

**THE BIOSYNTHESIS OF THE COFACTOR
PYRROLOQUINOLINE QUINONE**

**TR diss
1669**

MARIO VAN KLEEF

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus,
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ten overstaan van een commissie aangewezen
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door

MARIO VAN KLEEF
geboren te Rotterdam
Scheikundig ingenieur



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Dit proefschrift is goedgekeurd door de promotor Prof. dr. ir. J.A. Duine

Overige leden: Prof. dr. R. Verpoorte

Prof. dr. J.G. Kuenen

Prof. dr. ir. P. v.d. Putte

Dr. ir. J. A. Frank

Dr. J. P. van Dijken

Prof. dr. ir. G. D. Vogels (reserve)

This study was carried out at the Department of Microbiology and Enzymology of the University of Technology Delft, The Netherlands, and subsidized by the Netherlands Technology Foundation (STW).

STELLINGEN

1. Biosyntheseroutes zijn lijdenswegen.
2. De accumulatie van methanol in celsuspensies van Methylosinus trichosporium onder invloed van hoge concentraties fosfaationen wordt door Metha et al. ten onrechte toegeschreven aan de remming van het enzym methanoldehydrogenase.
 - Metha, P.K., Mishra, S. & Ghose, T.K. (1987) J. Gen. Appl. Microbiol. 33, 221-229.
 - Dijkstra, M. (1988) PhD thesis, Delft.
3. Het feit dat Acetobacter peroxidans - een catalase negatief organisme - in 1925 door Visser 't Hooft is geïsoleerd uit een oplossing waterstof peroxide maakt het uiterst onwaarschijnlijk dat catalase het belangrijkste enzym voor de afbraak van peroxiden is.
 - Visser 't Hooft (1925) Dissert. Delft.
 - Verduyn, C., Giuseppin, M.L.F., Scheffers, W.A. & van Dijken, J.P. (1988) Appl. & Env. Microbiol. 54, 2086-2090.
4. Het gebruik van een professionele goochelaar in het onderzoek van Nature naar de betrouwbaarheid van de resultaten van Davenas et al., waarbij biologische activiteit van antilichamen werd aangetoond in oplossingen zonder antilichaam moleculen, zal wellicht leiden tot de aanstelling van een groot aantal van deze lieden aan universiteiten en andere wetenschappelijke instellingen.
 - Davenas, E. et al. (1988) Nature 333, 816-818.
 - Maddox, J., Randi, J. & Stewart, W. (1988) Nature 334, 287-290.
5. Het feit dat massaspectrometrie van met semicarbazide gederivatiseerd methylamine dehydrogenase uit bacterie W3A1, na proteolyse en zuivering, aanleiding geeft tot een PQQ-achtige verbinding zonder de drie carboxylgroepen, zou erop kunnen duiden dat deze methode niet geschikt is voor cofactor identificatie.
 - van der Meer, R.A., Jongejan, J.A. & Duine, J.A. (1987) FEBS Lett. 221, 299-304.
 - McIntire, W.S. & Stults, J.T. (1986) Biochem. Biophys. Res. Commun. 141, 562-568.
6. Het feit dat Pseudomonas chlororaphis en P. aureofaciens geen aerobe denitrificerders zijn, vormt nog geen bewijs voor het niet bestaan van aerobe denitrificatie, zoals door Christensen en Tiedje wordt gesuggereerd.
 - Christensen, S. & Tiedje, J.M. (1988) FEMS Microbiol. Ecology 53, 217-221.
 - Robertson, L.A. (1988) PhD thesis, Delft.

7. Daar de binding van carrieramfolieten de fysische eigenschappen van een eiwit kan veranderen, dienen de metingen van Anthony et al. aan cytochroom c_L , opgezuiverd met behulp van preparatieve IEF, voorzichtiger geïnterpreteerd te worden.
 - Rodkey, L.S. (1988) J. Chrom. 437, 147-159.
 - Cross, A.R. & Anthony, C. (1980) Biochem. J. 192, 421-427.
 - O'Keefe, D.T. & Anthony, C. (1980) Biochem. J. 192, 411-419.
8. De methode van Fluckiger et al. om PQQ aan te tonen in bloedplasma is niet-specifiek en leidt daarom tot valse positieve resultaten.
 - Fluckiger, R., Woodtli, T. & Gallop, P.M. (1988) Biochem. & Biophys. Res. Commun. 153, 353-358.
9. De problemen bij het vaststellen van incorporatie van radioactieve isotopen in metaboliëten, alsmede de snelle ontwikkelingen bij instrumentele analysetechnieken als NMR en massaspectrometrie, zouden weleens tot gevolg kunnen hebben dat eerstgenoemde methode voor biosynthetisch onderzoek steeds minder gebruikt gaat worden.
 - Dit proefschrift
10. De slogan 'voorkomen is beter dan genezen' heeft voor werknemers van de TU Delft sinds 1976 een bijzondere betekenis.
11. De typische Britse volksaard wordt treffend geïllustreerd door het feit dat, als men in Harwich met de auto van de veerboot komt, de borden met de waarschuwing dat men hier links dient te rijden aan de linkerkant van de weg geplaatst zijn.
12. De winsten die behaald kunnen worden met grootschalige productie van PQQ lijken bij sommige researchgroepen een wel erg grote drang tot het vinden van toepassingen tot gevolg te hebben.

Schiedam, 11 oktober 1988

Mario van Kleef

"Een kleine stap voor de mensheid, maar een grote stap voor de mens"

Sorry, Neil

Aan mijn ouders

Woord van dank

Vijf jaar biosynthese van PQQ is natuurlijk niet niets. Een periode met ups, maar ook downs, waarin ik veel geleerd heb. Dit 'lustrumboekje' bewijst dat het tenslotte toch nog goed gekomen is.

De afgelopen jaren zijn natuurlijk geen 'one man show' geweest. Veel mensen hebben, direkt of indirekt, geholpen met het tot stand komen van dit proefschrift, en die mensen wil ik dan ook op deze plaats bedanken.

In de eerste plaats mijn promotor Hans Duine, voor de begeleiding bij het onderzoek en het schrijven van de artikelen. Verder al mijn collega's binnen en buiten de TU Delft, maar in het bijzonder natuurlijk van Enzymologie, voor alles wat zij de afgelopen jaren voor mij hebben gedaan. Uit angst iemand te vergeten zal ik geen namen noemen. De lijst zou bovendien wel erg lang worden.

Zeker niet vergeten mag ik ook mijn vrienden, die, soms waarschijnlijk zonder dat ze dat zelf wisten, steun en toeverlaat waren in de mindere periodes. En hoewel ik ook hier liever geen namen noem, wil ik toch een uitzondering maken voor Ineke, voor je begrip voor mijn onderzoek ("en met dat ene dingetje ben je nu vijf jaar bezig?") en de ontelbare gezellige uurtjes.

Hoewel dit stukje er eigenlijk mee had moeten beginnen, eindigt mijn dankwoord bij mijn ouders. Voor jullie belangstelling en ondersteuning, omdat jullie er altijd waren als ik jullie nodig had, en ik weet dat dit in de toekomst zo zal blijven. Ik draag mijn proefschrift dan ook aan jullie op.

PUBLICATIONS:

The publications designated 1 and 2, which are inserted in this thesis as Chapters II and V, respectively, are reprinted by permission from the relevant Journals:

- [1] van Kleef, M.A.G., Dokter, P., Mulder, A.C., and Duine, J.A. (1987) Detection of the cofactor pyrroloquinoline quinone. *Anal. Biochem.* 162, 143-149.
- [2] van Kleef, M.A.G., and Duine, J.A. (1988) Bacterial NAD(P)-independent quinate dehydrogenase is a quinoprotein. *Arch. Microbiol.* 150, 32-36.
- [3] van Kleef, M.A.G., Jongejan, J.A., and Duine, J.A. (1988) Factors relevant in the reaction of PQQ with amino acids. Submitted to *Eur. J. Biochem.*
- [4] van Kleef, M.A.G., and Duine, J.A. (1988) Factors relevant in bacterial PQQ production. Submitted to *Appl. & Env. Microbiol.*
- [5] van Kleef, M.A.G., and Duine, J.A. (1988) A search for intermediates in the biosynthesis of PQQ. Submitted to *Arch. of Microbiol.*
- [6] van Kleef, M.A.G., and Duine, J.A. (1988) L-tyrosine is the precursor of PQQ biosynthesis in *Hyphomicrobium* X. *FEBS Lett.*, accepted for publication.
- [7] Dokter, P., van Kleef, M.A.G., Frank, Jzn., J., and Duine J.A. (1985) Production of quinoprotein D-glucose dehydrogenase in the culture medium of *Acinetobacter calcoaceticus*. *Enzyme Microbiol. Technol.* 7, 613-616.
- [8] Groen, B.W., van Kleef, M.A.G., and Duine, J.A. (1986) Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*. *Biochem. J.* 234, 611-615.
- [9] Dokter, P., van Wielink, J.E., van Kleef, M.A.G., and Duine, J.A. (1988) Cytochrome b562 from *Acinetobacter calcoaceticus* LMD 79.41: its characteristics and role as electron acceptor for quinoprotein glucose dehydrogenase. *Biochem. J.*, accepted for publication.
- [10] Biville, F., Mazodier, P., Gasser, F., van Kleef, M.A.G., and Duine, J.A. (1988) Physiological properties of a PQQ⁻ mutant of *Methylobacterium organophilum*. *FEMS Microbiol. Lett.* 52, 53-58.

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ABBREVIATIONS

PQQ	pyrroloquinoline quinone, semisystematic name for 2,7,9-tricarboxy-1 <i>H</i> -pyrrolo(2,3- <i>f</i>)quinoline-4,5-dione
PQQH ₂	quinol form of PQQ
DCPIP	2,6-dichlorophenolindophenol
Wurster's blue	free radical of N,N,N',N'-tetramethyl- <i>p</i> -phenylenediamine
GDH	glucose dehydrogenase
QDH	quinone dehydrogenase
MDH	methanol dehydrogenase
MADH	methylamine dehydrogenase
ADH	alcohol dehydrogenase
AldDH	aldehyde dehydrogenase
BSA	bovine serum albumin
DAO	diamine oxidase
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
DOPA	3,4-dihydroxy phenylalanine
GOD-PAP	glucose oxidase-peroxidase aminophenazone
M _r	molecular mass
HPLC	high performance liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance
EDTA	ethylenediamine tetra-acetate
ATP	adenosine 5'-triphosphate
NAD	nicotinamide adenine dinucleotide
FAD	flavine adenine dinucleotide
E' ₀	midpoint potential at pH 7.0
OD	optical density
PTS	phospho-enolpyruvate phosphotransferase system

Chapter I

General introduction

1. THE BIOSYNTHESIS OF COFACTORS

1.1 Introduction

Whereas some enzymes depend for their activity only on protein structure as such, others require in addition one or more non-proteinaceous compounds. These components, called cofactors or cofactors, may be metal ions or organic molecules. They usually function as intermediate carriers of functional groups, of specific atoms, or of electrons that are transferred in the overall enzymatic reaction. Although cofactors are sometimes very tightly bound to the protein chain, they may also be loosely bound. The catalytically active enzyme-cofactor complex is called the holoenzyme. On removal of the cofactor the remaining protein, called apoenzyme, becomes inactive.

Since cofactors have catalytic functions, they are usually present in cells only in trace amounts. Usually, they are related to one of the vitamins, trace organic substances that are vital to the function of cells. The biological importance of vitamins was first recognized when it was found that some organisms cannot synthesize them and must therefore be supplied with them from exogenous sources.

The development of very sensitive methods for the analysis of minute amounts of sample has been closely followed by progress in the field of vitamin and cofactor research. After the isolation and establishment of the molecular structures of the vitamins a few decades ago - a great achievement at that time - studies on the biosynthesis of these compounds in species that do not need them in their diet were initiated. Vitamins and cofactors are formed as a result of a complex series of consecutive reactions, which are generally subjected to some form of regulation. Although the biosynthetic routes of bulk components of cells - amino acids, nucleotides, sugars and lipids - have been largely established, studies on the biogenesis of cofactors have been delayed, for quite some time, predominantly because of the minute quantities (of cofactor) produced by organisms. It is therefore not surprising that in some cases only in very recent years,

following the development of new, sensitive physical techniques, dramatic progress in this field has been made.

1.2 Experimental approaches

To unravel a biosynthetic pathway of a cofactor, three different approaches are generally used, as discussed in the following. In the past, it has been tried many times to establish precursor-product relationships by searching for compounds that stimulate the production of the cofactor. However, experiments of this type generally cannot provide evidence which has direct bearing on the biosynthesis of the product. An increased yield of a component whose biosynthesis is being studied may result not because the administered substrate is a direct precursor, but indirectly, as a consequence of a complex sequence of metabolic events induced by its presence. Conversely, administration of a real precursor does not necessarily enhance production of the cofactor, since this is generally regulated in some way, for instance by end-product inhibition. Experiments of this type can therefore at best serve to give directions to future investigations, in suggesting compounds or organisms that might be useful in mutant and/or incorporation studies (Hill and Spenser, 1986).

1.2.1 Mutant studies

These studies are usually performed with bacteria and other microorganisms due to the relative ease with which mutants can be obtained in these organisms. The approach is based on the fundamental concept that the chemistry of metabolic pathways proceeds in a series of discrete steps, each catalyzed by a single enzyme, which is, in turn, the functional expression of one discrete gene. This "one gene-one enzyme" relationship was formulated already in 1941 by Beadle and Tatum.

The effect of a mutation in a single enzyme of a certain biosynthetic pathway is illustrated in Fig. 1. From a generally available precursor A a product P is synthesized via intermediates B, C, D, and E. A genetic block, e.g. between C and D will prevent the production of P, unless an intermediate after the block (D or E) is supplied. Provided that a detection method can be developed (for example the inability to grow, unless product P is supplied), mutant strains of many organisms can be obtained more or less

easily by so-called "mutagenic treatments" (e.g. exposure to UV-irradiation or mutagenic chemicals). By isolating a large number of mutants, it can be expected that representatives of all steps are present. Since the biosynthesis of a compound is usually regulated by end-product inhibition (the presence of product P inhibits the first enzyme of the pathway, see Fig. 1), a genetic block in one of the steps does not prevent the functioning of the enzymes in the other steps. Therefore, a mutation between

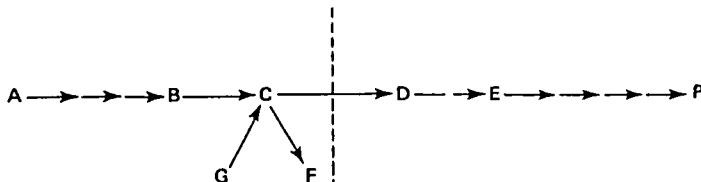


Fig. 1 : Intermediates in a biosynthetic pathway.

C and D results in accumulation of C, sometimes in fairly high amounts, together with lesser ones of B. If the quantity of C is sufficiently large, its structure can be elucidated. When no accumulation occurs, the chemical nature of the intermediates of a pathway may be established by testing the ability of different compounds to support the growth of a mutant in the absence of the end-product of the pathway. The major problem in such experiments is to decide which compounds should be tested, since a rational choice can only be made from the analogy with other pathways or by chemical reasoning, taking the structure of the end-product into account.

The mutual influence of a group of mutants on each other's ability to grow can often be assessed with cross-feeding experiments, in which one mutant is grown in the presence of medium obtained from the culture of a mutant belonging to another group. Alternatively, the two mutants can be grown together, in which case auxotrophic (or other genetic) markers can be used to establish which mutant feeds the other. These type of experiments allow the mutants to be placed in a functional sequence.

The use of mutants in investigations on biosynthetic precursor-product relationships is not without pitfalls, as sometimes many different interpretations are possible. Some problems encountered in the assessment of a given metabolite as an intermediate of a certain biosynthetic pathway are discussed below:

- a. Only those mutations concerning an enzyme that is directly involved in one of the steps in the biosynthetic pathway are of value. Sometimes, however, steps in the biosynthetic route are performed by a multi-enzyme complex, in which case different mutations do not give rise to accumulation of different intermediates.
- b. Certain mutations (especially deletions) may affect a gene which is not involved in any step of the biosynthetic pathway, but lies close together on the chromosome with the genes of the pathway under investigation. In this case, accumulation of compounds that are not intermediates in the route of biosynthesis being studied, may be observed (Chapter VI).
- c. A compound isolated from the culture medium of a given mutant may not be the actual biosynthetic intermediate, but the product of some further degradation (cf. F in Fig. 1). This situation can be recognized either by the failure of F to be used by other mutants, or sometimes simply by structural criteria (in cases where already more information on the pathway exists).
- d. A compound (e.g. G in Fig. 1) - not being a true intermediate - may have growth-factor activity for appropriate mutants by conversion to a true intermediate (C) by (a) reaction(s) which is (are) not part of the real biosynthetic sequence. In case this compound G is structurally related to C, this situation can be simply recognized. Otherwise, a more detailed analysis is required, for example the failure to isolate any mutants accumulating G, or the failure of G to act as growth-factor in other organisms, which carry the same mutation.
- e. A true intermediate may fail to be utilized by other mutants because permeability barriers prevent the compound from entering the cell. Especially strongly polar compounds are often unable to pass the bacterial membrane while the reverse process, excretion of highly polar intermediates, generally proceeds smoothly. The status of such nutritionally unavailable compounds can only be established by using permeabilized cells or cell-free extracts.
- f. The administration of high, nonphysiological concentrations of metabolites may cause deviations from the usual metabolism.
- g. Although the vast majority of reactions are mediated by enzymes, the fact should not be overlooked that this need not always to be the case.

Also spontaneous rearrangements do exist, e.g. the conversion of α -amino- β -carboxymuconate ϵ -semialdehyde to quinolinate in the biosynthesis of NAD from tryptophan (Keys and Hamilton, 1987).

