* strains. However, in this case it is still important to rule out effects of the marker gene. Many antibiotic resistance cassettes use strong constitutive promoters, which may, at least in theory, lead to the production of substantial amounts of the encoded proteins. Preferably, control experiments should be performed under growth conditions where no effect of the gene disruption is expected, to rule out interference of the marker-gene-

encoded proteins with cellular metabolism. The best available system for gene inactivation probably consists of a two-step approach, in which the expression cassette that has been used to replace a gene is itself removed from the genome by a recombination of two direct repeats flanking the marker gene.[1,119,152]

Special care is required when disruption mutants are grown in chemostat cultures. This holds in particular for disruptions in genes of which 'sleeping' isogenes or pseudogenes are present in the genome. The selective pressure in chemostats (or even in batch cultures as in the case of *PDC6*[56]) may confer a strong selective advantage to revertants in which these genes have been 'awoken' by recombination events or other mutations. It may therefore be necessary to also disrupt pseudogenes to construct stable null mutants for physiological studies.

Below, the physiological effects of disruptions in structural genes encoding pyruvate decarboxylase, pyruvate carboxylase and components of the pyruvate dehydrogenase complex will be discussed.

### *Disruption of genes encoding subunits of the pyruvate dehydrogenase complex*

Gene disruptions have been introduced in all four structural genes encoding subunits that are unique to the pyruvate dehydrogenase complex, but no disruption mutants have been described for the *LPD1* gene. Null mutations in the genes encoding the E1α, E1β, E2 and X subunits of the complex all result in complete loss of pyruvate dehydrogenase activity.[80,81,90,154] Nevertheless, these pyruvate dehydrogenase-negative ($Pdh^-$) strains were all viable in complex and defined media containing either glucose or non-fermentable carbon sources.

For the *lat1* and *pdx1* null mutants (lacking the E2 and X subunits, respectively), growth rates on glucose and other carbon sources were reported not to differ significantly from the isogenic wild-type strains. For the *pdh*β1 null mutant, the only phenotype reported was slightly retarded growth on glycerol plates.[90] Physiological effects of a null mutation in *PDA1* have been studied in most detail.[154] A null mutant constructed by replacement of the *PDA1* gene with the antibiotic resistance gene *Tn5ble* exhibited a growth rate on a glucose-containing mineral medium that was less than half that of the wild type. Peculiarly, when the same mutation was introduced in the auxotrophic strain M5, only a small difference in growth rate with the wild type was found. This difference was shown to be due to a partial leucine requirement of the $Pdh^-$ strains: when leucine was added to the growth medium of the prototrophic null mutant, its growth rate increased to a value close to the wild-type rate.[154] In the other mutant, this effect was masked by the *leu2* allele of the auxotrophic M5 strain. A partial leucine requirement has not been reported for strains carrying null mutations in *PDH*β1, *LAT1* or *PDX1*. The possibility therefore cannot be excluded that the partial leucine requirement in the *pda1* null mutant is caused by the absence of an active E1α subunit, rather than by the absence of a functional pyruvate dehydrogenase complex. However, it seems more likely that this phenotype has been overlooked because the other genes were disrupted in auxotrophic strains. This clearly illustrates the pitfalls of using auxotrophic yeast strains for physiological studies.

An explanation for the partial leucine auxotrophy of *pda1* null mutants was initially sought in production by the E1 subunit of active aldehyde, which is an early intermediate in the synthesis of valine, isoleucine and leucine (Figure 4). The fact that slow growth occurred in the absence of leucine already indicates that the pyruvate dehydrogenase complex is not the only source of active aldehyde in *S. cerevisiae*. Furthermore, the growth rate of the null mutants was not increased by addition of valine or isoleucine.[154] Synthesis of leucine branches off from that of the other branched-chain amino acids at the level of 2-oxoisovalerate (Figure 4). The first enzyme in the branch to leucine, the mitochondrial enzyme 2-isopropyl malate synthase (EC 4.1.3.12)[122] is inhibited at high CoA/acetyl-CoA ratios.[50] It has therefore been proposed that the partial leucine requirement of $Pdh^-$ *S. cerevisiae* may be due to an altered intramitochondrial CoA/acetyl-CoA ratio.[154]

A second unexpected phenotype of *pda1* null mutants was an increased frequency of respiratory-deficient mutants during batch cultivation on glucose-containing media.[154] This effect was not observed in glucose-limited chemostat cultures.[110] Loss of respiratory capacity was accompanied by loss of mitochondrial DNA ($rho^0$) and could be prevented by complementation with an intact copy of the *PDA1* gene.[154] The mechanism by which the *pda1* null mutation causes instability of the mitochondrial genome is unknown. *ILV5*, the structural gene encoding acetohydroxy-acid

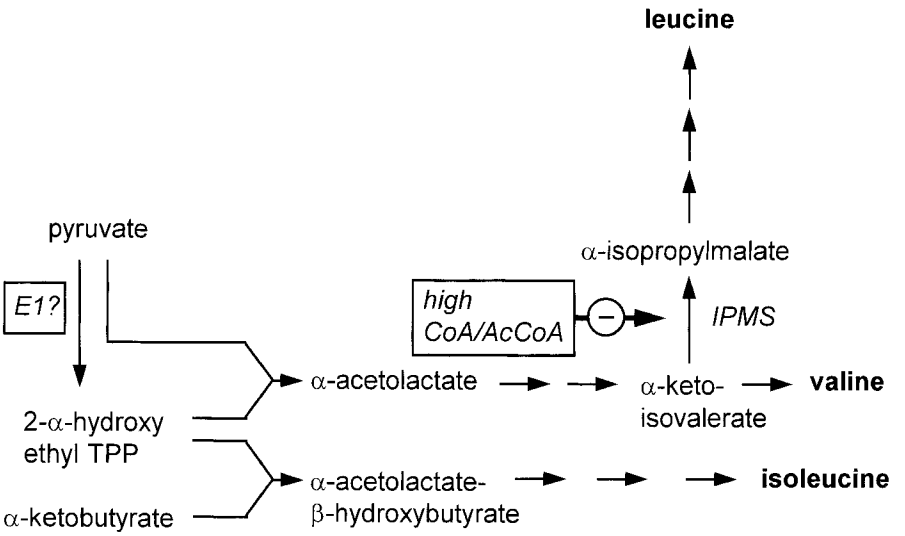


Figure 4. Possible explanations for the partial leucine requirement of *pda1* null mutants of *S. cerevisiae*. If the leucine requirement is caused by involvement of the E1 subunit of the pyruvate dehydrogenase complex in the synthesis of the intermediate 2-α-hydroxy-ethyl-thiamine pyrophosphate (2-α-hydroxy-ethyl-TPP; 'active aldehyde'), this would be expected to also affect synthesis of valine and isoleucine. Since no requirement of *pda1* null mutants for these amino acids was found, it has been proposed that absence of an active pyruvate dehydrogenase complex causes an increased intramitochondrial CoA/acetyl-CoA ratio.[154] This inhibits α-isopropyl malate synthase (IPMS), the first enzyme in the branch to leucine.

reducto-isomerase, a mitochondrial enzyme involved in branched-chain amino-acid biosynthesis,[106] acts as a multi-copy suppressor of mitochondrial DNA instability in cells lacking a functional *ABF2* gene.[162] This observation indicates that a link between branched-chain amino acid synthesis and stability of the mitochondrial genome is not unique to the *PDA1* gene.

The ability of pyruvate dehydrogenase-negative mutants to grow on glucose indicates that, in the absence of pyruvate dehydrogenase activity, the pyruvate decarboxylase by-pass (Figure 3) can meet the cellular demand for acetyl-CoA. An important difference between these two paths from pyruvate to acetyl-CoA is that the by-pass sequence, but not the pyruvate dehydrogenase reaction, involves hydrolysis of ATP (in the acetyl-CoA synthetase reaction). It was therefore anticipated that respiratory growth of a $Pdh^-$ strain should result in a lower biomass yield than in wild-type *S. cerevisiae*. Indeed, the biomass yield of a *pda1* null mutant in aerobic, glucose-limited chemostat cultures was substantially lower than that of the isogenic wild type (Table 3). The magnitude of the difference in biomass yield indicated that in wild-type *S. cerevisiae* grown aerobically under glucose limitation, conversion of pyruvate into acetyl-CoA occurs predominantly via the pyruvate dehydrogenase complex.[110]

### *Disruption of structural genes encoding pyruvate decarboxylase*

An *S. cerevisiae* strain in which all three structural *PDC* genes were disrupted, and which was consequently devoid of pyruvate decarboxylase activity, was first constructed in the auxotrophic strain M5. In complex media, its growth rate on glucose and galactose was about 20% of that of the wild type, whereas only a minor effect on growth rate was found in ethanol-grown cultures.[55] A reduced growth rate on complex media with glucose has also been reported for point mutants virtually devoid of pyruvate decarboxylase activity[79,127] and for a prototrophic strain in which the structural *PDC* genes had been replaced by dominant marker genes.[40]

In pyruvate decarboxylase-negative ($Pdc^-$) yeast, the NADH formed in glycolysis can no longer be regenerated by alcoholic fermentation, so sugar metabolism becomes critically dependent on respiration. Indeed, growth of $Pdc^-$ mutants

Table 3. Growth yields and protein contents of the wild-type *S. cerevisiae* strain T2–3D and the isogenic pyruvate dehydrogenase-negative mutant T2–3C (*pda1::Tn5ble*).

| Growth substrate | *S. cerevisiae* T2–3D (wild type) | | *S. cerevisiae* T2–3C (*Pdh*$^-$) | |
|---|---|---|---|---|
| | Biomass yield (g biomass g$^{-1}$) | Protein content (g [g biomass]$^{-1}$) | Biomass yield (g biomass g$^{-1}$) | Protein content (g [g biomass]$^{-1}$) |
| Ethanol | 0·59 ± 0·02 | 0·41 ± 0·01 | 0·59 ± 0·01 | 0·41 ± 0·02 |
| Glucose | 0·52 ± 0·01 | 0·40 ± 0·01 | 0·44 ± 0·01 | 0·40 ± 0·02 |

Cells were grown in aerobic, glucose-limited chemostat cultures (D=0·10 h$^{-1}$). Ethanol-limited chemostat cultures were included as a control. All cultures exhibited completely respiratory metabolism without significant excretion of metabolites.[110] Note that the decreased biomass yield of the *Pdh*$^-$ strain is not due to an altered biomass composition: the protein content of the biomass is essentially the same in all cultures.

on complex medium with glucose was arrested by the respiratory inhibitor antimycin A.[55] In *S. cerevisiae*, the synthesis of many respiratory enzymes is subject to glucose repression.[38,44,45,159] This offers a plausible explanation for the reduced growth rate of Pdc$^-$ mutants in complex, glucose-containing media. Repression appeared to be even stronger in defined mineral media containing glucose as the sole carbon source: the growth rate of a Pdc$^-$ strain in such a medium was negligible, although growth on ethanol was normal.[40]