From what has been said in the preceding paragraphs, it will be clear that identification of a compound as a true intermediate in a given biosynthetic pathway is by no means an easy or straightforward matter, and there are cases where a clear-cut decision is hardly possible at all. Definite answers to questions of precursor-product relationships can therefore be given only when mutant studies are accompanied by incorporation studies and/or enzymatic analysis.

1.2.2 Incorporation studies using radioactive or stable isotopes

To establish the precursors from which a certain metabolite is synthesized, the method generally applied is to study the incorporation of radioactive or stable isotopes in this metabolite when the organism is grown in the presence of specifically labeled compounds. In the past decades feeding experiments with radioactive compounds labeled with ^{14}C or ^3H have been performed predominantly. Many times, however, not the distribution of label within the product, but only percentage incorporation, specific radiochemical yield or dilution value was determined. There are several reasons why this approach is not satisfactory, especially in cases where only minute amounts of metabolite are produced, e.g. in studying the biosynthesis of cofactors: (i) Since the molar concentration of biosynthesized product is several orders of magnitude lower than that of labeled substrate which is applied, it is often necessary to dilute the biosynthesized product with non-radioactive carrier to obtain a sample with which chemical manipulations (purification, derivatization) can be performed. This method, called isotope dilution, is extremely susceptible to interference with contaminants. The problems become even greater when the newly biosynthesized product is not homogeneous, but consists of a mixture of two or more biologically active compounds, since only one of these compounds is diluted. (ii) The establishment of percentage incorporation (i.e., $100 \times \text{total activity (mCi) recovered in product} / \text{total activity (mCi) administered in precursor}$) depends on the yield of purified product, which is not an absolute parameter, but a function of the skill of the investigator. (iii) Although the determination of specific radiochemical

yield (100 x molar specific activity (mCi/mmol) of biosynthesized product before carrier dilution/ molar specific activity (mCi/mmol) of administered substrate), or its reciprocal, the dilution value, are independent of losses, reliable values require rigorous purification of end product and knowledge of the exact value of the specific activity of labeled substrate (which usually cannot be determined by the investigator). Moreover, the parameters will depend on the dilution of administered labeled substrate by endogenously biosynthesized unlabeled substrate.

From the foregoing, it will be clear that the only way to derive reliable precursor-product relationships in tracer experiments with intact organisms is the establishment of the distribution of label within the product. Using radioactive tracers, this is only possible following laborious stepwise degradation, which is often extremely difficult and time consuming (Hill and Spenser, 1986).

In recent years, there has been a pronounced increase in the use of stable isotopes (^{13}C and ^{15}N) for incorporation studies. This is due to the improved sensitivity, selectivity and reliability of mass spectrometry and NMR spectroscopy. Especially the application of ^{13}C and ^{15}N -NMR spectroscopy was hampered in the past by its low sensitivity, but the advent of high field superconducting magnets and recent advances in probe and coil design have led to dramatical improvements. Besides the advantage of absence of radioactivity, the distribution of labeled atoms within the product can be established by simple spectral interpretation, without the need for cumbersome sample degradation procedures. Contamination dangers do not exist, since only the labeled compound is seen by the method of measurement (Lapidot, 1984). The only drawback is that sufficient incorporation (> 1%) has to occur for these methods to be accurate. This is generally the case with bacterial and fungal cultures, but not with higher plants, where incorporations as low as 0.1-1.0% are usually found (Weiss and Edwards, 1980). Only an isotope ratio mass spectrometer offers the required precision in these cases (0.01%), but this requires relatively large samples (Lapidot, 1984).

1.2.3 Enzymatic analysis - in vitro studies

Once the precursors of a biosynthetic route and/or the nature of intermediates have been established, definite proof of postulated reactions

must be obtained by enzymatic analysis using *in vitro* (cell-free extract) systems. Incubation of (labeled) precursors/intermediates and isolation of the products from one or more enzymatic reaction(s), in combination with experiments with mutants unable to carry out a specific enzymatic step should give definite proof for the postulated route. Finally, the enzymes required for biosynthesis of the compounds under study may be purified and characterized (Cohen, 1967).

1.3 The biosynthesis of cofactors: selected examples

In this section, studies on the biosynthesis of several cofactors will be reviewed briefly. This paragraph should not be considered as a complete overview, but merely as an illustration of the different approaches used with this specific class of compounds. Emphasis will be laid on those cofactors which frequently occur in microorganisms, and have, or are supposed to have, relevance to the biosynthesis of pyrroloquinoline quinone (PQQ).

1.3.1 Thiamin (Vitamin B₁)

Thiamin consists of a substituted pyrimidine joined by a methylene bridge to a substituted thiazole. It occurs in cells mainly in its active cofactor form, thiamin pyrophosphate, formerly called cocarboxylase (Lehninger,

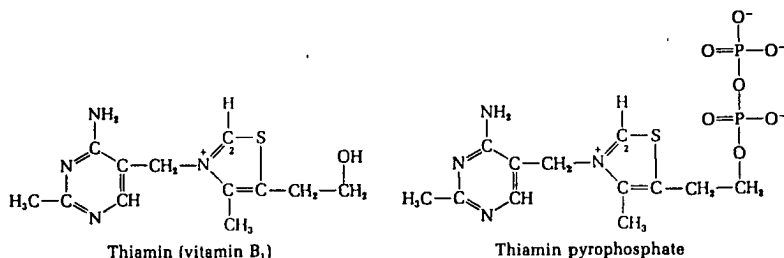


Fig. 2 : Structure of thiamin and thiamin-pyrophosphate.

1975). Although the major pathway for its synthesis from the preformed heterocyclic precursors, pyrimidine pyrophosphate and thiamin monophosphate, has been known for some time (Cheldelin and Baich, 1967), the biosynthetic route to the precursors has yet to be established. Most probably, different

pathways exist in both prokaryotes and eukaryotes for the synthesis of the precursors. Incorporation studies with radioactive and stable isotopes have been used to establish the origin of the thiazole moiety. The C₂ and N from the thiazole in *Saccharomyces cerevisiae* originate from glycine (Linnett and Walker, 1968, Ikami *et al.*, 1976, White and Spenser, 1979), but in *Escherichia coli* (Estramareix and Therisod, 1972) and *Salmonella typhimurium* (Bellion *et al.*, 1976) from tyrosine. As to the origin of C-4', -4, -5, -5' and -5" of the thiazole, different precursors have been proposed, i.e. pyruvate and glycerol (White, 1978) and pentulose in *E. coli* (David *et al.*, 1982) and *S. cerevisiae* (White and Spenser, 1982), and a 5-carbon compound such as ribose and ribulose in *Candida utilis* (Yamada *et al.*, 1985). Experiments with ³⁵S have indicated cysteine as the sulphur source of the thiazole, while the carbon skeleton is not incorporated (Tazuya *et al.*, 1987). Also for the biosynthetic pathway of the pyrimidine moiety, different pathways seem to exist in prokaryotes and eukaryotes (Tazuya *et al.*, 1987). Experiments with radioactive ¹⁴C have shown that in prokaryotes the pyrimidine ring is synthesized from 5-aminoimidazole ribotide [an intermediate in the purine synthesis, (Newell and Tucker, 1968)] and glycine (Estramareix and Lesieur, 1969, and Estramareix, 1970). In eukaryotes, however, glycine was not incorporated (Yamada *et al.*, 1983).

1.3.2 Riboflavin (Vitamin B2)

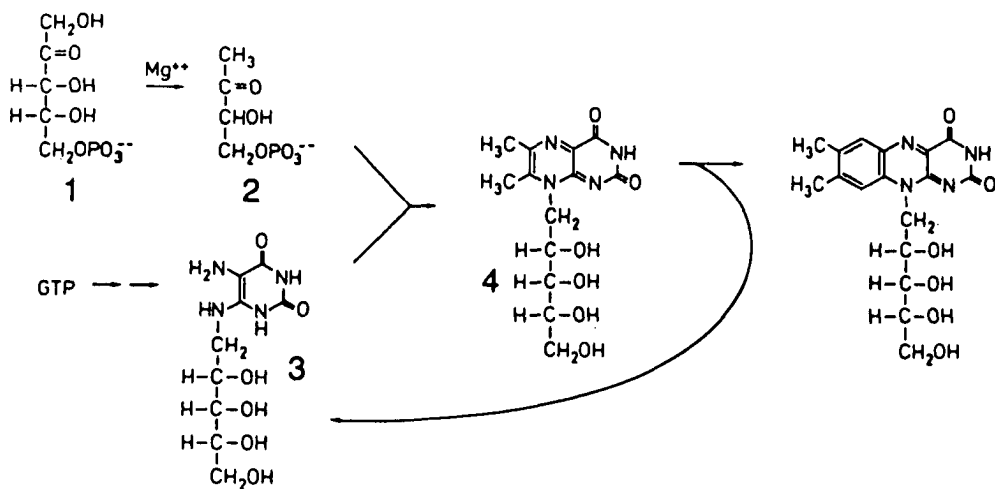


Fig. 3 : Biosynthesis of riboflavin.

Many microorganisms and plants, but not higher animals, synthesize riboflavin from simple precursors. Since certain organisms, such as *Candida* spp., produce unusually large quantities of this vitamin, these organisms have served frequently in studies aimed at the elucidation of the biosynthetic process (Cohen, 1967, and Cheldelin and Baich, 1967). The biosynthetic route to riboflavin is shown in Fig. 3. GTP and ribose phosphate lead to 3, which is converted to 4 by the addition of a 4-carbon unit. Dismutation of 4 yields riboflavin (Nielsen *et al.*, 1986). The origin of the 4-carbon unit has remained elusive for many years, but has recently been shown to be derived from ribose/ribulose phosphate, by performing *in vivo* and *in vitro* ^{13}C and ^{14}C experiments (Nielsen *et al.*, 1986). Incorporation studies with ^{13}C have established a similar mechanism in *Bacillus subtilis* (Le Van *et al.*, 1985). Very recently, it was demonstrated with a purified enzyme from *Candida guilliermondii*, catalyzing the formation of 4 from 3, that ribulose 5-phosphate is converted to 3,4 dihydroxy-2-butanone 4-phosphate, which is subsequently converted in the presence of 3, into 6,7-dimethyl-8-ribityllumazine (4, Volk and Bacher, 1988).

1.3.3 Nicotinamide adenine nucleotides

NAD and NADP function in numerous anabolic and catabolic reactions and are widely distributed through biological systems. Two main biosynthetic pathways have been observed. The so-called aerobic pathway, found in mammalian cells, a number of lower eukaryotes, and *Xanthomonas* spp., involves the aerobic degradation of tryptophan. In six steps, tryptophan is converted into quinolinic acid via 3-hydroxykynurenine. Through the use of radioactive tracers and by the characterization of the various intermediates, all enzymatic steps could be clarified. The so-called anaerobic pathway is found predominantly in prokaryotes. Quinolinic acid is formed here from its precursors L-aspartate and dihydroxyacetone phosphate in a sequence of reactions which has not been entirely clarified. Subsequent conversion of quinolinic acid to NAD occurs via a pathway common to all organisms that have been examined to date. All intermediates have been identified and all enzymes isolated. All approaches mentioned in section 1.2 have been used to elucidate the biosynthetic route to NAD, and excellent reviews exist on this subject (Chaykin, 1967, and Foster and Moat, 1980), so that NAD can be regarded as an illustrative example.

1.3.4 Pyridoxal phosphate (Vitamin B6)

Vitamin B6 occurs in several forms, of which pyridoxal phosphate and pyridoxamine phosphate are the ones operating as cofactor. These cofactors are extremely versatile, functioning in a large number of different enzymatic reactions in which amino acids or amino groups are transformed or transferred. Although the catalytic function of pyridoxal phosphate and its mode of action are well understood, biosynthetic investigations have led to relatively little insight so far. There are several reasons for this lack of progress.

Firstly, biosynthetic studies have been hampered for a long time by the minute amounts of cofactor that are produced by microorganisms and plant tissues. Although several yeasts (Scherr and Rafelson, 1962, Nishio *et al.*, 1973) and bacteria (Suzue and Haruna, 1970, Tani *et al.*, 1972) produce vitamin B6 in the mg/l concentration range (which seemingly make them ideally suited for biosynthetic studies), it is *E. coli*, an organism that produces less than 100 $\mu\text{g/l}$, that has served as the main microbiological tool for the study of vitamin B6 biosynthesis.

Genetic studies, predominantly carried out by Dempsey (Dempsey, 1980) have revealed that only a single biosynthetic route to pyridoxal phosphate exists in *E. coli*. Cross-feeding and complementation tests with pyridoxineless mutants isolated from this organism have shown that 9 or 10 enzymes may be required which can be classified into five unlinked groups that have since been shown (Bachmann *et al.*, 1976) to be widely separated from each other on the *E. coli* chromosome. Unfortunately, only very limited information has been forthcoming from these mutant studies with regard to precursors and intermediates of pyridoxal phosphate synthesis.

The second reason for the lack of progress in biosynthetic investigations is a basic one, namely the very simplicity of the pyridoxine structure. Whereas the structures of more complex natural products tend to suggest the identity of the precursor (e.g. tryptophan as the precursor of the indole alkaloids), there are no structural features within the vitamin B6 skeleton that suggest an obvious biogenetic atonomy and its derivation from multicarbon precursors.

Very simple molecules ($\text{C}_1\text{-C}_3$ units) are therefore likely precursors, giving rise to a large number of possible biosynthetic routes. However, simple molecules tend to be active participants in metabolic cycles which

causes the label to be scrambled in complex fashion, making the results of incorporation studies difficult to interpret. The results obtained with tracer experiments are discussed in an excellent review by Hill and Spenser (Hill and Spenser, 1986). Although the information obtained has been predominantly derived from radioactive ^{14}C tracer incorporation studies, the feasibility of ^{13}C NMR methodology have been demonstrated by these authors in a recent paper (Hill *et al.*, 1987). From this study, and previous ones, it follows that the pyridoxine skeleton is generated from two intact triose units and a triose derived 2-carbon unit, all derived from glycerol.

2. THE COFACTOR PYRROLOQUINOLINE QUINONE

2.1 General introduction, discovery, occurrence

Besides the well known cofactors NAD(P) and flavines, a new redox cofactor exists in a wide range of prokaryotic and eukaryotic organisms, namely pyrroloquinoline quinone (PQQ) (Fig. 4). Its chemical structure was solved independently by the groups of Forrest (Salisbury *et al.*, 1979) and Duine

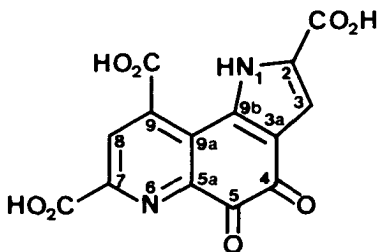


Fig. 4 : Structure of pyrroloquinoline quinone (2,7,9,-tricarboxy-1H-pyrrolo[2,3-*f*]-quinoline-4,5-dione.

(Duine *et al.*, 1980). Although the former group, in view of the methylotrophic origin of the cofactor, proposed methoxatin as trivial name, the more descriptive semi-systematic name pyrroloquinoline quinone, as proposed by Duine *et al.*, is more commonly employed now, since the presence of the cofactor has been demonstrated in a wide range of oxidoreductases,

occurring from microbe to man. Perhaps it also occurs as a cofactor in a nitrile hydratase (Nagasawa and Yamada, 1987). In analogy with flavoproteins, PQQ-dependent enzymes are designated as 'quinoproteins'. For a complete list, the reader is referred to recent reviews (Duine *et al.*, 1986 and 1987).

Evidence has been presented that several bacteria are unable to synthesize PQQ, but synthesize the apoenzyme part of the quinoprotein. *A. lwoffii* (van Schie *et al.*, 1984), *Escherichia coli* (Hommes *et al.*, 1984), *Agrobacterium* and *Rhizobium* spp. (van Schie *et al.*, 1985) produce glucose dehydrogenase apoenzyme. In addition, it has been shown that PQQ is a growth factor in a *Pseudomonas* sp. grown on polyvinyl alcohol (Shimao *et al.*, 1986), while the growth rate of *P. testosteroni* on alcohols is strongly enhanced on addition of PQQ to the culture medium (Groen *et al.*, 1986), due to the presence of quinoprotein polyvinyl alcohol- and alcohol dehydrogenase apoenzyme respectively. These observations suggest a role as growth factor for PQQ in these organisms. The observation that it is excreted by other organisms, and can be taken up and used by the organisms producing quinoprotein apoenzymes, supports this view. Whether the need for PQQ in higher organisms requires the uptake in the food of PQQ or a precursor-like vitamin (Jongejan *et al.*, 1986) is presently not known.

2.2 Physical properties

PQQ crystallizes as its sodium salt from NaCl containing solutions as tiny, brick-red needles. At pH 7, the solubility of the sodium salt in water is at least 20 g/l (20°C) (Frank Jzn, 1988). PQQ has a high redox potential ($E'_0 = +90$ mV at pH 7.0) as was demonstrated by potentiometric titrations (Duine *et al.*, 1981) and cyclic voltammetry (Eckert *et al.*, 1982). Three redox forms of PQQ participate in biological oxidations: PQQ, PQQ' and PQQH₂ (Duine *et al.*, 1986).

The absorption spectrum of PQQ (Fig. 5) is characterized by maxima at 249, 323 and 475 nm ($\epsilon = 22496, 9898$ and $742 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively). Since PQQ is partly hydrated in aqueous solutions (Dekker *et al.*, 1982), the absorption spectrum under such conditions is the sum of the contributions of PQQ and PQQ-H₂O. On pH variation, the absorption spectrum changes due to (i) pseudobase formation (Sleath *et al.*, 1985, and Rodriguez *et al.*, 1987) and (ii) deprotonation of the three carboxylic groups and protonation of the

pyridine nitrogen. The absorption spectrum of the reduced form of PQQ, the quinol PQQH₂, is characterized by a maximum at 302 nm ($\epsilon = 30484 \text{ M}^{-1} \cdot \text{cm}^{-1}$, at pH 7.0) (Fig. 4), which shifts to 317 nm at pH 9.5 and 315 nm at pH 1.5 (Duine *et al.*, 1981, unpublished results). Autoxidation of PQQH₂ readily occurs in water-containing solvents at pH values above 4. The absorption spectrum of PQQH[•] has been determined by Faraggi *et al.* (1986).

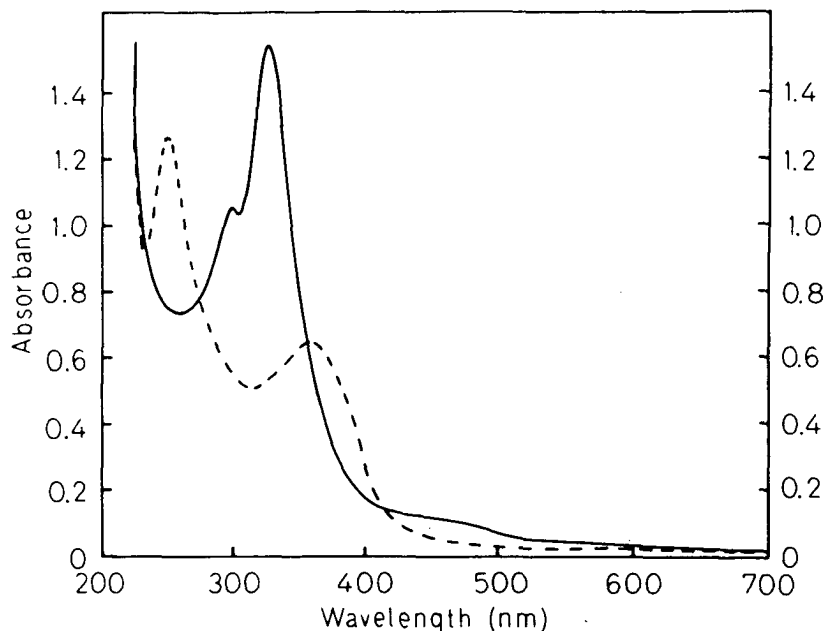


Fig. 5 : Absorption spectra of PQQ and PQQH₂.

Fluorescence excitation and emission spectra are shown in Fig. 6. Aqueous solutions of PQQ are fluorescent between pH 1 and 11. At pH 7, the fluorescence excitation spectrum differs significantly from the absorption spectrum. This is caused by the fact that only the hydrate of PQQ (PQQ.H₂O) is fluorescent, while the absorption spectrum is the sum of contributions of PQQ and PQQ.H₂O (Dekker *et al.*, 1982).

The most salient feature of the ¹H-NMR spectrum of PQQ is the low field position of the pyrrolo N-H signal (13.3 ppm), which is caused by the strong

interaction between this hydrogen and the C-9 carboxylic acid group. The C_3 -H and C_9 -H signals are found at approximately 7.4 and 8.6 ppm, respectively (Duine *et al.*, 1979, 1980, and 1981).

With the aid of U- ^{13}C -PQQ, obtained from the culture supernatant of *Hyphomicrobium* X grown on ^{13}C -methanol, all signals of the ^{13}C -NMR spectrum of PQQ could be assigned (Duine *et al.*, unpublished results; Chapter VII).

EI (Electron Impact) mass spectra of PQQ can be obtained only after this compound has been made volatile by derivatization to its trimethylester (Fig. 6). The spectrum shows at M^+ ion at m/e 374, while Field Desorption

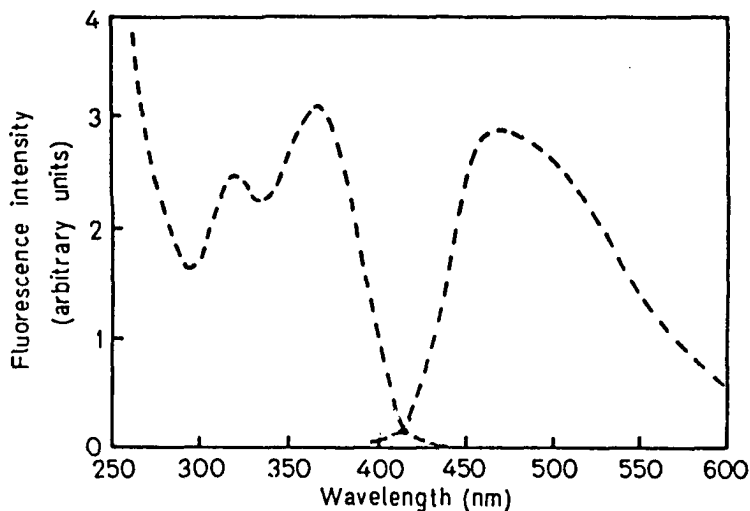


Fig. 6 : Fluorescence spectrum of PQQ (4 μ M PQQ in 0.02 M Potassium phosphate, pH 7.2).

(FID) mass spectra show a M^+ ion at m/e 372. This difference can be explained by assuming that the compound is reduced by residual water vapour in the inlet system, generating a $(M+2)^+$ peak, a behaviour which is common to many quinones, especially o-quinones (Zeller, 1974). Other fragments in the EI mass spectrum are seen at m/e 342 (344), 314, 286, 254, and 195, all due to losses of the esterified groups (Duine *et al.*, 1980). Recently, FAB (Fast Atom Bombardment) mass spectra of PQQ have been obtained (J. Greve, personal communication).

2.3 Chemical properties

One of the most interesting features of PQQ is its remarkable stability towards aggressive chemicals, such as concentrated H_2SO_4 and 1 M NaOH, as compared to the more labile cofactors such as NAD and flavines. PQQ is stable both in hot concentrated H_2SO_4 and HCl, as well as 1 M NaOH, and resists temperatures of at least 120°C . As far as known, only in 10 M NaOH (J. Frank, unpublished results) and mixtures of HNO_3 and H_2SO_4 (unpublished results), PQQ is degraded to biologically inactive compounds.

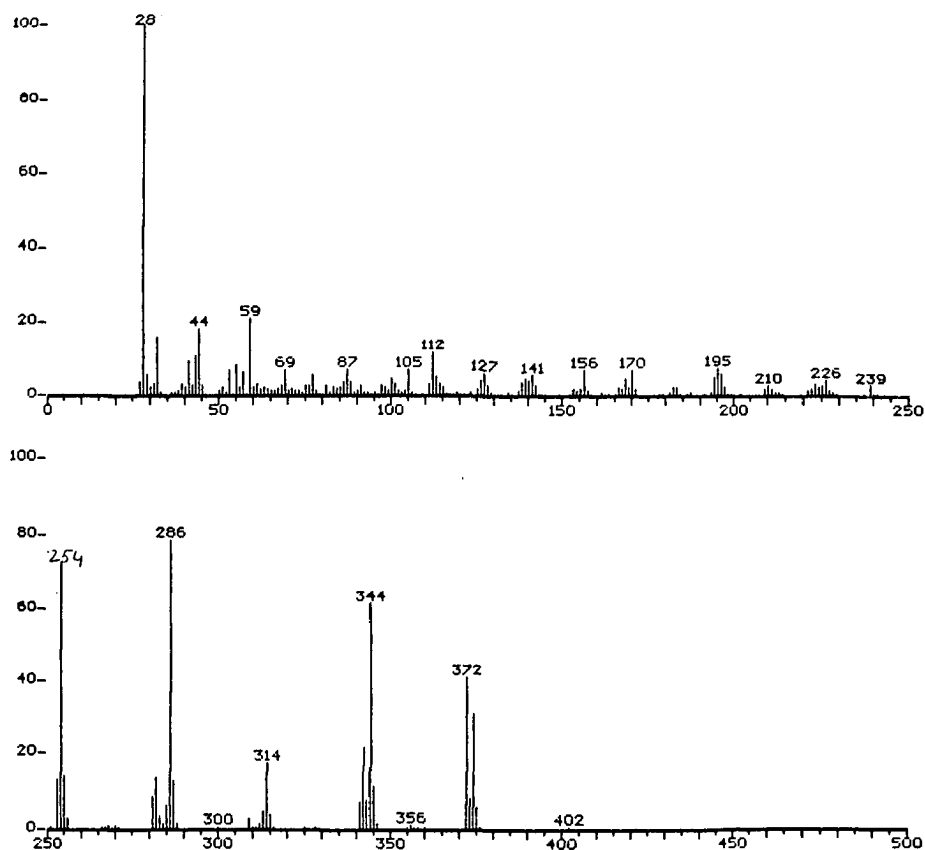


Fig. 7 : EI Mass spectrum of the PQQ trimethyl ester.

Despite this stability, PQQ shows considerable reactivity, residing mainly in the quinone carbonyls. The C₅-carbonyl is the target of nucleophilic attack, resulting in the formation of more or less stable adducts (Duine *et al.*, 1979 and 1983).

Reduction of PQQ to its quinol form is easily achieved by a variety of agents, such as hydrazines, NaBH₄, β -mercaptoethanol and cysteine (Duine *et al.*, 1981, Itoh *et al.*, 1986a), the oxidation of these agents occurring even more rapidly in the presence of PQQ than in the presence of certain artificial electron acceptors (unpublished results). The kinetics of the oxidation of free thiol groups by PQQ (Itoh *et al.*, 1986a), and reoxidation of PQQ by molecular oxygen (Itoh *et al.*, 1986b) have been described.

Several reports have appeared on the oxidation of primary amines (Eckert *et al.*, 1982, Oshiro *et al.*, 1983) and amino acids by PQQ. In view of the omnipresence of amino acids, the reaction of these compounds with PQQ seems most interesting. Itoh *et al.* (1984) have reported that efficient conversion to the corresponding aldehydes can be achieved with catalytic amounts of PQQ. The group of Bruce on the other hand has shown (Sleath *et al.*, 1985) that PQQ is progressively lost in this reaction due to formation of oxazoles and other non-identified products. This subject is further discussed in Section 3.2 and Chapter III.

3. THE BIOSYNTHESIS OF THE COFACTOR PQQ

3.1 Introduction

In 1983, when the present investigations on the biosynthesis of PQQ were started, nothing was known about precursors and intermediates. However, examining the chemical structure of the cofactor, at first sight the amino acids tyrosine (or phenylalanine), glutamate and alanine come to question as precursors. In fact, these compounds have meanwhile attracted the attention to develop a biomimetic route for chemical synthesis (Buchi *et al.*, 1985). Another possibility is that the quinoline ring of PQQ is derived from kynurenic acid or a closely related compound. Since kynurenic acid is formed from L-tryptophan in several microorganisms (Behrman, 1962), this amino acid

would be a likely precursor too. As the quinoline ring of pseudanes (2-n-alkyl-4-hydroxyquinolines) resembles the quinoline moiety of PQQ, the precursors of these compounds, namely anthranilic acid and acetate (Ritter and Luckner, 1970), are also possible precursors for PQQ.

Two major approaches have been used in trying to establish the precursors and intermediates of PQQ. In discussions with the University of Leiden (Prof. P. van de Putte and Dr. N. Goosen), where the genes for PQQ biosynthesis were to be cloned, it was decided to use *Acinetobacter calcoaceticus* LMD 79.41 in our studies, for reasons discussed in Section 3.4. Using mutants defective in the biosynthesis of PQQ, it would be attempted to establish the intermediates of PQQ biosynthesis, by studying growth performances of the mutants and carrying out cross-feeding experiments. As problems arose in the unambiguous assessment of the inability of PQQ⁻-mutants, and organisms producing quinoprotein apoenzymes, to produce PQQ, this had first to be solved (Section 3.2, Chapter II).

In order to establish the precursors of PQQ, labeling procedures had to be developed. Since the PQQ skeleton is very resistant to degradation, incorporation experiments were not performed with radioactive tracers, but with stable ¹³C and ¹⁵N isotopes. The incorporation of these compounds into PQQ would then be followed using mass spectrometry (MS) and/or NMR spectroscopy. Since media containing amino acids are deleterious to PQQ, and the labeling experiments had to be carried out in their presence, conditions to prevent degradation of PQQ in culture media were investigated simultaneously (Section 3.2, Chapter III and VII).

Unfortunately *A. calcoaceticus*, the organism used in mutant studies, appeared to synthesize only very low amounts of PQQ. In view of the amounts of cofactor needed for reliable and accurate MS or NMR analysis, it appeared to be impossible to perform incorporation studies with this organism. Therefore, other organisms were screened for their PQQ production. *Pseudomonas* strains and methylotrophs appeared to be the best candidates (Section 3.3, Chapter IV).

3.2 Purification and analysis of PQQ

In view of the polar character of PQQ, ion-exchange chromatography and HPLC methods using reversed phase chromatography are generally used for its purification from biological samples (Duine *et al.*, 1986). Since the

majority of PQQ synthesized by microorganisms is excreted (Duine *et al.*, 1986, Ameyama *et al.*, 1987), spent culture media are a good source. A two-step purification procedure, using Amberlyst A-21 anion exchanger, followed by a clean up with a reversed phase column (Seppak) is sufficient for most purposes. In more complex situations, this is followed by an HPLC step.

Detection of PQQ in purified samples by physical methods can be achieved using UV-Vis absorption spectrophotometry or fluorescence detection, which is more sensitive and selective (Duine *et al.*, 1986). It should be noted here that fluorescence spectroscopy is not selective enough to detect PQQ in a protein hydrolysate, due to interfering fluorescence of compounds present in the protein hydrolysate (Chapter II). This method has been used repeatedly in demonstrating the presence of PQQ in samples from natural sources (Ameyama *et al.*, 1984, 1985a, and 1985b). Upon reduction of PQQ with NaBH_4 in the presence of oxygen, followed by oxidation of the resulting vicinal diol group formed with NaIO_4 , a highly fluorescent compound is formed (Duine *et al.*, 1980). This method has been used for purposes where a more sensitive detection is required. Particularly informative is HPLC coupled to photodiode array detection, permitting identification and additional resolution, with chemometric techniques such as multi-component analysis and curve resolution (Duine *et al.*, 1986).

Extremely sensitive biologically assays, based on activity measurements of samples incubated with reconstitutable quinoprotein apoenzymes, have been developed. Originally, quinoprotein glucose dehydrogenase apoenzyme from *Acinetobacter calcoaceticus* and *P. aeruginosa* have been used for this purpose (Duine *et al.*, 1983). An easier and more sensitive way is the use of quinohaemoprotein alcohol dehydrogenase apoenzyme from *P. testosteroni* (Groen *et al.*, 1986).

The demonstration of the presence or absence of a PQQ synthesizing capacity in microorganisms is dealt with in Chapter II. In that chapter, procedures are described which are effective in removing contaminating PQQ from equipment and media. These procedures have been used to demonstrate that under normal laboratory conditions of growth, *E. coli* does not synthesize PQQ. A very sensitive and reliable assay for PQQ has been developed using *A. calcoaceticus* PQQ⁻-mutants. Using this assay, samples containing 0.1 nM PQQ can be analyzed. In applications requiring an extremely sensitive and reliable method, this assay is strongly recommended.

In view of the problems met in microbial PQQ production and purification of PQQ in the presence of amino acids, and the controversial findings in the literature (Section 2.3), it was decided to perform further research on this topic (Chapter III). It appears that with the majority of amino acids, both a cyclic reaction, apparent in the catalytic conversion of amino acids, and a linear reaction, leading to formation of oxazoles, take place. The structure of the oxazole products depends on the amino acid used. Under anaerobic conditions, PQQ functions as oxidant in the formation of oxazole from oxazoline. Ammonium salts and divalent cations were shown to be important activators for this reaction. Since the activity of several quinoproteins is highly stimulated by these compounds, it is very likely that these cations are relevant for the catalytic mechanism of these enzymes. Hydrolysis of biologically inactive oxazoles into PQQ was achieved under strongly acidic conditions. With the aid of the results obtained in Chapter III, it was possible to avoid losses of PQQ and amino acids in cultures of *Hyphomicrobium* X grown in the presence of synthetic mixtures of amino acids, by replacement of ammonium salts by nitrate as a nitrogen source (Chapter VII).

The determination of covalently bound PQQ in quinoproteins also presents special problems. Proteolytic degradation generates large amounts of amino acids which react with PQQ to practically unidentifiable products (Chapter III), while tryptophan has a deleterious effect on PQQ under conditions of acid hydrolysis (Chapter III). This may (partly) explain why it is not possible to detect covalently bound PQQ in quinoproteins after acid hydrolysis. Protection of PQQ has been successfully achieved by derivatization *in situ* with hydrazines (Lobenstein-Verbeek *et al.*, 1984, van der Meer *et al.*, 1986). Identification of the products formed from the reaction of tryptophan with PQQ under strongly acidic conditions, as well as other derivatization procedures under denaturing conditions, are well under way.

3.3 Factors relevant in bacterial PQQ production

In screening microorganisms suitable for labeling studies, quinoprotein content and levels of external PQQ have been determined in a number of bacteria under a variety of growth conditions (Chapter IV). Since nothing had been reported on the regulation of PQQ biosynthesis and the factors

which govern PQQ production in bacteria, it was attempted to obtain some insight by performing experiments and using the data from the literature. It appeared that PQQ synthesis occurred only on induction of quinoproteins, but reversibly, quinoprotein synthesis did not depend on PQQ synthesis.

Quinoprotein substrates were not required for both quinoprotein and PQQ synthesis. Although PQQ production was determined by the type of organism and quinoprotein produced, coordination between quinoprotein and PQQ synthesis is weak since the former seems to depend on the growth rate while the latter does not. Some organisms continued to synthesize PQQ *de novo* when this cofactor was administered exogenously. Probably PQQ can not be taken up by either passive diffusion or active transport mechanisms, and is therefore not able to exert feed-back regulation on PQQ biosynthesis in these organisms (Chapter IV).