In glucose-limited chemostat cultures, the low residual substrate concentrations alleviate glucose repression.[37,65,132] Therefore, if glucose repression of respiratory enzymes were the sole cause of the inability of a Pdc$^-$ strain to grow on glucose in defined mineral media, growth in glucose-limited chemostat cultures should be possible. However, when ethanol-limited chemostat cultures of a Pdc$^-$ strain were shifted to a feed containing glucose as the growth-limiting carbon source, growth came to a stand-still and the culture washed out. When ethanol-limited chemostat cultures were instead shifted to a feed containing a mixture of glucose and ethanol, glucose was completely consumed and steady-state cultures were obtained. These mixed-substrate cultures exhibited the same growth efficiency as wild-type *S. cerevisiae* grown under identical conditions.

It subsequently appeared that growth of a Pdc$^-$ strain in glucose-limited chemostat cultures was only possible when small amounts of acetate or ethanol (3– 5% of the total carbon supplied) were also added to the reservoir media (Flikweert *et al.*, unpublished).[40] Omission of these $C_2$-compounds from the medium invariably resulted in complete cessation of growth (Figure 5). Glucose-acetate mixtures that supported growth under carbon-limited conditions did not do so in batch cultures.[40] These observations show that two factors contribute to the inability of Pdc$^-$ *S. cerevisiae* to grow on glucose in batch cultures: glucose repression of the synthesis of respiratory enzymes prevents energy transduction and, secondly, absence of pyruvate decarboxylase causes a requirement for $C_2$-compounds. A Pdc$^-$ mutant grew in batch cultures on galactose-acetate mixtures, albeit slowly, whereas growth on galactose alone was

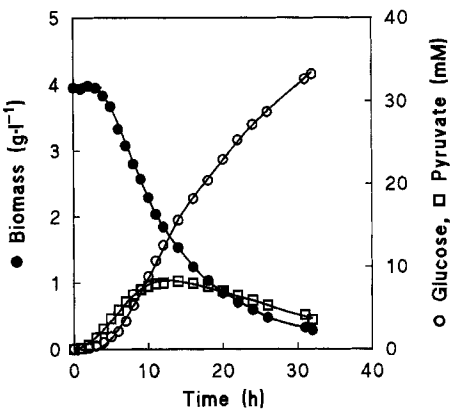


Figure 5. $C_2$-requirement of a pyruvate decarboxylase-negative mutant of *S. cerevisiae*. Concentrations of biomass, glucose and pyruvate after switching a steady-state chemostat culture (D=0.10 h$^{-1}$) of a Pdc$^-$ *S. cerevisiae* strain to a medium feed with glucose as the sole carbon source. The culture was pre-grown on a mixture of glucose and acetate, with acetate accounting for 5% of the total carbon in the feed. Note that, initially, growth appears normal as the biomass concentration remains approximately constant. Cessation of growth and wash-out occur after ca. 5 h, probably as a result of depletion of endogenous lipid reserves (Flikweert *et al.*, unpublished).

negligible (Pronk *et al.*, unpublished). This is consistent with the proposed role of glucose repression in the phenotype of Pdc$^-$ *S. cerevisiae*, since galactose represses respiration to a lesser extent than glucose.[44,78]

The $C_2$-requirement of Pdc$^-$ *S. cerevisiae* indicates that the mitochondrial pyruvate dehydrogenase complex is unable to provide all acetyl-CoA required for growth. Acetyl-CoA is a precursor for a number of biosynthetic processes including lipid synthesis, which, in *S. cerevisiae*, is a cytosolic process.[111] It has been proposed that the pyruvate dehydrogenase by-pass may be essential for the provision of acetyl-CoA in the cytosolic compartment.[40] Four observations are consistent with this hypothesis: 1. The minimum amount of acetate required to sustain carbon-limited growth on glucose-acetate mixtures (ca. 2 mmol g biomass$^{-1}$) is close to the amount of acetyl-CoA required for lipid synthesis (Pronk *et al.*, unpublished). 2. In the pyruvate dehydrogenase by-pass, conversion of acetate into acetyl-CoA is catalysed by acetyl-CoA synthetase (EC 6.2.1.1). *S. cerevisiae* contains two structural genes encoding acetyl-CoA synthetase isoenzymes, *ACS1* and *ACS2*.[10,28] The *ACS1* gene is subject to glucose repression, whereas *ACS2* is expressed constitutively.[11,74] Disruption mutants in which the constitutively expressed *ACS2* gene has been inactivated, and which therefore do not synthesize acetyl-CoA synthetase in the presence of glucose, fail to grow on this sugar.[10] 3. The three enzyme activities of the pyruvate dehydrogenase by-pass have all been reported to occur either exclusively or at least partly in the cytosol.[63,70,143] 4. ATP-citrate lyase (EC 4.1.3.8), a key enzyme in the export of acetyl-CoA units from the mitochondrial matrix to the cytosol in lipid-accumulating yeasts, is absent in *S. cerevisiae*.[111]

An interesting implication of this hypothesis is that the carnitine shuttle, which facilitates the entry of acetyl-CoA units into the mitochondria,[72,126] is apparently not reversible in growing *S. cerevisiae* cells. We are currently testing this hypothesis by *in vivo* labelling studies using $^{13}C$ nuclear magnetic resonance.