3.4 Mutant studies

In view of the participation in a multi-disciplinary project for which *A. calcoaceticus* was the model organism, in the first instance this organism was chosen for mutant investigations. *A. calcoaceticus* has the following attractive properties for this type of studies:

1. It is relatively easy to isolate PQQ⁻-mutants from this organism. *A. calcoaceticus* LMD 79.41 converts glucose and other aldoses to their corresponding aldonic acids by means of a non-specific quinoprotein aldose dehydrogenase (Dokter *et al.*, 1986). In most strains, the aldonic acid formed cannot be degraded further by *Acinetobacter* species, and therefore, this strain is not able to grow on aldoses. For *A. calcoaceticus* LMD 79.41, one exception is L-arabinose, which can be used as a sole carbon and energy source (Goosen *et al.*, 1987). The absence or presence of acid production from glucose, or growth with L-arabinose can be used for the isolation of PQQ⁻-mutants. It should be noted that it is much more difficult to obtain PQQ⁻-mutants from other organisms. The reason is that, generally, more than one pathway is present in organisms for substrates that are degraded by quinoproteins (Duine *et al.*, 1986). Therefore, in these cases screening, e.g. for the absence of growth on a special carbon source, is not possible.

Until very recently (Biville *et al.*, 1988) there has been no report on the isolation of PQQ⁻-mutants from methylotrophic bacteria. Since these

organisms excrete large amounts of PQQ, they would be ideally suited for mutants studies. However, apart from problems met in the isolation of mutants from methylotrophic bacteria, in order to obtain mutants defective solely in the dissimilation of methanol most investigators have always screened for mutants unable to grow on methanol, but still able to grow on methylamine (Nunn and Lidstrom, 1986). Since methylamine is degraded via quinoprotein methylamine dehydrogenase in most methylotrophs [in which PQQ is covalently bound (van der Meer *et al.*, 1987)], PQQ⁻-mutants may never be found, provided that no other route of PQQ biosynthesis exists for covalently bound PQQ. An exception to this rule, however, is *M. organophilum* XX, as in this organism methylamine is not dissimilated via quinoprotein methylamine dehydrogenase, but via methylglutamate dehydrogenase (Biville *et al.*, 1988), which is a flavohaemoprotein (Boulton *et al.*, 1980). Consequently, on screening for mutants unable to grow on methanol, but able to grow on methylamine, several PQQ⁻-mutants could be isolated, belonging to five different genetic classes (F. Gasser, personal communication).

2. Genetic techniques (transformation, conjugation, transduction) are rather well documented for *Acinetobacter* species (Juni, 1982).

3. *A. calcoaceticus* is a very versatile organism, able to use a large number of organic compounds as sole source of carbon and energy (Baumann *et al.*, 1968). Although not as versatile as *Pseudomonas* species, the organism is a very attractive one in order to examine differences between wild type and PQQ⁻-mutants in metabolic performances.

Genetic studies on the biosynthesis of PQQ have been reported already (Goosen *et al.*, 1987). By using PQQ⁻-mutants from *Acinetobacter calcoaceticus* LMD 79.41, the genes for PQQ biosynthesis were cloned. Complementation experiments revealed four different classes of PQQ⁻-mutants located in three operons. Genes I and II probably code for one enzyme consisting of two subunits with M_r 29,700 and M_r 10,800. Gene III codes for an enzyme containing a subunit of M_r 37,400 or M_r 43,600. Gene IV appears to be very small, equivalent to a protein of only 23 amino acids. The function of this small DNA fragment is not clear at the moment, that is whether it functions on RNA or protein level. Since the cloned PQQ genes come to expression in *A. lwoffii* (Goosen *et al.*, 1987) and *E. coli* (N. Goosen, unpublished results), both non PQQ-producers, this could be interpreted that

bacterial PQQ biosynthesis starts with (a) frequently occurring metabolite(s) and is completed in four steps.

The results obtained with PQQ⁻-mutants from *A. calcoaceticus* are described in Chapter VI. A major problem encountered in our search for intermediates is the very low amount of PQQ that is synthesized by this organism under normal conditions of growth (< 10 nM). However, during our investigations it was found that significant amounts of PQQ were present in the medium of quinate or shikimate grown cultures. Studies with PQQ⁻-mutants from *A. calcoaceticus* in PQQ-free media have revealed that quinate dehydrogenase (QDH) (EC 1.1.99.-), catalyzing the first step in the degradation of these growth substrates, is a quinoprotein (Chapter V). Since several other bacteria were found to contain quinoprotein QDH, it is assumed that all bacterial NAD(P)-independent quinate dehydrogenases are quinoproteins (Chapter V).

Since it was found that higher amounts of PQQ were excreted when a fed-batch method of culturing was applied, higher levels of PQQ production were achieved using this method with quinate as a carbon source. However, using these conditions, or stress conditions with a limiting amount of cofactor, excretion of intermediates by PQQ⁻-mutants could not be established (Chapter VI). Neither did cross-feeding experiments, using extensive variations in combinations and conditions, result in stimulation or reconstitution of PQQ synthesis. A tentative explanation, accounting for these findings, is that biosynthesis proceeds on a matrix, for instance the peptide containing 23 amino acids, so that no free intermediates are present during synthesis (Chapter VI).

3.5 Incorporation studies

It has already been mentioned that PQQ is a very stable molecule. Only after periodate oxidation of PQQH₄, the C₄-C₅ bond is broken (Duine *et al.*, 1980). Opening of the pyrrole ring can be achieved only by treatment with a mixture of H₂SO₄ and HNO₃ (unpublished results). So far no reactions are known in which the pyridine ring is degraded, possibly because this ring is stabilized by the two carboxyl groups (Frank Jzn, 1988). Since reliable precursor-product relationships can only be achieved by establishing the distribution of label within the product, the approach using radioactive tracers seemed very unattractive, as this requires extensive degradation of

the product, PQQ. Therefore, incorporation studies were performed with stable isotopes, after which MS or NMR analysis could be performed. As amino acids appeared to be likely precursors, a replacement-approach was developed in which microorganisms were grown in the presence of either a relatively cheap ^{13}C -carbon- or ^{15}N -nitrogen source, replacement occurred with unlabeled amino acids, and incorporation was determined by following the ^{12}C or ^{14}N content in PQQ and protein.

In order to obtain an adequate amount of PQQ for MS or NMR analysis, incorporation studies had to be performed with good PQQ-producers. Unfortunately, at the start of our investigations, no conditions were known for *A. calcoaceticus* to provide these amounts. As is discussed in Chapter IV, *Pseudomonas* species (producing quinoprotein ADH when grown on alcohols) or *Methylobacter* (producing quinoprotein MDH when grown on methanol) are the better candidates. *Pseudomonas* species seemed most attractive, since they are usually very versatile organisms, able to grow on a larger number of different carbon sources. Therefore it was reasoned that if incorporation studies with amino acids would not yield positive results, many other presumed precursors could be administered. Unfortunately, this property has also its negative aspects. Preliminary incorporation studies with $^{15}\text{NH}_4\text{Cl}$ in the presence of amino acids and ethanol as a carbon and energy source showed that the amino acids were used preferentially as a nitrogen and carbon source, resulting in complete scrambling of the administered compounds (unpublished results).

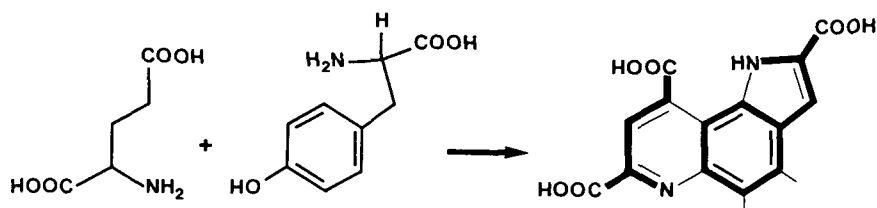


Fig. 8 : Tyrosine and glutamic acid as precursors of PQQ.

Initially, the presumed incorporation was followed by mass spectrometry, which requires a derivatization step of PQQ to the trimethyl ester in order to obtain mass spectra. However, the derivatization of low amounts of

purified PQQ (less than 1 mg.) appeared to give low yields (10-25%) and the resulting PQQ trimethyl ester was unstable in many solvents, giving rise to unidentifiable degradation products. The presence of these degradation products severely hampered the interpretation of the mass spectra. Assessment of distribution of label within PQQ was further hindered by the fact that the mass spectra predominantly consisted of large pyrroloquinoline ring fragments.

Because of these problems, analysis of PQQ by ^{13}C -NMR was attempted in order to establish incorporation. To avoid possible problems with degradation of amino acids, a methylotrophic organism, *Hyphomicrobium* X, was used. This organism is not able to use amino acids as a carbon source (Harder and Attwood, 1978). The results of these experiments are described in Chapter VII. Tyrosine, and not phenylalanine, was established as direct precursor in this organism. It should be noted that Unkefer and coworkers (personal communication) have obtained evidence for tyrosine and glutamate as precursors for PQQ in *Pseudomonas* AM1, also using ^{13}C -NMR spectroscopy.

3.6 Conclusions and perspectives

From the results presented in this thesis, it appears that much progress in both the analysis as well as in the elucidation of the biosynthesis of PQQ has been made. Knowledge has been gained on the factors that are relevant in the production of PQQ and its reaction with amino acids. Reliable methods for the establishment of a PQQ synthesizing capacity in microorganisms have been developed, as well as an extremely sensitive assay for PQQ.

Although, with the aid of PQQ⁻-mutants isolated from *Acinetobacter calcoaceticus*, the genes involved in PQQ biosynthesis have been cloned and sequenced, and have been shown to come to expression in several non PQQ-producing organisms, still nothing is known about the intermediates and the enzymes catalyzing the biosynthesis of PQQ. The failure to demonstrate cross-feeding between mutants or accumulation of intermediates can possibly be explained by assuming that PQQ biosynthesis occurs on a template (e.g. a short polypeptide) on or in which amino acids - the precursors of PQQ - are modified and assembled to PQQ. Studies with recently isolated PQQ⁻-mutants from *Methylobacterium organophilum* XX may be very helpful to test this hypothesis. Also the location of the enzymes of PQQ biosynthesis, as well as

the assemblage (in the periplasmic space) of quinoprotein apoenzymes with PQQ, remains to be solved.

¹³C incorporation studies with amino acids have established tyrosine as precursor of PQQ biosynthesis in *Hyphomicrobium* X. If evidence can be provided for glutamate as precursor of PQQ, at least in methylotrophs, the PQQ molecule is synthesized completely from amino acids. Whether PQQ is synthesized from the same precursors in other organisms, especially in mammals, where PQQ has been found solely in a covalently bound form, remains to be established. Another interesting question is whether higher organisms, including man, synthesize PQQ themselves, or require it in their diet. In view of the recent findings of PQQ as a cofactor in amine oxidases and dopamine- β -hydroxylase, enzymes regulating the concentrations of important bioregulators, elucidation of the biosynthesis of covalently bound PQQ may have important implications in pharmacology.

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Chapter II

Detection of the cofactor pyrroloquinoline quinone

1. SUMMARY

In order to demonstrate the presence or absence of a pyrroloquinoline quinone (PQQ) synthesizing capacity in microorganisms, we have found that media and equipment must be treated to remove contaminating PQQ. Procedures are described to be effective for that purpose. These have been used with *Acinetobacter calcoaceticus* PQQ⁻ strains to develop a sensitive and reliable assay for PQQ. They have also been used to show that under our conditions of growth *Escherichia coli* does not synthesize PQQ. Fluorescence spectroscopy is not selective enough to detect PQQ in a protein hydrolysate due to background fluorescence in the same spectral regions as PQQ. In addition, PQQ reacts with amino acids to give products that cannot be detected by either fluorescence spectroscopy or biological assay. In this regard, claims that several materials originating from plants or animals contain PQQ should be reexamined. Moreover, PQQ cannot be detected with these methods in hydrolysates of enzymes containing covalently bound PQQ.

2. INTRODUCTION

PQQ is a redox cofactor in several enzymes (quinoproteins) and is widely distributed among bacterial species [1]. Systematic investigations on the occurrence of PQQ have not been published yet. However, since the organic cofactor in mammalian Cu²⁺-containing amine oxidases is covalently bound PQQ [2], it could be imagined that most, if not all, organisms synthesize PQQ.

On the other hand, many bacteria produce the protein part of quinoproteins but not the cofactor [1]. One striking example is *Escherichia coli* where the well known laboratory strains all contain the quinoprotein, glucose dehydrogenase (GDH) apoenzyme [3]. Functionality can be achieved, however, as was demonstrated for an *E. coli* C PTS⁻ mutant (which is unable to metabolize glucose via the phosphorylative route) since it could grow on glucose only in the presence of PQQ [3]. The finding of these apoenzymes could be explained by assuming that commonly applied growth conditions are

unable to induce the synthesis of PQQ. Otherwise, these species could lack the genetic information for PQQ biosynthesis. In the latter case, the ability of the laboratory strains to produce (sometimes even constitutively) these apoenzymes could be explained by assuming that PQQ is provided as a vitamin by other organisms in the natural environment.

Recently, it was reported that PQQ is a growth stimulator for several microorganisms, e.g., for *Saccharomyces cerevisiae*, reducing the lag time for growth on a mineral medium [4]. These authors also reported that PQQ was produced in large amounts by *E. coli* [5], as the growth stimulator in the culture fluid of this bacterium was chromatographed and its identity as PQQ was proven by fluorescence spectroscopy, a technique which was also used to reveal PQQ in protein hydrolysates [6]. Furthermore, they claimed the presence of PQQ [7] in many substances originating from animal and plant materials, suggesting that PQQ is ubiquitous. This was supported by the observation that the microorganisms used to test the stimulating effect produced PQQ themselves because they first had to be rinsed from adhering PQQ in order to show the stimulatory effect [8].

Since we found that (a) *E. coli* does not produce PQQ [3], (b) chromatographic columns and glassware become easily contaminated with PQQ (this report) and (c) PQQ reacts with, e.g., several amino acids to generate a large number of compounds which cannot be identified by fluorescence spectroscopy [9], biological assays, or HPLC ([10], and this report), it therefore seemed worthwhile to evaluate the methods to detect PQQ and describe the precautions to be taken in order to avoid contamination with PQQ.

3. MATERIALS AND METHODS

"PQQ-free" cultivation of the organisms

A. calcoaceticus PQQ⁻ mutants LMD 82.43 and 1332 (to be described elsewhere), derived from strain LMD 79.41 (originally obtained from Professor J. Hauge [11]), were cultivated on an inorganic medium containing per liter: 4.6 g KH₂PO₄, 11.5 g K₂HPO₄, 2.5 g (NH₄)₂SO₄, 0.2 g MgSO₄·7 H₂O, 1 ml spore solution according to Vishniac [12], and 0.2% (w/v) succinate as

a carbon source. The organisms were grown at 30°C with shaking (200 rpm) for about 17 h.

E. coli type strains NCTC 9001 (ATCC 11775), PC 886, and PC 1000 and strains B/r H266 and PPA41 (a mutant of *E. coli* PC 1000 which lacks Enzyme I activity of the PTS system [3]) were precultured on an inorganic medium as described [13] at 37°C with shaking (200 rpm), using 0.2% (w/v) sodium succinate as a carbon source. After preculturing overnight, the strains were transferred to a mineral medium with low buffering capacity (0.01 M potassium phosphate, pH 7.0) containing glucose (50 mM) with or without PQQ (20 µM).

The following precautions were taken to avoid PQQ contamination. All glassware was heated for 12 h at 560°C. To the solutions (except the calcium, iron, and spore solutions) Amberlyst A21 (50-100 µm, p.A. from SERVA, Heidelberg, FRG) in the chloride form (0.3 g/liter) was added since this anion exchanger strongly binds PQQ [14]. After incubation with stirring (1 h), the mixtures were filtered to remove the ion-exchanger material and the filtrates were sterilized by autoclaving.

The effectiveness of the precautions taken was checked by testing the cultured cells by method 1 or 2, as described below.

PQQ analysis

(a) Biological assay. (1) PQQ was detected via *A. calcoaceticus* PQQ⁻ mutants by measuring GDH activities in whole cells and in cell-free extracts. For detection of GDH holoenzyme activities in whole cells due to contamination with PQQ, 10-ml portions of the culture were centrifuged, washed, and suspended in the mineral salts medium (10 ml). Incubation occurred in 100-ml flasks at 37°C after addition of D-glucose (10 mM final concentration). The rate of disappearance of glucose was determined by measuring glucose concentrations (Boehringer GOD-PAP method) at marked time intervals. Using these cells for PQQ determinations, samples containing PQQ were added before the addition of glucose. GDH activities in cell-free extracts were determined as reported elsewhere [15].

(2) PQQ with *E. coli* was detected by measuring GDH activities of whole cells. The presence of GDH holoenzyme in strains of *E. coli* was determined by incubating the cells with glucose as described above and measuring the production of gluconic acid [16] after 24 h.

(b) Fluorescence spectroscopy. Fluorescence spectra were measured with a home-built spectrophosphorimeter [17] with right-angle illumination. Excitation spectra were automatically corrected using a rhodamine B quantum counter. The emission spectra were uncorrected.

Protein hydrolysates were prepared by heating the samples (10 mg protein) in 6 M HCl (2 ml) for 4 h at 100°C. The hydrolysates were neutralized with 6 M NaOH.

4. RESULTS

Contamination with PQQ

A. calcoaceticus LMD 79.41 oxidizes glucose to gluconic acid via its quinoprotein GDH, but is unable to grow on glucose or gluconate, indicating that other routes of glucose metabolism are absent. Therefore it could be expected that PQQ⁻ mutants derived from it are not able to metabolize glucose. However, when no precautions were taken to avoid PQQ contamination, it was routinely found that 2-25% of the GDH was in its holoenzyme form. The effect of using PQQ-free glassware and media is illustrated in Table 1. GDH

Table 1: PQQ detection with *A. calcoaceticus* PQQ⁻-mutants

Strains LMD 82.44 and 1332 were grown in a PQQ-free mineral medium. GDH holoenzyme activities were measured in whole cells (*in vivo*) and in cell-free extracts (*in vitro*). The effectiveness of the PQQ-removal procedures is indicated as a percentage of the activity obtained in the presence of 20 μ M PQQ.

PQQ ⁻ mutants	GDH activity <i>in vivo</i> (mmol glucose/l/h)			GDH activity <i>in vitro</i> (U/g wet cells)		
	-PQQ	+PQQ	%	-PQQ	+PQQ	%
LMD 82.44	0.0158	> 10	< 0.16	0.30	37.6	0.8
1332	0.0279	> 10	< 0.28	0.12	12.3	1.0

holoenzyme activity in whole cells is reduced to less than 0.3%, while this value in cell-free extracts is reduced to 1.0% (the higher value of the cell-free extracts compared to whole cells is probable due to contamination with PQQ during the preparation of the extract).

Wild-type *E. coli* strains metabolize glucose with a PTS system and gluconate via the Entner-Doudoroff route, suggesting that detection of PQQ (based on glucose conversion via GDH) by glucose consumption or gluconate production is not possible. It was found, however, that when *E. coli* was grown on glucose in a mineral medium with a low buffering capacity, the pH of the culture fluid decreased gradually. Furthermore, in the presence of PQQ, even lower pH values were obtained and gluconic acid production could be demonstrated (Table 2), most probably because the conversion of gluconic

Table 2: PQQ detection with *E. coli* strains

E. coli strains were precultured on a PQQ-free mineral medium and transferred to a medium with low buffering capacity (0.01 M potassium phosphate, pH 7.0), containing 50 mM glucose with or without PQQ (20 μ M). After incubation with shaking for about 24 h at 37°C, the pH of, and the gluconate concentration in the culture fluid were measured. n.d. = not detectable; n.p. = not performed.

Strain	PQQ		Gluconic acid	
	(20 μ M)	Growth	pH	(mM)
NCTC 9001	-	++	3.51	n.d.
	+	++	3.23	13.0
PC 886	-	++	3.58	n.d.
	+	++	3.32	5.4
PC 1000	-	++	3.60	n.d.
	+	++	3.28	8.4
B/r H266	-	++	3.67	n.d.
	+	++	3.28	8.0
PPA41 (PTS ⁻)	-	-	6.91	n.p.
	+	++	3.60	4.0

acid is inhibited at these low pH values. As is apparent from Table 2, no gluconic acid was produced when cells were cultured in a PQQ-free medium. Addition of PQQ (20 μM) to the medium resulted in production of gluconic acid (4-13 mM), while in its absence (no precautions to avoid PQQ contamination being taken) gluconic acid concentrations as high as 1-8 mM were found. As reported before [3], the PTS^- mutant could only grow on glucose in the presence of PQQ.

Biological assay for PQQ

In order to check the sensitivity and reliability of PQQ detection via glucose consumption with whole cells, varying amounts of PQQ were added to cell suspensions of a PQQ-free cultivated PQQ^- -mutant of *A. calcoaceticus*.

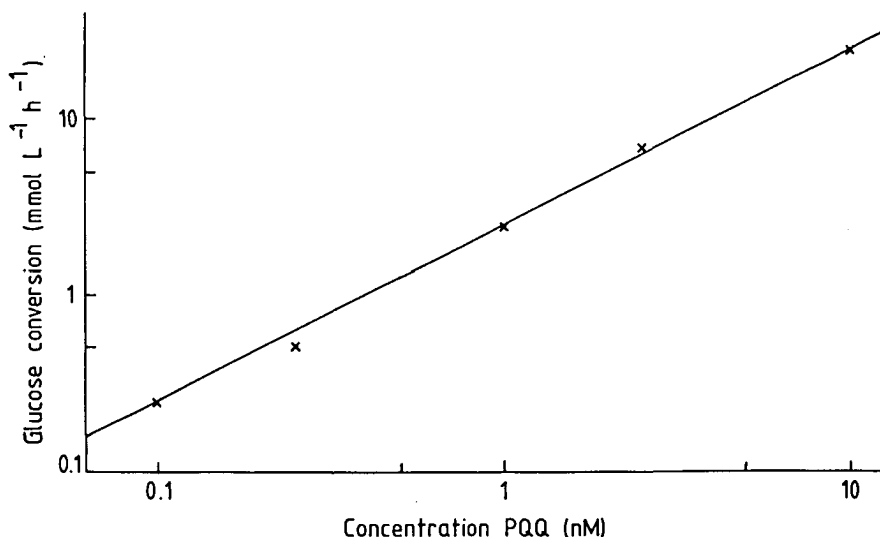


Fig. 1: Biological assay for PQQ.

Double logarithmic plot of PQQ concentration versus the amount of glucose converted by *A. calcoaceticus* PQQ^- -mutant LMD 82.44.

As seen in Fig. 1, a linear relationship exists between PQQ concentration and the rate of glucose oxidation over several decades and a response can be detected easily at the nanomolar level.

Detection of PQQ with fluorescence spectroscopy

PQQ in water-containing solutions is hydrated to a large extent and the adduct formed is responsible for the fluorescence of a PQQ solution [9].

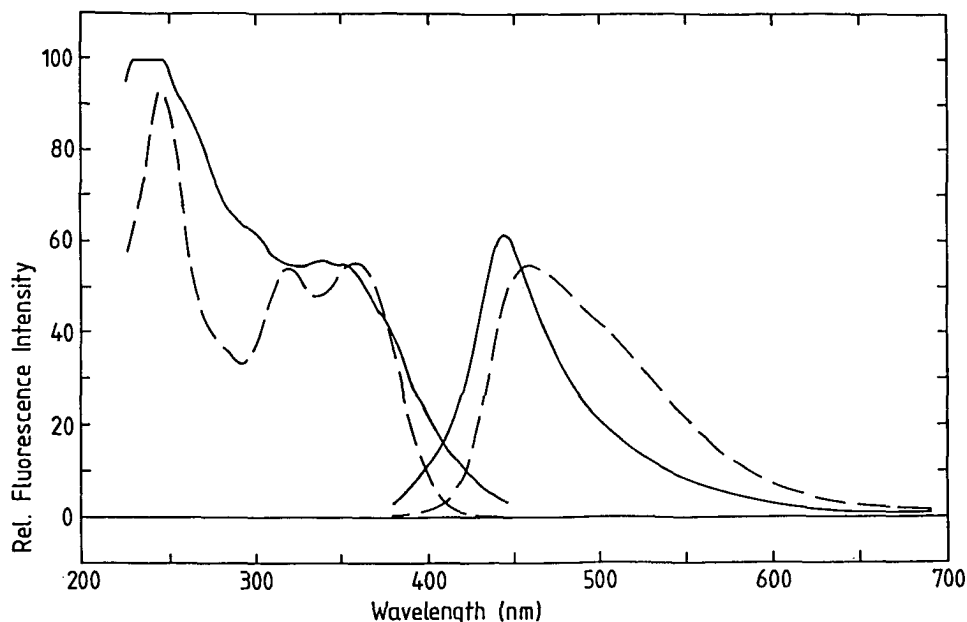


Fig. 2: Fluorescence spectra of casamino acids, 0.5 g/liter (—) and PQQ, 3 μ M (- - -), both in 10 mM potassium phosphate, pH 7.0.

The emission spectra were obtained by excitation at 365 nm and the excitation spectra were obtained by monitoring the emission at 460 nm.

However, as seen in Fig. 2, a casamino acid solution shows fluorescence in the same spectral regions as PQQ, albeit with somewhat different maxima.

A similar result was found for the hydrolysates of bovine serum albumin (BSA) and diamine oxidase (DAO) from porcine kidney, an enzyme which contains covalently bound PQQ [20], since they showed fluorescence properties similar to those of the casamino acid solution (Fig. 3). No PQQ was detected in the casamino acid solution or the BSA and DAO hydrolysates using the biological assay described elsewhere [19] (PQQ concentration <2 nM).

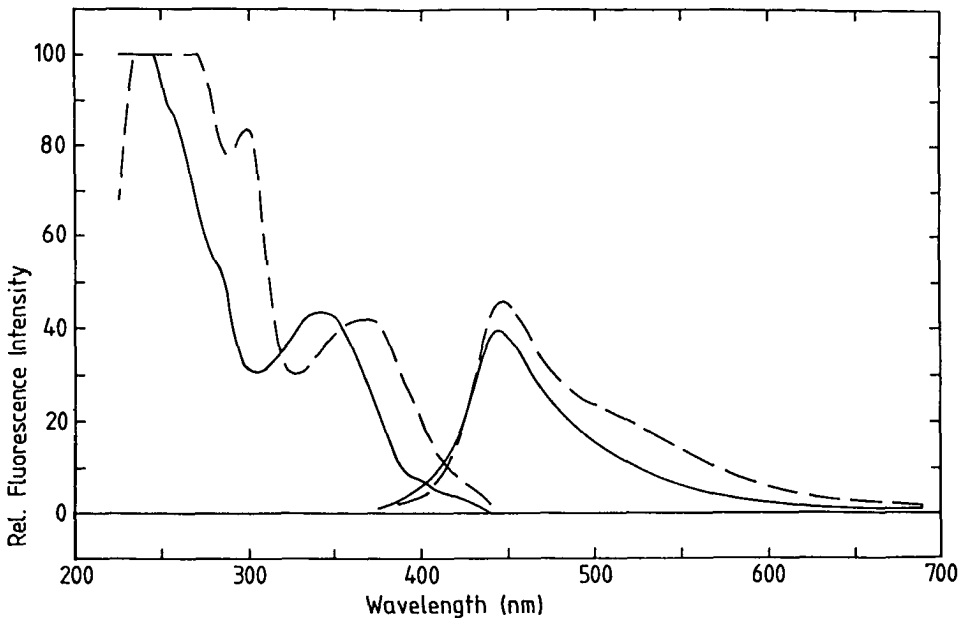


Fig. 3: Fluorescence spectra of hydrolysates of diamine oxidase, 0.13 g/liter (—) and bovine serum albumin, 0.14 g/liter (- - -) at pH 7.0.

The emission spectra were obtained by excitation at 365 nm and the excitation spectra were obtained by monitoring the emission at 460 nm.

5. DISCUSSION

Vitamins and coenzymes are already effective in biological systems at very low concentrations. Therefore, in order to establish whether such a compound is synthesized by an organism, one must be sure that even the slightest contamination with that compound has been excluded.

Since we frequently found that *A. calcoaceticus* PQQ⁻mutants and *E. coli* strains contained varying amounts of GDH holoenzyme, an attempt was made to remove PQQ which might be present in the media and on the glassware. Both heating of glassware as well as treatment of the media with an ion exchanger were necessary to achieve consistently low levels of GDH activity (Tables 1

and 2). These results imply that, in practice, contamination with PQQ is not imaginary and that the common *E. coli* laboratory strains do not produce PQQ under our conditions of growth; the latter is in accordance with our earlier findings [3]. Although Ameyama *et al.* originally claimed that *E. coli* produced large amounts of PQQ [5], in a recent report [21] they mentioned that variable amounts of GDH holoenzyme were found in this organism. In view of these contradictory findings and the fact that they did not mention precautions taken to avoid PQQ contamination, it seems questionable whether the *E. coli* strain they used synthesizes PQQ at all.

The biological assay developed with PQQ⁻-mutants of *A. calcoaceticus* is very sensitive. From Fig. 1 it can be concluded that 1 ng PQQ in a 10-ml sample can be detected easily. This is 20 times more sensitive than other biological assays based on reconstitution of quinoprotein apoenzymes [19,21]. In this context it also should be realized that contamination of a culture fluid with 0.1 nM PQQ already gives rise to a background of 1% GDH holoenzyme activity.

Because spontaneous reversion in *A. calcoaceticus* PQQ⁻-mutants does not occur (reversal was reported for a *P. aeruginosa* PQQ⁻-mutant [21]), and variable background GDH activity can now be avoided, a reliable test is available which can be used for the detection of PQQ, especially for large samples. Although a similar test could be developed with *E. coli* PPA41 (this mutant lacks the route for glucose metabolism via the PTS system) no attempts were made to examine this strain for that purpose.

In order to prove the presence of PQQ in enzymes and materials derived from animals or plants, Ameyama *et al.* used fluorescence spectroscopy for identification [5,7,18]. Our results (Figs. 2, 3) reveal that protein hydrolysates show fluorescence in the same spectral regions as PQQ. Although spectral differences are manifest, they are too small to apply this technique for the detection of PQQ in protein hydrolysates. Furthermore, it should be noted that PQQ reacts with amino acids to yield a number of weakly fluorescing compounds which are inactive in a biological assay [2,9]. The biological activity of PQQ is reduced to less than 1% of the original value after incubation for 24 h at room temperature in a solution of casamino acids (unpublished results). In view of these findings, it seems necessary to reexamine the materials which Ameyama *et al.* claim contain PQQ and, therefore, are stimulatory in growth experiments with certain microorganisms [4].

It will also be clear that it is highly improbable that PQQ as such is present in hydrolysates of proteins which contain covalently bound PQQ and that it is certainly not possible to use fluorescence spectroscopy for identifying PQQ in such mixtures. Although Ameyama *et al.* claim that they were able to do so [18], we believe that PQQ should be converted to a hydrazone with hydrazine before hydrolysis of the enzyme occurs. By following this procedure we were able to demonstrate that PQQ is the organic prosthetic group in bovine plasma amine oxidase [2], in diamine oxidase from porcine kidney [20], and in human lysyl oxidase [22].

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Chapter III

Factors relevant in the reaction of PQQ with amino acids.

Analytical and mechanistic implications.

1. SUMMARY

The reaction of PQQ on incubation with amino acids was followed spectrophotometrically, by monitoring oxygen consumption, and with a biological assay for PQQ. For several α -amino acids, the formation of a yellow coloured compound ($\lambda_{\text{max}} = 420 \text{ nm}$) was accompanied by oxygen uptake and disappearance of biological activity from the reaction mixture. The yellow product appeared to be an oxazole of PQQ, the exact structure depending on the amino acid used. Oxazole formation also occurred under anaerobic conditions with concomittant formation of PQQH_2 , suggesting that PQQ is used as oxidant in the formation of oxazole from the corresponding oxazoline. Since oxygen uptake under aerobic conditions was higher than required for oxazole formation, there also exists a cyclic reaction in which the supposed aldimine adduct of PQQ and the amino acid, is converted into the aminophenol form of the cofactor and an aldehyde resulting from decarboxylation of the amino acid. Addition of NH_4^+ -salts as well as that of certain divalent cations greatly stimulated the reaction rate. With basic amino acids, oxazole formation scarcely occurred. However, as oxygen consumption was high (provided that special divalent cations were present), degradation of these compounds occurred. A reaction scheme is proposed explaining the role of activators in these reactions. Since the activity of several quinoproteins (PQQ-containing enzymes) is highly stimulated by NH_4^+ -ions, and divalent cations (Ca^{2+} , Fe^{2+} , and Cu^{2+}) are additional cofactors in certain metallo-quinoproteins, the outcome of the present investigation is compared with the mechanistic features of these enzymes. The results described here explain why analysis methods for PQQ can fail in case the samples contain amino acids. Although hydrolysis of model oxazoles into PQQ was achieved in good yield under strongly acid conditions, unfortunately hydrolysis of PQQ-oxazoles, formed from a complex mixture of amino acids, did not lead to PQQ, because tryptophan had a deleterious effect on PQQ under this condition.

2. INTRODUCTION

Analysis of the cofactor pyrroloquinoline quinone (PQQ) is feasible with biological assays as well as with chromatographic procedures [1]. Although these methods are in principle sensitive and reliable, problems are met in cases where the compound does not occur in its free form. PQQ is known to react with a variety of nucleophiles [1] so that it cannot be analysed in a protein hydrolysate. Therefore, to detect and quantify the cofactor in enzymes where it exists in covalently bound form, the so-called "hydrazine method" was developed, derivatizing PQQ *in situ* to the stable C(5)-hydrazone before hydrolysis is carried out [2,3]. The PQQ-hydrazone can then be isolated from the hydrolysate and quantified by chromatographic procedures. Although this method has been successfully applied on several of these quinoproteins (PQQ-containing enzymes) [2,3,4], the procedure relies on the initial formation of the hydrazone derivative. Therefore, this approach may not be applicable to all enzymes. Moreover, the question remains to be settled whether PQQ can be analyzed by this method in those cases where stable adducts already exist.

Reactions of PQQ with nucleophiles are also interesting from a physiological point of view. Quinoproteins appear to be widely distributed among prokaryotes as well as eukaryotes [1]. In addition, several bacteria produce quinoprotein apoenzymes (e.g. glucose dehydrogenase by *Escherichia coli* [5], alcohol dehydrogenase by *Pseudomonas testosteroni* [6]). Attempts to induce PQQ synthesis in these organisms have failed so far but the apoenzymes become functional on addition of PQQ to the bacteria. The fact that several other bacteria excrete PQQ into their culture medium (e.g. methylotrophic bacteria, acetic acid bacteria, *Pseudomonas* species) could be interpreted that PQQ is a growth factor for those organisms producing quinoprotein apoenzymes. Also the occurrence of quinoproteins in mammalian tissues [3] raises the question whether PQQ is taken up from the diet as a vitamin or is a compound which is produced by the organism itself. Considering the rapid reaction of PQQ with nucleophiles *in vitro* [1], it is highly improbable that the compound is available in its free (oxidized) form under *in vivo* conditions, where substantial concentrations of nucleophiles are normally present. Thus, also for this aspect it is very important to know the factors which are relevant for adduct formation of PQQ. In addition, it could be imagined that it is possible to convert the adducts

into PQQ. Since this could provide insight into the mechanism of transport and storage of PQQ *in vivo* as well as providing clues for analysis of PQQ in complex biological systems, development of ways to decompose PQQ-adducts is also important.