### *Disruption of structural genes encoding pyruvate carboxylase*

The effect of disruption of the two *PYC* genes was first investigated in the multiply auxotrophic strain W303.[137] In mutants expressing a single *PYC* gene, no clear phenotype was observed. However, Gancedo and coworkers found that when both the *PYC1* and *PYC2* genes were disrupted, the resulting strain was devoid of pyruvate-carboxylase activity and unable to grow in a defined medium containing glucose as the sole carbon source. Growth of this pyruvate-carboxylase-negative (Pyc$^-$) mutant was possible when aspartate instead of ammonium was added, thus by-passing the anaplerotic function of pyruvate carboxylase.[137] In a separate study, a similar phenotype was found for a *pyc1 pyc2* double null mutant constructed in the auxotrophic strain DBY746.[18] In this background, a partial aspartate requirement was also observed for a strain in which only *PYC1* had been disrupted. This suggests that the relative contribution of isoenzymes to the overall pyruvate-carboxylase activity may be a strain-dependent property.

An observation difficult to interpret at present is the report by Brewster *et al.*[18] that their Pyc$^-$ strain requires aspartate for growth on ethanol. The glyoxylate cycle is generally assumed to be the exclusive source of oxaloacetate during growth on $C_2$-compounds, since an *S. cerevisiae* strain in which *ICL1*, the structural gene encoding isocitrate lyase, had been inactivated was unable to grow on ethanol.[129] Even from a theoretical point of view, pyruvate carboxylase cannot meet the requirement for oxaloacetate during growth on ethanol: pyruvate, its substrate, has itself to be synthesized via oxaloacetate during growth on $C_2$-compounds. Also the aspartate requirement of Pyc$^-$ mutants during growth on ethanol appears to be strain-specific, as it has been reported that the Pyc$^-$ strain constructed by Stucka *et al.*[137] does not require aspartate during gluconeogenic growth.[13] Further research is required to investigate a possible role of pyruvate carboxylase during growth of *S. cerevisiae* on ethanol (e.g. by providing 'sparking' amounts of oxaloacetate to allow proper induction of glyoxylate-cycle enzymes during the initial stages of batch growth).

The inability of Pyc$^-$ strains to grow on glucose[18,137] confirms that the glyoxylate cycle cannot by-pass pyruvate carboxylase under these conditions. This is in line with the fact that synthesis of the glyoxylate-cycle enzymes is repressed by glucose and that isocitrate lyase is even inactivated in the presence of glucose[85] Interestingly, a dominant suppressor mutation has been isolated which allows Pyc$^-$ *S. cerevisiae* to grow on glucose in batch cultures.[13] The suppressor mutation was

shown to be allelic to a previously isolated mutation called *DGT1*, which causes a reduced uptake of glucose and thus alleviates glucose catabolite repression.[43]

$Pyc^-$ mutants containing the suppressor mutation exhibited significant levels of isocitrate lyase, indicating that the glyoxylate cycle had taken over the role of pyruvate carboxylase.[13] This should have substantial implications for glucose metabolism, not only because of changed metabolic fluxes, but also because the glyoxylate cycle occurs in a separate metabolic compartment (the glyoxysome). In theory, it should be possible to alleviate glucose repression in $Pyc^-$ mutants lacking the suppressor mutation by growing them in glucose-limited chemostat cultures. This may be a useful method to study the consequences of this drastic re-routing of carbon metabolism on growth efficiency and metabolic compartmentation.

## REGULATION OF METABOLIC FLUXES AT THE PYRUVATE BRANCH-POINT

### *Competition of key enzymes for pyruvate*

The contribution of any reaction in a metabolic network can be controlled at three levels: (i) synthesis of relevant enzymes, (ii) (covalent) modification of these enzymes, and (iii) the intracellular concentration of substrates and effectors. All three mechanisms can, in principle, affect pyruvate metabolism in *S. cerevisiae*. Nevertheless, it is logical that the concentration of the common substrate of the enzymes at the pyruvate branch-point has received special attention.

Holzer[59] first proposed that the intracellular concentration of pyruvate might be an important parameter in the regulation of its fermentative and respiratory dissimilation. This was based on the observation, later confirmed by others,[14,75] that the $K_m$ of the pyruvate dehydrogenase complex is an order of magnitude lower than that of pyruvate decarboxylase. As already indicated by Holzer,[59] the pyruvate dehydrogenase complex and pyruvate decarboxylase occur in different subcellular compartments and therefore cannot directly compete for pyruvate. The $K_m$ of isolated mitochondria for pyruvate is, however, similar to that of the pyruvate dehydrogenase complex.[143] According to Holzer's model, pyruvate decarboxylase is largely by-passed at low intracellular pyruvate concentrations, thus enabling respiratory dissimilation of pyruvate via the pyruvate dehydrogenase complex. In contrast, high intracellular concentrations of pyruvate will involve pyruvate decarboxylase in its dissimilation and thus trigger alcoholic fermentation. Indeed, in aerobic, glucose-limited chemostat cultures, the onset of respirofermentative metabolism ('long-term Crabtree effect'; Table 1) coincides with an increase of the extracellular pyruvate concentration (taken as an indicator for the intracellular concentration).[109]

In discussions on competition of enzymes for a common substrate it should be considered that affinity is not solely determined by $K_m$. From the Michaelis-Menten equation ($v = V_{max}\, s/(K_m + s)$) it follows that, at low substrate concentrations ($s \ll K_m$), the relation between reaction rate and substrate concentration can be approximated by the first-order equation $v = (V_{max}/K_m)\, s$. Since the amount of an enzyme in the cell also determines its contribution to metabolism at limiting substrate concentrations, affinity is equivalent to $V_{max}/K_m$ rather than $1/K_m$. As will be discussed below, this seems relevant for the competition between pyruvate decarboxylase and the pyruvate dehydrogenase complex.

In *S. cerevisiae*, pyruvate decarboxylase is present at high levels, even during glucose-limited, respiratory growth.[109,110] It is not possible to estimate the *in vivo* capacity of mitochondrial pyruvate oxidation from studies on isolated organelles. However, an indication can be obtained from experiments in which the respiratory capacity of *S. cerevisiae* is saturated by adding excess glucose to respiring, glucose-limited chemostat cultures. In such experiments, performed with either wild-type or pyruvate decarboxylase-negative strains, only a relatively small increase of the respiration rate is observed directly after a glucose pulse.[107,144] This indicates that the capacity of mitochondrial pyruvate oxidation is close to the *in vivo* rate of pyruvate oxidation in these cultures. This capacity is at least ten-fold lower than the *in vitro* pyruvate decarboxylase capacity, as calculated from activity measurements with cell extracts (Flikweert *et al.*, unpublished).

Based on the above, the high capacity ($V_{max}$) of pyruvate decarboxylase would be expected to compensate for its high $K_m$ and to allow the enzyme to compete efficiently with mitochondrial pyruvate oxidation. Nevertheless, the pyruvate dehydrogenase complex is predominantly responsible for pyruvate dissimilation in aerobic, glucose-limited chemostat cultures grown at a low dilution rate.[110] The cooperativity of pyruvate decarboxylase with

respect to pyruvate[14,62] may well be a crucial factor in preventing a major involvement of pyruvate decarboxylase under these conditions; at low pyruvate concentrations, cooperativity causes a lower conversion rate than would be predicted on the basis of Michaelis-Menten kinetics.

As discussed above, even under respiratory growth conditions, pyruvate decarboxylase plays an essential role by providing cytosolic acetyl-CoA. It is as yet unclear how, in respiring, glucose-limited cultures of *S. cerevisiae*, sufficient pyruvate is diverted via pyruvate decarboxylase to meet this assimilatory requirement, while at the same time avoiding the occurrence of alcoholic fermentation.

In the literature, discussion on competition for pyruvate by enzyme systems in *S. cerevisiae* has consistently focussed on pyruvate decarboxylase and the pyruvate dehydrogenase complex. Peculiarly, the third key enzyme at the branch-point, pyruvate carboxylase, has escaped attention. As discussed in one of the preceding sections, the *in vivo* activity of this enzyme appears to be predominantly regulated by the concentrations of substrate and effectors, rather than by changes in enzyme synthesis, for instance as a function of growth rate.

As with all assimilatory reactions, the rate of oxaloacetate formation has to increase linearly with the growth rate. One way to meet this objective is an increase of the cytosolic pyruvate concentration with increasing growth rate. In view of the $K_m$ values of pyruvate carboxylase (ca. 0·8 mM)[121] and pyruvate decarboxylase (ca. 5 mM),[109] this will inevitably lead to an increased flux through pyruvate decarboxylase. Thus, increased *in vivo* fluxes through pyruvate decarboxylase might be a direct consequence of the requirement for an increased rate of oxaloacetate formation via pyruvate carboxylase. This may play a role in the switch to respirofermentative metabolism at high growth rates in sugar-limited, aerobic chemostat cultures of *S. cerevisiae*.

Pyruvate dissimilation via pyruvate decarboxylase is not tantamount to alcoholic fermentation: acetaldehyde can also be dissimilated by respiration (Figure 3). Also, at this level, kinetic properties of key enzymes seem to favour respiratory dissimilation; the $K_m$ of acetaldehyde dehydrogenase for acetaldehyde is two orders of magnitude lower than that of alcohol dehydrogenase.[109]

At neither the pyruvate branch-point nor the acetaldehyde branch-point, is affinity for the carbon substrate the sole factor affecting its metabolic fate. In addition to the concentration of cofactors and effectors, regulation of the synthesis of key enzymes may be involved. For example, it has been proposed that the switch to respirofermentative metabolism, observed at high growth rates in glucose-limited cultures, is at least partly due to a limited synthesis of acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase. This reduces the capacity of the pyruvate dehydrogenase by-pass, thereby limiting the ability of the cells to respire pyruvate once it has been diverted via pyruvate decarboxylase.[109]

### *Pyruvate decarboxylase: a glycolytic pacemaker?*

In glycolysis, conversion of glucose to pyruvate is coupled to reduction of $NAD^+$ and to phosphorylation of ADP. Since only small amounts of these two coenzymes are available in the cytosol, prolonged glycolytic activity requires their efficient regeneration. Consequently, the rate of glycolysis can be controlled by the following three processes: (i) the linear reaction pathway leading from extracellular glucose to intracellular pyruvate (this encompasses synthesis and activity modulation of sugar carriers and enzymes of the glycolytic pathway); (ii) hydrolysis of the ATP generated in glycolysis, either by growth-associated events, futile cycles, or maintenance processes; and (iii) the reoxidation of the NADH formed in glycolysis, either by respiration or by fermentation.