Reaction of PQQ with some amines [7,8,9], amino acids [10, Flueckiger *et al.*, to be published], and thiols [11] has been described recently. In view of the omnipresence of amino acids, the reaction of these compounds with PQQ seems most interesting. Oshiro and coworkers [10] reported decarboxylation of amino acids to the aldehyde with PQQ as a catalyst. On the other hand, Bruce and coworkers [9] found that the reaction of 7,9-didecarboxy-PQQ with glycine gives an oxazole of PQQ (Fig. 1). In view of these controversial

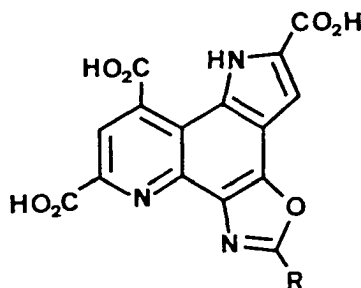


Fig. 1: Structure of PQQ-oxazoles.

findings and their significance for the elucidation of the role of PQQ as a vitamin and its analysis in biological materials, it was decided to investigate decarboxylation as well as oxazole formation with several amino acids, to determine the factors which could influence these reaction, and to develop methods to convert the presumed oxazole adducts into PQQ.

3. MATERIALS AND METHODS

Chemicals

PQQ, its C_5 -acetone adduct, and PQQ-trimethyl ester were prepared as described [12]. 3-methyl-PQQ and 3-propyl-PQQ were synthesized according to a published procedure [12] with modifications (J.A. Jongejan, unpublished results). 5-OH-PQQ was prepared by dehydration of $PQQH_4$ (to be published

elsewhere). 4-OH-PQQ was a kind gift of Prof. C.W. Rees. N₁-methyl- and N₁-ethyl-PQQ were prepared by a method to be published elsewhere, according to a published procedure [13]. All other chemicals were from commercial sources.

Oxazole formation

Oxazole formation was monitored by measuring the rate of absorption increase at 420 nm. The type of oxazole was determined afterwards by HPLC and photodiode array detection, giving the retention time and absorption spectrum. Unless otherwise indicated, oxazole formation was studied in a standard mixture consisting of the following components (total volume 3 ml): 0.033 M Tris/HCl, pH 8.3; 0.3 M NH₄Cl; 0.4% (w/v) amino acid; 10 μ M EDTA, 110 μ M Ca²⁺; 50 μ M PQQ. EDTA was added to minimize interference of metal ions which bind stronger to EDTA than Ca²⁺. The reaction was started by addition of amino acid. Analysis by HPLC occurred after at least 2 h of incubation. HPLC was performed on a Waters RCM 100 module containing a 10 μ M C₁₈ RCM reversed phase cartridge. The eluent (flowrate 1.5 ml/min) consisted of a linear gradient (20 min) of 10-90% methanol containing 0.4% (v/v) of concentrated H₃PO₄. The eluting peaks were scanned (at 250 nm) with a Hewlett-Packard 1040A photodiode-array detector, taking spectra at the apex of the peaks.

Oxygen consumption

The rates of oxygen uptake were measured at 25°C with a Clarck type oxygen electrode in the standard mixture for oxazole formation. The reaction was started by addition of PQQ and oxygen consumption rates were calculated on the basis of an initial oxygen concentration in the mixture of 250 μ M.

Biological assay

Samples were analyzed with a biological assay, measuring the extent of reconstitution of the quinoxaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni* [6].

Anaerobic experiments

Solutions were made anaerobic by degassing under reduced pressure and subsequently leading a stream of N_2 (<3 ppm O_2) through the solution for 15 min.

Oxazole model compounds

a. From glycine, L-serine and L-threonine.

100 mg PQQ.1 H_2O was dissolved in 0.05 M NH_4Cl (100 ml), and the pH adjusted to 8.2 with 0.1 M NaOH. Amino acid (350 mg) was added, and the mixture was incubated with stirring at room temperature while leading a stream of O_2 through the solution, the pH being kept at 8.2 with 0.1 M NaOH. After 2 h, the pH was brought to 1.5, and the solution stored at 4°C. The precipitated product was filtered off, and redissolved in water (pH 4.0) which was then heated to 90°C. After acidification to pH 1.5, the solution was precipitated at 4°C, and filtered. This procedure was repeated once to remove all mineral salts, and the precipitate was dried under vacuum above P_2O_5 to give 110 mg of orange-brown product. 50 mg was recrystallized from DMSO, and applied to NMR analysis.

b. From glycine-methyl ester.

100 mg PQQ.1 H_2O was dissolved in 0.3 M NH_4Cl plus 3 μM EDTA (110 ml), and the pH adjusted to 8.3. Glycine ester (250 mg) was added, and the mixture was incubated with stirring for 2 h. Adjusting the pH on incubation was not necessary in this case. The solution was acidified to pH 1.0 at 4°C, and the precipitate filtered off and redissolved in 5 ml DMF at slightly elevated temperature. After two days, the precipitate was recrystallized once again from DMF to yield 10-20 mg product, and washed with ether before being air-dried. The product was dissolved in DMSO and applied to NMR analysis.

Hydrolysis of oxazoles

Alkaline hydrolysis was performed by dissolving the oxazole in 2 M NaOH and incubating with 1% glycine under exclusion of O_2 at 100°C for at least 8

h (the inclusion of glycine improved the yields, probably because it keeps the cofactor in its reduced form under the employed conditions). Acid hydrolysis was performed by dissolving the oxazole in 2 M HCl and incubating at 100°C for 2 h. After incubation, samples were brought to pH 5 to 6, either with HCl or NaOH, and diluted five times with water before carrying out the biological assay. To stimulate oxazole formation and decomposition in complex media, a synthetic mixture was used consisting of the following amino acids (g/l): L-lysine (0.53), L-histidine (0.27), L-arginine.HCl (0.52), L-aspartic acid (0.70), L-threonine (0.34), L-serine (0.26), L-glutamic acid (0.84), L-proline (0.34), glycine (0.42), L-alanine (0.59), L-valine (0.47), L-isoleucine (0.35), L-leucine (0.58), L-cysteine (0.09), L-methionine (0.14), L-tyrosine (0.18), L-phenylalanine (0.34), L-tryptophan (0.30).

NMR spectroscopy

¹H-NMR spectra were recorded on a Varian EM 360 spectrometer operating at 60 MHz, using tetramethylsilane as an internal reference.

4. RESULTS

Oxazole formation

The reaction of PQQ with glycine in the standard mixture resulted in the formation of a yellow product ($\lambda_{\text{max}} = 420 \text{ nm}$) with concomitant O₂ uptake and disappearance of biological activity. Since the isolated product was not biologically active, it could be calculated that 3.2 moles of O₂ were consumed per mole of PQQ converted. L-serine and L-threonine also gave a yellow product in good yield. All the three yellow products appeared to be identical as judged from HPLC (retention time of 17.5 min), absorption spectra, and ¹H-NMR analysis [signals at 7.30 ppm (3-H), 8.36 ppm (8-H), and 9.29 ppm (methenyl-H)]. In view of the NMR signals, the most likely structure is as indicated in Fig. 1, analogous to that proposed by Bruce et al. [8] for the product of 7,9 didecarboxy PQQ ethyl ester and glycine (R=H). Most probably, the product formed from L-tryptophan and L-tyrosine has the same structure (indicated as type I), in view of the similar

Table 1: Different types of oxazoles.

Samples were taken from the standard mixture after incubation for 2 h and subjected to HPLC. Products were detected by monitoring the eluate by UV detection at 250 nm. Spectra were taken with the photodiode array detector at the apex of the peaks. Yields were calculated by using the model oxazoles as a standard.

Amino acid	Retention time t_R (min)	Yield (%)	Oxazole type

Glycine	17.7	100	I
L-serine	17.6	75	I
L-threonine	17.7	86	I
L-tryptophan	17.5	70	I
L-tyrosine	18.3	9	I
L-alanine	17.5	5	I
	15.4	8	III
L-valine	18.0	6	II
L-leucine	17.7	10	II
L-isoleucine	18.7	10	II
L-phenylalanine	19.8	24	II
L-methionine	18.0	40	II
L-glutamate	15.8	13	III
L-glutamine	15.5	32	III
L-aspartate	15.9	22	III
L-asparagine	15.6	30	III
L-histidine	14.9	9	III
Glycine methyl ester	16.9	54	IV
Glycine ethyl ester	17.7	56	IV

retention times (Table 1) and absorption spectra (Fig. 2). Other amino acids tested gave yellow products with different retention times and/or absorption spectra (Table 1, Fig. 2). Based on the absorption spectra, two classes can be indicated (type II and III). Since the differences are marginal, it is assumed that the yellow products indicated as type II and III have the structure as indicated in Fig. 1, with the residue R being the R of the

amino acid used. L-alanine behaved exceptional since several yellow coloured products were found, two of these with characteristics of oxazole type III, and one with characteristics of type I.

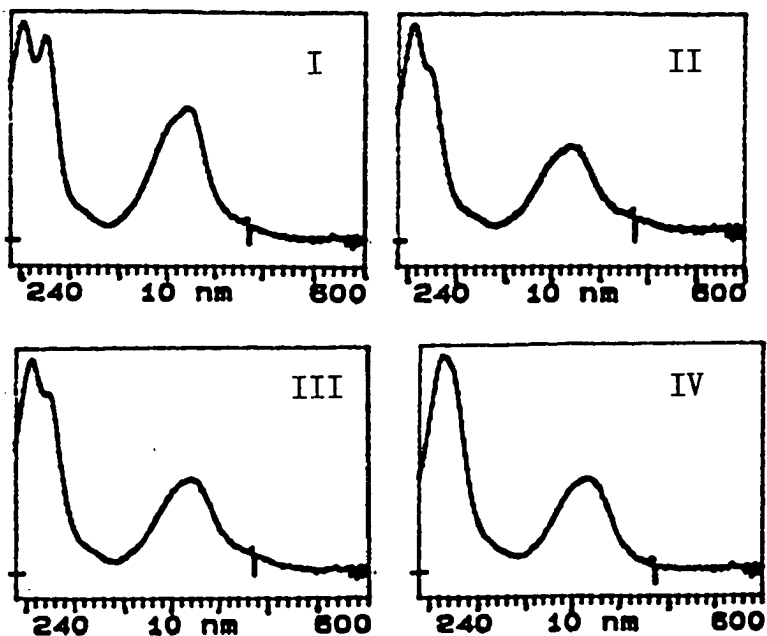


Fig. 2: Absorption spectra of different types of oxazoles.

Samples were taken from the standard mixture after incubation for 2 h and subjected to HPLC. Products were detected by monitoring the eluate by UV detection at 250 nm. Spectra were taken with the photodiode array detector at the apex of the peaks.

No yellow compounds were detected for L-proline, L-lysine and L-ornithine. However, the latter two reacted with PQQ to other products, as revealed by HPLC.

PQQ became reduced to PQQH₂ on incubation with L-cysteine. In addition, a precipitate was formed, most probably L-cystine.

Table 2: Oxygen consumption and oxazole formation rates for several amino acids and related compounds.

In some cases, Ca^{2+} was replaced by Cu^{2+} in the standard mixture, as indicated. Compounds showing no activity in the standard mixture were further tested with both divalent cations, and in the presence and absence of NH_4^+ .

Amino acid	Stimulation by		O_2 consumption $v_{\text{init}}^{\text{init}}$ (nmol.l ⁻¹ .s ⁻¹)	Oxazole formation $v_{\text{init}}^{\text{init}}$ (nmol.l ⁻¹ .s ⁻¹)	Moles of O_2 consumed/ moles of oxazole formed
	NH_4^+	cations			
Glycine	+	Ca^{2+}	245	130	3.2
L-serine	+	Ca^{2+}	16	26	0.75
L-threonine	+	Ca^{2+}	15	28	0.67
L-tryptophan	+	Ca^{2+}	36	23	1.6
L-alanine	+	Ca^{2+}	6	1.1	n.d.
L-leucine	+	Ca^{2+}	8	2.5	n.d.
N-methylserine			0	0	
L-lysine	-	Cu^{2+}	152	< 0.1	
L-ornithine	-	Cu^{2+}	245	< 0.1	
L-glutamine	-	Cu^{2+}	248	< 0.1	
L-asparagine	-	Cu^{2+}	123	< 0.1	
L-arginine	-	Cu^{2+}	378	< 0.1	
δ -aminovalerate			0	0	
ϵ -aminocaproate			0	0	
Glycinamid	+	-	n.d.	80	n.d.
Glycine Me-ester	+	-	21	95	0.67
Glycine Et-ester	+	-	25	60	0.74

n.d. = not determined

Glycinamide and glycine esters also gave yellow products (type IV), but with absorption spectra and retention times different from the product of glycine (type I). NMR spectroscopy revealed that the ester group is retained ($R = \text{COOCH}_3$ in Fig. 1) since, in addition to the signals of the aromatic protons (at 7.43 and 8.55 ppm, respectively), a singlet was found at low field (3.78 ppm), originating from the $-\text{OCH}_3$ moiety. On the other hand, amino acids with derivatized amino groups (N-methyl serine, Table 2) did not react.

Conditions for α -amino acids

Glycine was used as a model compound to study the conditions and kinetics of the aerobic oxidation of α -amino acids with PQQ. The addition of NH_4^+ -salts (0.3 M) stimulated the rate of oxazole formation and oxygen consumption at least 30-fold. The optimum pH was 8.3 (Tris/HCl buffer). Decreasing the glycine concentration (added in large excess) did not substantially affect the reaction rate, since a tenfold decrease resulted in only a twofold lowering of the reaction rate.

Since it was found that low concentrations (10 μM) of EDTA almost completely inhibited the reaction of PQQ with glycine, the influence of metal ions on oxygen consumption and oxazole formation was investigated. Only Ca^{2+} and Cu^{2+} , and to a slight extent Mg^{2+} ions, stimulated the reaction (Table 3), whereas Ba^{2+} , mono- or trivalent cations did not. With Mn^{2+} , oxygen consumption was very low and a different yellow coloured compound was formed ($\lambda_{\text{max}} = 383 \text{ nm}$). In the presence of Fe^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} , type I oxazole compound and the 383 nm-compound were observed, as was revealed by the absorption spectra of the mixtures. Oxygen consumption in the standard mixture was inhibited by these metal ions (results not shown). In the case of Mn^{2+} supplementations to the standard mixture, O_2 consumption linearly decreased and complete abolishment required the addition of one mole of Mn^{2+} per 2 moles of PQQ.

The 383 nm-product was also formed in the presence of high (1 mM) Ca^{2+} or Cu^{2+} concentrations. Boiling of the mixture or acidification to pH 4 resulted in a complete disappearance of the 383 nm-product and concomittant formation of type I oxazole. With Mn^{2+} , oxazole formation could also be demonstrated after acidification or boiling the mixture for longer periods.

Addition of excess EDTA to the 383 nm-product resulted in the reappearance of PQQ.

To study only the oxazole formation process, kinetic experiments were performed with low concentrations (20 μM) of Ca^{2+} . The reaction between PQQ, glycine and O_2 proceeds as a pseudo-first-order reaction ($k = 26 \cdot 10^{-3} \cdot \text{s}^{-1}$). Conversion rates with serine, threonine and tryptophan were much lower (Table 2). In addition, the number of O_2 molecules consumed per oxazole molecule formed were lower than for glycine. Other α -amino acids (indicated in Table 2) showed even lower reaction rates.

Conditions for basic amino acids and ω -amides of acid amino acids

For studies on the conditions for basic amino acids, the α, ω -diamino acids, lysine and ornithine, were used. In the standard mixture (with Ca^{2+}), O_2 uptake was very low compared with glycine, but replacing Ca^{2+} by Cu^{2+} , Mn^{2+} , or Zn^{2+} resulted in high stimulations, while replacement by Ni^{2+} inhibited the reaction completely (Table 3). The absence of NH_4Cl in the mixture had scarcely any effect. Only traces of oxazoles or other coloured products were observed and PQQ could be recovered quantitatively from the mixture.

Table 3: The effect of divalent cations on O_2 consumption.

Replacement of Ca^{2+} in the standard mixture occurred, as indicated.

Cation (100 μM)	Oxygen consumption (v_{init} (nmol.l ⁻¹ .s ⁻¹))					
	L-Lys	L-Orn	L-Gln	L-Asn	L-Arg	Gly
-	20	25	-	-	-	-
EDTA (1 mM)	-	13	-	-	-	0
Cu^{2+}	152	245	248	123	378	198
Ca^{2+}	12	23	32	70	33	245
Mn^{2+}	93	97	-	-	-	8
Ni^{2+}	0	0	-	-	-	-
Zn^{2+}	117	155	-	-	-	-
Mg^{2+}	-	-	-	-	-	13

Curiously, ω -amino carboxylic acids, like δ -amino valeric acid and ϵ -amino caproic acid, were unreactive. On the other hand, the ω -amide forms of the acid α -amino acids (L-glutamine and L-asparagine) behaved as the basic amino acids (Table 3). With L-asparagine in the presence of Ca^{2+} , O_2 consumption was somewhat higher (Table 3), and some oxazole was found (results not shown).

Conditions for glycinamide and glycine esters

To study the phenomenon of decarboxylation, amino acids with an esterified or amidated carboxylic acid group were investigated. Glycinamide and glycine esters all gave yellow products with the characteristics of an oxazole (Table 1, type IV), while the reaction rates were comparable to those with glycine under standard conditions. Interestingly, this only occurred in the absence of divalent cations, and much less oxygen was consumed (less than 1 mole O_2 / mole oxazole formed). In the presence of divalent cations

Table 4: Anaerobic conversion of amino acids in the presence of different divalent cations (as indicated).

Yields were determined from the areas of the peaks in a HPLC chromatogram.