It is now generally accepted that the flux through a metabolic pathway can hardly ever be described in terms of a single enzyme that limits the overall rate (so-called 'bottleneck' or 'pacemaker' reactions). Instead, it has emerged that control of metabolic flux may be distributed over more than one reaction in a pathway (for a review see ref. 68). A practical implication of this is that, in order to optimize flux capacity, it may be necessary to simultaneously increase the levels of more than one enzyme in a pathway.[97]

In *S. cerevisiae*, high glycolytic fluxes are invariably accompanied by alcoholic fermentation.[35] This implies that, particularly at high glycolytic fluxes, pyruvate decarboxylase becomes a pivotal enzyme in NADH reoxidation. It has therefore been investigated whether, and to what extent, this enzyme exerts control over the magnitude of the glycolytic flux in growing *S. cerevisiae* cells. Schmitt and Zimmermann[127] grew a series of *pdc* mutants with different levels of pyruvate decarboxylase in shake-flask cultures on a mineral

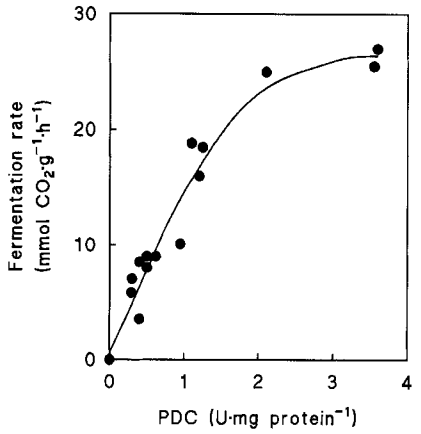


Figure 6. Correlation between pyruvate decarboxylase activity and fermentation rate. Pyruvate decarboxylase activities determined in cell extracts (U mg protein$^{-1}$) and fermentation rates (mmol ethanol $g^{-1}$ per h) in glucose-grown shake-flask cultures of various *S. cerevisiae* strains (wild-type and *pdc* mutants). Data from Schmitt and Zimmermann.[127]

medium with glucose. Over a wide range of pyruvate decarboxylase activities, fermentation rates exhibited a linear correlation with the enzyme activity measured in cell extracts (Figure 6). However, saturation set in below the wild-type pyruvate decarboxylase level, suggesting that (under the experimental conditions) pyruvate decarboxylase was not a major factor in controlling glycolytic flux in growing cells of wild-type *S. cerevisiae*. Indeed, in a now classical study on fermentation and growth rates in a set of *S. cerevisiae* strains overexpressing individual glycolytic enzymes, a four-fold overexpression of pyruvate decarboxylase did not enhance alcoholic fermentation or growth rate.[124]

The experiments discussed above seem to argue against a significant role of fermentative NADH reoxidation in controlling fermentative capacity of *S. cerevisiae*. However, the extent to which a single reaction exerts control over the flux through a pathway depends on external conditions and the overall make-up of the metabolic network. In this respect, it is important to note that the experiments of Schmitt and Zimmermann[127] and Schaaff *et al.*[124] were performed in growing shake-flask cultures. These systems are characterized by a very high glycolytic activity of the cells and by a coupling of fermentative dissimilation and biomass formation.

One of the goals of applied research on glycolytic flux in *S. cerevisiae* is improvement of the fermentative capacity of bakers' yeast. In the bakers' yeast process, biomass is grown in fed-batch cultures that are essentially aerobic and carbon-limited.[12] Subsequently, the yeast is transferred to an anaerobic dough environment, where sugar is present in excess. During the initial phase of the dough fermentation, the fermentative capacity will to a large extent depend on the conditions in the fed-batch reactor which, obviously, differ substantially from those in shake-flask cultures. Furthermore, the dough environment is probably far from optimal for balanced glycolytic activity and growth. Therefore, to identify rate-controlling steps during the initial stages of a dough fermentation, glycolytic flux measurements should be performed under conditions that resemble the dough environment, with cells pregrown under conditions that mimic those extant during the industrial production of bakers' yeast. Published data on the regulation of fermentative capacity of *S. cerevisiae* during aerobic, sugar-limited growth are few. In glucose-limited, aerobic chemostat cultures the fermentative capacity, determined under anaerobic conditions, has been reported to increase sharply above the critical dilution rate at which alcoholic fermentation sets in. This increase of fermentation capacity was parallelled by an increase of the pyruvate decarboxylase activity.[32] Although not substantiated by experiments in which pyruvate decarboxylase was overexpressed, these results indicated that pyruvate decarboxylase should not yet be eliminated as a potential target for attempts at increasing the fermentative capacity of bakers' yeast in dough.

### *Does phosphorylation of the pyruvate dehydrogenase complex occur in* S. cerevisiae*?*

In mammalian cells, activity of the pyruvate dehydrogenase complex is modulated by a reversible phosphorylation of the E1α subunit. Phosphorylation and dephosphorylation are catalysed by a pyruvate dehydrogenase-specific kinase and phosphatase, respectively, which act on three serine residues in the E1α peptide. Phosphorylation of all three serines leads to complete inactivation, whereas phosphorylation of one or two serines merely causes a decrease of enzyme activity. The activity of the mammalian pyruvate dehydrogenase kinase and phosphatase is regulated by a

number of physiological parameters, including the intramitochondrial $NAD^+$/NADH, CoA/acetyl-CoA and ADP/ATP ratios (for reviews see refs 113,161). This reversible modulation of the activity of the pyruvate dehydrogenase complex provides a mechanism to adapt the flux through this key enzyme to the carbon, redox and energy status of mammalian cells.

If the pyruvate dehydrogenase complex in *S. cerevisiae* were subject to a similar rapid activation-inactivation mechanism, this could (in principle) be highly relevant during transient growth conditions. For example, if the pyruvate dehydrogenase complex were inactivated after exposure of respiring cells to excess glucose, this might contribute to the redirection of pyruvate metabolism via pyruvate decarboxylase that is seen during the short-term Crabtree effect (Table 1).

During the original studies of Kresze and Ronft[75,76] on the isolation and characterization of the pyruvate dehydrogenase complex from *S. cerevisiae*, no indications were obtained for the existence of a specific kinase in this yeast. However, it has been demonstrated that the *S. cerevisiae* E1α subunit can be phosphorylated and inactivated by pyruvate dehydrogenase kinases from bovine kidney and rat liver.[91,142] Although these studies did not show the presence of a pyruvate dehydrogenase kinase in *S. cerevisiae*, it was demonstrated that the sequence around the phosphorylated serine residue (at position 313 from the first methionine)[136] was highly homologous to that of bovine and porcine E1α phosphorylation sites.[142] Replacement of ser313 by a his residue abolished pyruvate dehydrogenase activity (Wenzel and Steensma, unpublished).

Upon incubation of isolated *S. cerevisiae* mitochondria with radiolabelled ATP or pyrophosphate, phosphorylation of a 40 kDa mitochondrial peptide was observed, which was tentatively identified as the pyruvate dehydrogenase E1α subunit.[105] This result should be interpreted carefully, since the estimated size of the labelled peptide is smaller than that predicted from the nucleotide sequence of the *PDA1* gene.[7,136] More convincing evidence that a pyruvate dehydrogenase kinase is present in *S. cerevisiae* was obtained by James *et al.*[64] After incubation of isolated mitochondria with radioactive phosphate, a labelled 45 kDa peptide could be immunoprecipitated with a specific antiserum against the *S. cerevisiae* E1α subunit. Moreover, it was demonstrated that pyruvate dehydrogenase activity of isolated mitochondria could be reversibly inhibited by ATP. A peculiar observation in this study was that phosphorylation could only be reproducibly observed in exponentially growing cells when galactose was the carbon source.[64] This seems difficult to reconcile with the fact that respiration rates during growth on galactose are higher than during growth on glucose.[78]

It is clear that the mechanisms involved in short-term regulation of pyruvate dehydrogenase activity require further study. In this respect, it is interesting to note that the systematic sequencing of the *S. cerevisiae* genome has revealed an open reading frame that exhibits 28% homology with rat pyruvate dehydrogenase kinase (YIL042C; Barrell and Rajandream, GenBank Z46861). It will be interesting to study whether the *in vitro* phosphorylation phenomena discussed above are abolished when this open reading frame is disrupted.

In wild-type *S. cerevisiae*, inactivation of the pyruvate dehydrogenase complex does not necessarily preclude respiratory dissimilation of pyruvate, as this may still occur via the pyruvate dehydrogenase by-pass (Figure 3). In a pyruvate decarboxylase-negative strain, only the pyruvate dehydrogenase complex can act as an interface between glycolysis and TCA cycle. This makes $Pdc^-$ *S. cerevisiae* strains excellently suited to study the *in vivo* regulation of pyruvate dehydrogenase activity. We have therefore used a $Pdc^-$ strain to investigate if phosphorylation of E1α is a relevant mechanism during the exposure of respiring *S. cerevisiae* cultures to excess glucose (Flikweert *et al.*, unpublished). In a $Pdc^-$ strain, inactivation of the pyruvate dehydrogenase complex should largely abolish respiration of glucose. However, such a decrease of the respiration rate was not observed after a glucose pulse to carbon-limited, respiring chemostat cultures (Figure 7).

An additional advantage of $Pdc^-$ *S. cerevisiae* is that pyruvate decarboxylase does not interfere with assays of pyruvate dehydrogenase activity in cell extracts. As a result, activity of the complex could easily be measured as pyruvate- and CoA-dependent reduction of $NAD^+$ by cell extracts (Flikweert *et al.*, unpublished). After a glucose pulse, no strong decrease of pyruvate dehydrogenase activity was observed (Figure 7), thus confirming that, at least under these conditions, phosphorylation of E1α is not a relevant regulatory mechanism.

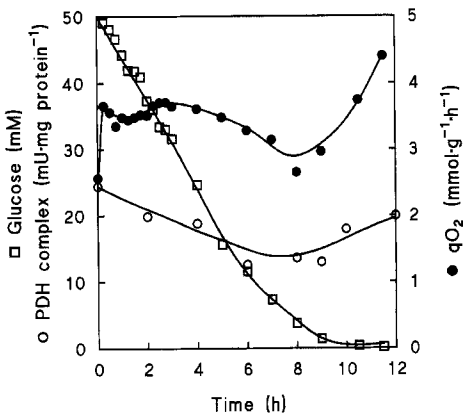


Figure 7. The pyruvate dehydrogenase complex is not inactivated after a glucose pulse to a respiring chemostat culture. At time zero, 50 mM-glucose was added to a carbon-limited chemostat culture ($D=0{\cdot}1\ h^{-1}$) of a pyruvate decarboxylase-negative *S. cerevisiae* strain, growing on a mixture of glucose and acetate (see Figure 5). Oxygen uptake by the culture was determined on-line, pyruvate-dehydrogenase activities were determined in crude cell extracts (Flikweert *et al.*, unpublished).