Condition	PQQ (%)	PQQH ₂ (%)	oxazole	
			type I (%)	type III (%)
Glycine/ Ca^{2+}	0	31	39	0
Lysine/ Ca^{2+}	7	25	9	3.5
Lysine/ Cu^{2+}	2.2	51	8	3
Ornithine/ Cu^{2+}	4	60	2	5

(Ca^{2+} , Cu^{2+} and Mn^{2+}) a yellow coloured compound ($\lambda_{\text{max}} = 307$ and 410 nm) was formed immediately. With Mn^{2+} , the compound was stable, and oxazole (type IV) was formed only after addition of excess EDTA. With Ca^{2+} and Cu^{2+} , the compound was not stable, as concluded from the decreases in absorbance at 307 and 410 nm on prolonged incubation. However, oxazole (type IV) was not formed unless excess EDTA was added.

Anaerobic conditions

Experiments under anaerobic conditions showed that O_2 is in fact not required for oxazole formation. HPLC analysis of the products showed that with glycine, PQQH₂ and oxazole (type I) were formed in a 1:1 molar ratio (Table 4). With lysine or ornithine, the main product was PQQH₂ and low amounts (up to 10%) of yellow coloured compounds were detected (Table 4).

PQQ derivatives and analogues

To obtain more information on the mechanisms, various PQQ analogues and derivatives were screened for their activity. Oxygen consumption was measured with glycine and L-lysine (in the case of L-lysine, Ca^{2+} in the

Table 5: Oxygen consumption and oxazole formation with PQQ-derivatives and analogues.

Compound	v_{init} (nmol.l ⁻¹ .s ⁻¹)		Oxazole formation Gly/Ca ²⁺	Moles of oxygen consumed/moles of oxazole formed
	O ₂ consumption			
	Gly/Ca ²⁺	Lys/Cu ²⁺		
PQQ	245	152	130	3.1
3-methyl-PQQ	133	82	165	2.2
3-propyl-PQQ	223	68	480	3.6
N ₁ -methyl-PQQ	150	80	125	-
N ₁ -ethyl-PQQ	250	87	-	-
PQQ-trimethyl ester	161	53	90	2.1
PQQ-acetone adduct	-	0	1.4	-
4-OH-PQQ	-	0	0	-
5-OH-PQQ	-	0	0	-
Phenanthroline-dione	< 5	< 5	-	-
Phenanthraquinone	< 5	< 5	0	-

standard mixture was replaced by Cu^{2+}), and oxazole formation for glycine only. Phenanthraquinone, PQQ-acetone adduct, 4-OH-PQQ and 5-OH-PQQ were

neither active with glycine nor with lysine (Table 5). 3-Methyl PQQ, 3-propyl-PQQ, N₁-methyl-PQQ, N₁-ethyl-PQQ and the trimethyl ester of PQQ were active with both amino acids (Table 5). Oxazole formation was extremely rapid for 3-propyl-PQQ. With 4,7-phenanthroline-5,6-dione and glycine, formation of a compound was observed with an absorption maximum at 402 nm. Oxygen consumption was insignificant with both amino acids, either in the presence or absence of NH₄⁺-salts.

Alkaline hydrolysis of PQQ oxazoles

Reasonable good yields of PQQ (up to 50% of the original amount), were obtained upon alkaline hydrolysis of oxazole type I, prepared from glycine with PQQ, but only when comparatively high concentrations of oxazole were used (>10 μM) and the solution was held semi-anaerobic by O₂ removal and addition of excess glycine. Because acid hydrolysis looked more promising, the alkaline hydrolysis was not investigated further.

Table 6: Stability of PQQ under strongly acidic conditions in the presence of amino acids.

Amino acid added	[PQQ] before hydrolysis (μM)	recovery (%)
---	1-50	100
Glycine (0.2%)	1-50	100
Mixture amino acids ^{a)}	5.0	79
"	2.0	70
"	1.0	45
"	0.5	29
Tryptophan (0.1%)	1.0	0

a) Described in Materials and methods

Acid hydrolysis of PQQ oxazoles

When the PQQ-glycine-oxazole was hydrolyzed in 2 M HCl at 100°C for 2 h, PQQ was routinely recovered from the mixture in a 70-100% yield, even when the concentration of oxazole in the hydrolysis mixture was as low as 0.5 μ M. This also applied to the PQQ-methionine-oxazole. However, when PQQ (5-50 μ M) was converted completely to oxazoles in nutrient broth (10 g/l) under standard conditions (the amino acid was replaced by nutrient broth), upon subsequent hydrolysis by adding an equal volume of 4 M HCl, no PQQ was detected. A synthetic mixture of amino acids behaved similarly, although some PQQ could be detected in this case (10-50% recovery depending on the PQQ concentration). Since oxazoles were obviously formed and degraded, but recovery of PQQ was variable, the stability of PQQ itself (in the presence of amino acids and 2 M HCl) was checked (Table 6). This experiment showed that severe losses occurred with mixtures of amino acids. Testing the amino acids separately, L-tryptophan was found to be the causative factor. No PQQ could be detected after incubation of a 1 μ M PQQ solution with 0.1 % L-tryptophan for 2 h at 100°C in either 2 M or 6 M HCl.

5. DISCUSSION

Some aspects of the reaction of PQQ with amino acids has been investigated by Oshiro and coworkers [10]. Since GLC-analysis revealed the formation of amino acid-derived aldehydes, they concluded that PQQ behaves as a catalyst in a Strecker-type oxidative decarboxylation reaction. In an earlier report by Bruice and coworkers [9], the conversion of a PQQ analogue to the corresponding pyrroloquinoline oxazole upon incubation with glycine and some amines was demonstrated. From the results described in the present paper it follows that interaction of PQQ with the majority of naturally occurring amino acids under aerobic conditions comprises both a cyclic reaction, apparent in the catalytic degradation of amino acids, and the formation of stable PQQ-amino acid adducts, including oxazoles. Since the interaction of PQQ with amino acids and related substances is a very complicated topic, it is beyond the scope of this article to evaluate all reactions and intermediates individually. Neither can all kinetic implications of a model system be accounted for at the moment. Several important features, however,

have been established, which are (partly) accommodated for in the working hypothesis given in Fig. 3.

Entry into both the linear and the cyclic pathway is governed by the primary addition of the nucleophilic amino group either at C_5 or at C_4 . Since earlier studies [14] have revealed a substantial difference in electrophilic character of the two carbonyl groups, favoring C_5 as the site of primary nucleophilic addition, it can be safely assumed that the reaction starts at the C_5 position of PQQ also in this case. The observation that the rates of the linear and the cyclic pathway are equally affected by ammonia and metal ions favours this assumption. Structure elucidation of the resulting pyrroloquinoline oxazoles should give a definite answer to this question with respect to the exact orientation of the oxazole.

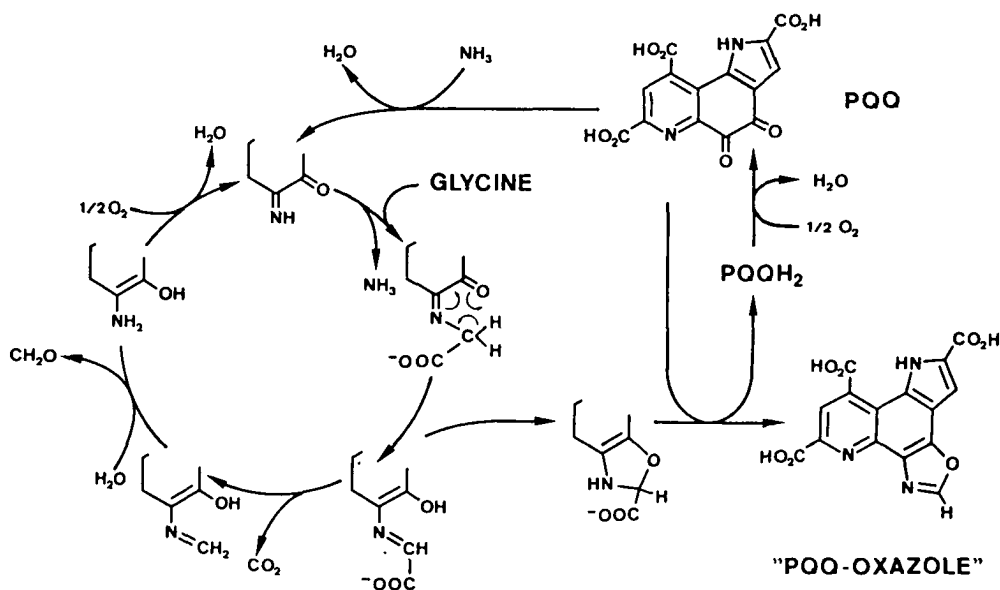


Fig. 3: Proposed reaction mechanism for PQQ and α -amino acids.

Oxazole formation as well as the cyclic pathway, leading to amino acid decarboxylation and reduction of PQQ to PQQH₂, are shown.

The oxidation of α -amino acids was enormously stimulated by ammonia. Since the 5-imino form of PQQ is formed on addition of ammonia to PQQ [14], this stimulation can be explained on the basis of the enhanced electrophilic

character of the C_5 -imino group. Particularly the addition of the amino group of α -amino acids will take place more readily on C_5 -imino PQQ than on the parent C_5 -carbonyl PQQ. This is in accordance with the observation that the oxidation of PQQ with amines and α,ω -diamino acids is not dependent on ammonia.

In the proposed scheme (Fig. 3) the quinone-imine formed after addition of the amino acid to C_5 -imino PQQ tautomerizes spontaneously to the Schiff base. Evidence for this rearrangement has been obtained recently for the reaction of o-quinones with secondary amines [15]. An issue of special interest is the location of the step in which actual branching between the cyclic and linear pathways take place. Knowledge of the chemical nature of the branch point intermediate might eventually enable us to devise conditions favoring suppression of stable adduct formation or, in the opposite case, promote the rapid and complete conversion of PQQ to a limited set of pyrroloquinoline oxazoles. Since glycineamide and glycine esters give rise to oxazoles which have retained the amide or ester moiety, it is obvious that oxazole formation has not necessarily to be preceded by a decarboxylation. Therefore, the Schiff base seems an attractive candidate as branching point intermediate. The tendency of this intermediate to lose the carboxyl group primarily governs the distribution between the cyclic and the linear pathway.

Decarboxylation leads to PQQ-alimine which is hydrolyzed to formaldehyde (in the case of glycine) and PQQ-aminophenol. Ring closure of the Schiff base leads to PQQ-oxazoline. It has been shown that oxazole formation from quinones and quinone imines and amines proceeds via an oxazoline [16]. Another molecule of the quinone was assumed to function as oxidant in the final dehydrogenation step [16]. The results of experiments under anaerobic conditions support this view. The presence of $PQQH_2$ and oxazole in a 1:1 molar ratio in the reaction mixture can be explained only by assuming that PQQ is the hydrogen acceptor in the final dehydrogenation process of PQQ-oxazoline to PQQ-oxazole. The failure to detect any PQQ-aminophenol in the anaerobic mixture is rather surprising. Since under aerobic conditions the cyclic pathway operates effectively (in the case of glycine, 3.2 moles of O_2 were consumed per mole of PQQ converted), one would expect PQQ-aminophenol as the main product, together with some $PQQH_2$ and oxazole. Apparently, the cyclic pathway does not operate at all under anaerobic conditions,

presumably owing to an unfavourable equilibrium situation, preventing the formation of PQQ-aldimine and/or PQQ-aminophenol.

Finally, under aerobic conditions, the reduced products from the cyclic (PQQ-aminophenol) and linear (PQQH₂) are regenerated by oxygen.

HPLC and ¹H-NMR analysis have shown that different types of oxazoles can be formed, depending on the amino acid used. The appearance of unsubstituted (type I) oxazole with serine, threonine, tryptophan and tyrosine illustrates that side chains having even a very modest leaving group character will eventually give rise to the branching point intermediate depicted in Fig. 3 for the reaction of PQQ with glycine. It should be noted that pyridoxal phosphate shows a similar type of reaction with amino acids having an electron attractive group [17].

Reaction rates of PQQ with alanine and the other α -amino acids are very low compared to the rate with glycine, probably because the tautomerization equilibrium involving the quinone imine and Schiff base favors the quinone imine intermediate more strongly for the alanine adduct. Presumably, the ring-closure as well as the decarboxylation reaction are also hampered by steric factors.

No oxazole formation is observed with lysine and related α,ω -diamino acids. These compounds are presumed to behave as amines, in which case the ω -, and not the α -amino group is oxidized.

The presence of certain metal ions resulted in enormous stimulatory as well as inhibitory effects. Since oxazole formation with glycinamide and glycine esters occurred rapidly in their absence, the primary action of the metal ions seems to be their depolarizing effect on the charged carboxyl group. Whether they play a role in facilitating (stable) complex formation between PQQ and the amino acid can not be conclusively answered at the moment, but the inhibitory effect of special cations, notably Mn²⁺, may well be the result of a particularly tight complex involving ligands provided by PQQ and the amino acid. Also the high oxidation rates of α,ω -diamino acids in the presence of certain cations (and the inhibitory effect of other cations), in contrast with the complete absence of oxidation with ω -amino carboxylic acids, strongly favours the assumption that metal ion chelates play an important role in this reaction. Again it should be noted that metal ions are supposed to catalyze the *in vitro* reaction of amino acids with pyridoxal phosphate by the mechanisms mentioned above [17].

The involvement of NH_4^+ and metal ions in the oxidation of amino acids with PQQ is also interesting from a mechanistic point of view. Quinoprotein methanol dehydrogenase (EC 1.1.99.8) has been shown to be activated by NH_4^+ salts *in vitro* [18]. In addition, the activity of quinoprotein methylamine dehydrogenase (EC 1.1.99.--) is highly stimulated by NH_4^+ salts [19]. Quinoprotein glucose dehydrogenase from various bacteria, and quinate dehydrogenase from *A. calcoaceticus* require divalent cations (Mg^{2+} or Ca^{2+}) for cofactor binding [19,20,21], whereas quinohaemoprotein alcohol dehydrogenase from *P. testosteroni* is strictly dependent on Ca^{2+} ions for anchoring of the cofactor [6]. Lipoxxygenase contains a complex of Fe^{3+} and PQQ [22], while Cu^{2+} occurs in copper-containing oxidoreductases. Therefore, the strong effects observed on the reaction of PQQ with amino acids make it very likely that these cations are highly relevant for the catalytic mechanism of these enzymes.

The results with PQQ derivatives show that the o-quinone group is essential for the reaction, but not the only decisive factor, since phenanthraquinone was not active. The fact that only very low oxidation rates were found with 4,7-phenanthroline-5,6-dione may be an indication for the involvement of the 7-COOH group of PQQ, although it may also result from the absence of a difference in reactivity of the two carbonyl groups in this compound (leading to occupation of both carboxyl groups).

All model PQQ-oxazoles tested were easily and nearly quantitatively converted into PQQ in 2 M HCl at 100°C. In contrast, no PQQ was recovered after hydrolysis of oxazoles made from nutrient broth. The reason for this is the reactivity of PQQ towards L-tryptophan under strongly acidic conditions. Since this phenomenon can also occur during hydrolysis in 6 M HCl of quinoprotein enzymes in which PQQ is covalently bound, analysis procedures comprising such a step cannot be reliable. The reaction product(s) formed from PQQ and L-tryptophan under acidic conditions is (are) presently under investigation.

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Chapter IV

Factors relevant in bacterial PQQ production

1. SUMMARY

Quinoprotein content and levels of external PQQ were determined in several bacteria under a variety of growth conditions. Using these data and those from the literature, a number of factors can be indicated which are relevant for PQQ production. Synthesis of PQQ is only started if synthesis of a quinoprotein occurs but reversibly, quinoprotein synthesis does not depend on PQQ synthesis. Quinoprotein substrates are not necessarily required for both quinoprotein and PQQ synthesis. Although PQQ production was determined by the type of organism and quinoprotein produced, coordination between quinoprotein and PQQ synthesis is loose since under-production as well as over-production of PQQ with respect to quinoprotein is observed. The results obtained can be interpreted that quinoprotein synthesis depends on the growth rate while PQQ synthesis does not. In that view, highest PQQ production can be obtained under limiting growth conditions, as was shown indeed by comparing levels of PQQ in fed-batch and batch experiments. The presence of nucleophiles, esp. amino acids in culture media may cause losses of PQQ due to transformation to biologically inactive compounds. Some organisms continued to synthesize PQQ *de novo* when this cofactor was administered exogenously. Probably PQQ can not be taken up by either passive diffusion or active transport mechanisms, and is therefore not able to exert feedback regulation on PQQ biosynthesis in these organisms.

2. INTRODUCTION

From current knowledge on the occurrence of PQQ, it can be concluded that its distribution ranges from microbes to man. PQQ is the cofactor of the so-called quinoproteins, enzymes belonging to the class of oxidoreductases (Duine *et al.*, 1986). To develop this novel branch of enzymology, one of the important aspects to investigate is the production of the cofactor. Knowledge on this aspect is not only relevant for the performance of commercial PQQ fermentation processes but also to obtain insight into

biosynthesis and the possible role as vitamin of the cofactor. Since the mechanisms of induction and regulation are completely unknown, as a first attempt a search was made for the factors which are relevant in bacterial PQQ production.