## CONCLUSIONS

### *Methodology*

Introduction of defined mutations in structural genes is an indispensable tool for studies on function and regulation of branched metabolic pathways. However, functional or regulatory effects of such mutations on amino acid or nucleotide synthesis may easily be overlooked in auxotrophic strains (as illustrated by the leucine requirement of *pda1* null mutants). The yeast strains used in this type of study should therefore lack auxotrophic lesions. The multitude of nutrients and intermediates present in complex media may obscure effects of mutations on intermediary carbon metabolism (for example, the aspartate requirement of $Pyc^-$ mutants might easily have been overlooked in media containing peptone). Therefore, defined mineral media should be used for growth studies. Shake-flask cultures, however useful for the initial phenotypic characterization of yeast strains, have major drawbacks for physiological research, especially with *S. cerevisiae*. Chemostat cultivation, although more laborious, allows independent manipulation of key culture parameters and enables studies on sugar metabolism without the major interference of glucose repression (as illustrated by the analysis of the phenotype of $Pdc^-$ mutants).

### *Physiological role of key reactions at the pyruvate branch-point*

The pyruvate dehydrogenase complex is predominantly responsible for the conversion of pyruvate to acetyl-CoA during glucose-limited, respiratory growth. This enzyme complex is to some extent redundant: in $Pdh^-$ mutants, this role can be taken over by the pyruvate dehydrogenase by-pass. However, the latter route involves ATP hydrolysis and therefore leads to lower biomass yields in respiring, glucose-limited cultures. Additional phenotypic characteristics of *pda1* null mutants apparent at high growth rates in batch cultures (a requirement for leucine and an increased frequency of respiratory-deficient mutants) indicate that the role of the pyruvate dehydrogenase complex is not limited to respiratory pyruvate dissimilation.

The by-pass route is indispensable for growth of *S. cerevisiae* on glucose. Two factors contribute to the inability of $Pdc^-$ mutants to grow on glucose in batch cultures: glucose repression of respiration and a requirement for $C_2$-compounds. The latter requirement is probably caused by an inability of the mitochondrial pyruvate dehydrogenase complex to provide the cytosolic acetyl-CoA needed for lipid synthesis.

The inability of $Pyc^-$ mutants to grow on glucose in batch cultures indicates that, in wild-type *S. cerevisiae*, the glyoxylate cycle cannot by-pass the anaplerotic pyruvate carboxylase reaction. Isolation of a suppressor mutant of a $Pyc^-$ strain, in which glucose repression of glyoxylate-cycle enzymes is alleviated, shows that this is due to regulatory rather than physiological constraints.

### *Regulation of fluxes at the pyruvate branch-point*

Although their levels are not constant, the key enzymes at the pyruvate branch-point are expressed under all growth conditions investigated. Different isoenzymes contribute to the overall activity of pyruvate decarboxylase (*PDC1, PDC5* and, possibly, *PDC6*) and pyruvate carboxylase (*PYC1* and *PYC2*). Although the regulation of the structural genes encoding these isoenzymes may differ, there is no conclusive evidence that they have distinct physiological functions.

High intracellular concentrations of pyruvate favour its dissimilation via pyruvate decarboxylase, which has a high $K_m$ for pyruvate and a high capacity. Since the capacity of the further reactions

of the pyruvate dehydrogenase by-pass is limited, diversion of pyruvate metabolism via pyruvate decarboxylase will ultimately lead to alcoholic fermentation in aerobic cultures. The assimilatory enzyme pyruvate carboxylase may play a key role in this diversion of pyruvate dissimilation if its *in vivo* activity is controlled by the intracellular pyruvate concentration.

*In vitro* experiments have indicated that the *S. cerevisiae* pyruvate dehydrogenase complex can be inactivated by phosphorylation. The physiological function of this short-term regulatory mechanism is, as yet, unclear. Inactivation of the complex does not occur after exposure of respiring cells to excess glucose.

## OUTLOOK

Research on pyruvate metabolism has come a long way since Neuberg's work in the early years of this century. Integration of molecular genetics and quantitative physiology continues to generate new and exciting results, which pay off the time investments required for construction of suitable yeast strains and implementation of techniques for controlled cultivation. Recent studies illustrate the intricacy of the *S. cerevisiae* metabolic network: absence of pyruvate-metabolizing enzymes affects processes as diverse as amino-acid biosynthesis, stability of the mitochondrial genome and lipid synthesis.

In future research, genetic modification of pyruvate metabolism should be extended from straightforward elimination or overexpression of key enzymes to more subtle changes of their catalytic and regulatory properties. Quantitative analysis of the physiology of *S. cerevisiae* strains in which native genes are replaced by heterologous analogs (e.g. replacement of pyruvate decarboxylase by a mammalian lactate dehydrogenase)[108] will yield further interesting results. Techniques for accurate determination of metabolite concentrations in growing yeast cultures[73,140] have to be implemented to translate *in vitro* enzyme characteristics to the regulation of *in vivo* fluxes. Wherever possible, such measurements should take into account subcellular compartmentation of pyruvate metabolism. Finally, a deeper understanding of the regulation of fluxes at the pyruvate branch-point will have to encompass the signalling pathways involved in modulation of enzyme synthesis and activity in *S. cerevisiae* (for a review, see ref. 141).

Achievement of the ultimate (and ambitious) aim of research in this area, a complete and quantitative description of the regulation of metabolic fluxes at the pyruvate branch-point *in S. cerevisiae*, will enable a better control of product and by-product formation in the industrial applications of this yeast. Furthermore, unravelling of the function and regulation of this comparatively well-known area in the metabolic network of *S. cerevisiae* may be helpful in the development of research strategies for the functional analysis of other genes and gene products whose functions are, at present, less understood.

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