Quite different situations appear to exist with respect to bacterial PQQ production. Gram-negative methylotrophs not only synthesize PQQ to provide their quinoprotein apoenzymes with PQQ, but they also excrete the compound (amounting to the micromolar level) into their culture media (Duine and Frank, 1980). It is therefore not surprising that such a type of organism is mentioned in all the recent patent applications on the fermentative production of PQQ (Uragami, 1986, Ameyama and Adachi, 1987, and 1988, Uragami, 1987). The same phenomenon is found for some bacteria growing on ethanol, e.g. *Pseudomonas* spp. and acetic acid bacteria, the latter responsible for the substantial amounts of PQQ in vinegar (Duine *et al.*, 1985). However, it appears that the provision with PQQ is not always adequate in these cases. Thus, it was reported (Ameyama *et al.*, 1984, 1985a) that the lag time in growth of *Acetobacter* species can be shortened by supplementation of the culture media with PQQ. Whether this effect is related to uncoordinated synthesis of PQQ and apo-quinoprotein or to removal of PQQ from the holo-enzyme during manipulation of the organism is not clear. The latter explanation is supported by the fact that detectability of the effect required repeated washings of the cells (Ameyama *et al.*, 1985b). On the other hand, several bacteria have been shown to have a variable ratio of apo- to holo-quinoprotein enzyme (Hommes *et al.*, 1985, Duine *et al.*, 1986, van Schie *et al.*, 1987).

A very curious property, shown by many bacteria and unexplained so far, is the phenomenon of production of apo-quinoprotein enzyme but not of PQQ. Originally, this effect was found for glucose dehydrogenase in *Acinetobacter lwoffii* (van Schie *et al.*, 1984), and later on in many *Escherichia coli* strains (Hommes *et al.*, 1984). When these organisms are provided with PQQ, it appears that aldose sugars can be used as an auxiliary energy source via this enzyme (van Schie *et al.*, 1985, Müller and Babel, 1986). A more direct role is obvious in the case of quinoprotein alcohol dehydrogenase of *Pseudomonas testosteroni*. On supplementing the medium with PQQ, growth rates on alcohols were stimulated significantly (Groen *et al.*, 1986). A crucial role has been observed for polyvinyl alcohol dehydrogenase in a *P.* species, the organism only able to grow on this substrate if it is provided with PQQ

(Shimao *et al.*, 1984). In all these cases, the question can be posed why the organisms do not produce the holoenzyme. The answer could be that they lack (part) of the information for PQQ biosynthesis. This view is supported by the recent finding that transfer of four genes of PQQ biosynthesis, cloned from *A. calcoaceticus*, led to glucose dehydrogenase holo-enzyme production in *A. lwoffii* (Goosen *et al.*, 1987) and *E. coli* (Dr. N. Goosen, personal communication).

From the foregoing it will be clear that PQQ synthesis by bacteria is a complicated matter. Based on the present work and data from the literature, factors are indicated which are relevant for the production of the cofactor.

3. MATERIALS AND METHODS

Cultivation

Acinetobacter calcoaceticus LMD 79.41 was cultivated on an inorganic medium (van Kleef *et al.*, 1987). *Pseudomonas putida* biovar B LMD 84.56, *P. stutzeri* LMD 26.48, and *P. aureofaciens* ATCC 15926 [obtained from Professor F. Lingens (Salcher and Lingens, 1980)] were cultivated on an inorganic medium containing per liter: 4.6 g KH_2PO_4 , 11.5 g K_2HPO_4 , 2.5 g NH_4Cl , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and calcium, iron and spore solutions according to Duine *et al.*, 1978. *Hyphomicrobium* X was grown on methanol according to Duine *et al.* (1978) and *Methylobacterium organophilum* XX according to Biville *et al.*, (1988). In batch experiments, mineral media were supplemented with a carbon and energy source as indicated. In fed-batch experiments, strains were precultured on 20 ml mineral medium supplemented with a carbon source (0.2%) as indicated. When good growth was obtained ($\text{OD}_{610} > 1.0$), the culture was diluted ten times with fresh mineral medium and feeding started with the carbon source in mineral medium at a flow rate of 10 ml/h. All organisms were grown at 30°C on a rotary shaker (200 rev min⁻¹).

Enzyme assays

Cell free extracts of *A. calcoaceticus* and *P. putida* were prepared as reported elsewhere (Duine *et al.*, 1979). Cell-free extracts of *Hyphomicrobium* X were prepared according to Dijkstra *et al.*, 1988.

Glucose dehydrogenase activity was measured as described previously (Duine *et al.*, 1979), measuring the reduction rate of Wurster's Blue in 0.1 M Tris/HCl buffer, pH 7.0. Quinate dehydrogenase was assayed as described (van Kleef and Duine, 1988), measuring the reduction rate of Wurster's Blue in 0.1 M Tris/HCl buffer, pH 7.5. Methanol dehydrogenase was assayed in a reaction mixture containing (total volume 1 ml): 0.06 M borate plus 0.06 M NH_4Cl , pH 9.0, 100 μM Wurster's Blue, 5 mM methanol and cell-free extract. All measurements were performed at room temperature. One unit of enzyme activity was defined as the amount of enzyme required to convert one micromole of substrate per minute under the assay conditions.

PQQ assay

PQQ was assayed enzymically using PQQ dependent alcohol dehydrogenase apoenzyme from *P. testosteroni* (Groen *et al.*, 1986).

4. RESULTS

Induction of PQQ synthesis

In Table 1, a compilation is given showing the production of quinoproteins (as a rough estimate) and the production of PQQ in the culture medium under a variety of conditions, and with different bacteria. The following observations are relevant with respect to induction: (i) in the absence of quinoprotein synthesis, there is no production of extracellular PQQ (results not shown), and (ii) production of quinoprotein, and thus of PQQ, can occur in the absence of the quinoprotein substrate. The latter observation applies for instance to *A. calcoaceticus* and several *Pseudomonas* species, showing constitutive glucose dehydrogenase holo-enzyme production, and *M. organophilum* XX synthesizing appreciable amounts of methanol dehydrogenase with succinate as sole carbon and energy source. Growth on p-hydroxybenzoate induces quinate dehydrogenase (QDH) in *A. calcoaceticus*. This carbon source is not degraded via quinate, but via protocatechuic acid, which has been shown to be the inducer of all the enzymes of both the β -ketoadipate and hydroaromatic pathways, including QDH, in *A. calcoaceticus* (Tresguerres *et*

Table 1: Bacterial quinoprotein and PQQ production.

Organism	Carbon source	Quinoprotein(s) synthesized		Extracellular PQQ production (nmol/l)
		Type ¹	Amount	
		U/g wet cells		

a. In batch cultures				
<i>A. calcoaceticus</i>	Succinate	GDH	10-20	<5
	Quinate	GDH	20-30	
		QDH	5-10	50-200
		p-OH benzoate	GDH	20-30
		QDH	5-10	50-700
<i>A. lwoffii</i> RAG-1 ²	Peptone	apoGDH	2-10	0
	Quinate	apoGDH	2-10	0
		apoQDH	0.27	0
<i>E. coli</i> ³		apoGDH	1-2	0
<i>Agrobacterium</i> spp. ⁴	Glucose	apoGDH	n.p.	0
<i>R. leguminosarum</i> ⁴	Glucose	apoGDH	n.p.	0
<i>Acetobacter aceti</i>	Alcohols	ADH ⁵	195	
		AldDH ⁶	250	n.p.
<i>Gluc. suboxydans</i>	Glucose	GDH ⁷	140	n.p.
	Alcohols	ADH ⁵	375	
		AldDH ⁶	160	n.p.
<i>P. aeruginosa</i>	Gluconate	GDH	n.p.	<5
	Alcohols	ADH	40 ⁸	500-3000
<i>P. fluorescens</i> ⁹	Glucose	GDH	70	<5
<i>P. testosteroni</i> ¹⁰	Alcohols	apoADH	34	0
<i>P. stutzeri</i>	Alcohols	ADH	n.p.	2000
<i>P. putida</i> biovar B	Alcohols	ADH	n.p.	2000-3000
	Glucose	GDH	2	<5
	Quinate	QDH	1-2	<5
<i>P. aureofaciens</i>	Benzoate	GDH	n.p.	<5
	Quinate	QDH	3	<5
<i>Hyphomicrobium</i> X	Methanol	MDH	48	3000-6000

Organism	Carbon source	Quinoprotein(s) synthesized		Extracellular PQQ production nM
		Type ¹	Amount U/g wet cells	

<i>M. organophilum</i> XX	Methanol	MDH	2.2 ¹¹	2000-8000
	Succinate	MDH	2.6 ¹¹	400
<i>P. BB1</i> ¹²	Methanol	MDH	32	2000-4000
<i>Nocardia</i> sp. 239 ¹³	Methanol	MDH	?	2000
<i>Arthrobacter</i> Pl ¹⁴	Methylamine	MeAO	3.7	0
<i>Thiobacillus versutus</i> ¹⁵	Methylamine	MADH	6	3000-6000

b. In fed-batch cultures

<i>A. calcoaceticus</i>	Succinate	GDH	20-60	50-150
	Quinate	GDH	20-80	
		QDH	5-10	50-700
<i>P. stutzeri</i>	Alcohols	ADH	n.p.	4000-10000
<i>P. putida</i> biovar B	Alcohols	ADH	n.p.	3000-6000
<i>Nocardia</i> sp. 239 ¹³	Methanol	MDH	?	6000

¹ GDH= Glucose dehydrogenase; QDH= Quinate dehydrogenase; ADH= Alcohol dehydrogenase; AldDH= Aldehyde dehydrogenase; MDH= Methanol dehydrogenase; MADH= Methylamine dehydrogenase; MeAO= Methylamine oxidase.

² van Kleef and Duine, 1988.

³ Hommes *et al.*, 1984.

⁴ van Schie *et al.*, 1987.

⁵ Ameyama *et al.*, 1982a.

⁶ Ameyama *et al.*, 1982b.

⁷ Ameyama *et al.*, 1981.

⁸ Groen *et al.*, 1984.

⁹ Ameyama *et al.*, 1981.

¹⁰ Groen *et al.*, 1986.

¹¹ Biville *et al.*, 1988.

¹² Dijkstra M., personal communication.

¹³ P. van Ophem, personal communication.

¹⁴ van Iersel *et al.*, 1986, R. van der Meer, personal communication.

¹⁵ J. Frank and J. van Wielink, personal communication.

n.p. = not performed.

al., 1970, Ingledew *et al.*, 1971). In all cases where quinoprotein synthesis is observed in the absence of its substrate, biosynthesis of PQQ could be

demonstrated (Table 1), either directly when the cofactor is excreted in the culture fluid, or indirectly, measuring quinoprotein holoenzyme activity.

Factors relevant for the production rate

On the basis of the results presented in Table 1, and numerous batch and fed batch experiments in which growth and PQQ production was followed simultaneously (results not shown), the rate of external PQQ synthesis in several organisms has been estimated. From Table 2 it appears that substantial differences are found, according to the organism and carbon source used.

Table 2: Comparison between type of quinoprotein, and rate of PQQ biosynthesis.

Organism	Carbon source	Quinoprotein synthesized ¹	Specific activity U/mg protein	Rate of PQQ synthesis ²

<i>A. calcoaceticus</i>	Succinate	GDH	635 ³	0.5-1.0
	Quinate	QDH + GDH	-	2.5-3.0
<i>P. fluorescens</i>	Glucose	GDH	386 ⁴	< 2.0
<i>P. aeruginosa</i>	Ethanol	ADH	35 ⁵	50
<i>P. stutzeri</i>	Ethanol	ADH	-	55
<i>Hyphomicrobium</i> X	Methanol	MDH	8.1 ⁶	70

¹ GDH = Glucose dehydrogenase; QDH = Quinate dehydrogenase; ADH = Alcohol dehydrogenase; MDH = Methanol dehydrogenase.

² Expressed as nanomoles excreted PQQ per g wet weight of cells per h.

³ Dokter *et al.*, 1986.

⁴ Ameyama *et al.*, 1981.

⁵ Groen *et al.*, 1984.

⁶ Duine *et al.*, 1978.

Coordination between quinoprotein and PQQ synthesis

The fact that several organisms produce apo-quinoprotein but not PQQ, indicates that there is not a dependency of the first process on the second. The absence of a tight coupling of the processes is also apparent from the over-production of PQQ with respect to quinoprotein synthesis, resulting in

excretion of PQQ into the medium. Reversibly, situations exist in which PQQ is under-produced. In experiments with fast exponentially growing batch cultures of *A. calcoaceticus* (e.g. grown on ethanol) no PQQ can be detected in the culture fluid, and only 25% of the GDH in the cell-free extract is in its holoenzyme form (results not shown). Examining the very low rates of PQQ biosynthesis found in *A. calcoaceticus* grown on carbon sources on which QDH is not induced (Table 2), and the appreciable amounts of GDH found under this condition (Table 1), this is not surprising. The same observation applies to *P. putida* biovar B grown on glucose in batch culture. Only 25% of the GDH in the cell-free extract is in the holoenzyme form (results not shown).

Regulation of PQQ synthesis

Some bacteria which excrete large amounts of PQQ when grown on alcohols were used to study the influence of exogenously added PQQ on its

Table 3: Effects of exogenously added PQQ on PQQ biosynthesis in *Hyphomicrobium X*.

Hyphomicrobium X was precultured on 0.3% (v/v) methanol, and subcultured on the same medium to which various concentrations of PQQ were added.

PQQ (μM) added before growth	PQQ (μM) determined after growth	PQQ (μM) synthesized by the organism
0.2	3.8	3.6
1.5	5.9	4.4
3.9	8.0	4.1
8.1	12.1	4.0
9.3	12.8	3.5
12.0	15.4	3.4

biosynthesis. Thus, *Hyphomicrobium X* was precultured in the presence of methanol, inducing quinoprotein and PQQ synthesis, and subcultured (1 ml/ 50 ml) on the same medium, to which known amounts of PQQ were added. Before and

after growth, PQQ concentrations in the culture fluid were measured, and the amount of biosynthesized PQQ calculated. As shown in Table 3, synthesis of PQQ by *Hyphomicrobium* X was unimpaired by the additions, the amount of biosynthesized PQQ being about $4.0 \mu\text{mol.l}^{-1}$ in all cases. The same results were obtained with *P. putida* growing on ethanol and, in one experiment, with *A. calcoaceticus* grown fed-batch on quinate (results not shown). Only ethanol-grown *P. stutzeri* LMD 26.48 behaved differently, since a significant decrease of synthesis occurred in media supplemented with PQQ (results not shown).

Effect of amino acids in culture media

When PQQ producing organisms are grown in media containing amino acids, (e.g. peptone, yeast extract) variable losses of PQQ (excreted by the organism) occur, depending on the incubation time, composition and pH of the medium (results not shown).

5. DISCUSSION

The concentration of PQQ in culture media will be governed by (i) the rate of PQQ biosynthesis, (ii) excretion of PQQ from the cells into the culture medium, and (iii) losses of PQQ due to reaction with nucleophilic compounds, esp. amino acids.

From the preliminary experiments described here, it appears that PQQ synthesis is only induced if quinoprotein synthesis is initiated. However, this relationship does not hold for the reverse case, as quinoprotein synthesis can proceed in the absence of PQQ synthesis. Although the events which trigger the synthesis processes are presently unknown, the presence of a quinoprotein substrate is not essential for induction.

The rate of PQQ biosynthesis seems to be connected with the type of quinoprotein induced. Although no linear relationship exists, it is evident from Table 2 that quinoproteins having a low specific activity (but constituting 10-20% of the total cellular protein, so that they will be synthesized at a relatively high rate) give rise to high PQQ production rates, whereas the rate of PQQ synthesis with quinoproteins having a high

specific activity (e.g. GDH, usually constituting only 0.01-0.05% of the total cellular protein) is low. On the other hand, quinoprotein and PQQ synthesis are not well coordinated, since over-production as well as under-production of PQQ occurs. For *Hyphomicrobium* X it can be calculated from Table 2 (M_r of MDH = 120,000 (Duine *et al.*, 1978); one MDH contains two PQQ's (Duine *et al.*, 1981)) that a tenfold excess of PQQ is excreted into the culture medium. However, the presence of apoenzyme GDH in ethanol grown batch cultures of *A. calcoaceticus* and glucose grown batch cultures of *P. putida* shows that under-production of PQQ may occur as well. It has been shown that addition of PQQ to whole cells or cell-free extract is sometimes stimulatory, depending on the growth conditions applied, indicating that quinoproteins sometimes occur in the apoenzyme form (Hommes *et al.*, 1984, van Schie *et al.*, 1987, J.A. Duine, unpublished results). The data mentioned in Table 1 can therefore well be explained by assuming that quinoprotein and PQQ synthesis proceed at different rates and are differently influenced by cellular growth rate. In view of the ascribed role of some quinoproteins as an auxiliary energy providing system in some organisms, PQQ and quinoprotein synthesis rates can also be influenced by certain limiting growth conditions (Hommes *et al.*, 1985, van Schie *et al.*, 1987).

It should be realized that external PQQ production might not reflect overall PQQ production. Excretion of PQQ into the culture medium occurring only in the late exponential and stationary phase of growth has been observed with methylotrophic bacteria (McIntire *et al.*, 1987, and Prof. F. Gasser, personal communication), and *Nocardia* sp. 239 (Mr. P. van Ophem, personal communication). Measurements of cellular PQQ concentrations in the exponential phase of growth suggest, however, that PQQ is already present, but excreted only at the end of the growth, for reasons unknown (Prof. F. Gasser, personal communication). On the other hand, with *Acinetobacter* and *Pseudomonas* species PQQ is excreted gradually during incubation (results not shown). Finally, it should be mentioned in this context that the determination of PQQ in culture fluids is severely biased under conditions in which nucleophiles occur, the adducts formed with PQQ being undetectable with the common procedures of PQQ analysis (van Kleef *et al.*, 1987).

The fact that quinoprotein dehydrogenases occur in Gram-negative bacteria, and are mostly (if not always) located in the periplasm, could explain excretion of PQQ into the medium. If it is assumed that assemblage of the quinoproteins occurs in the periplasm, holo-enzyme formation might simply

proceed by recombination with PQQ transported from the cytoplasm to the periplasm from where it easily escapes to the medium [this process will be more complicated for methylamine dehydrogenase since PQQ is covalently bound to the protein chain in this case (van der Meer *et al.*, 1987)]. This is in accordance with the observation that the Gram-positive bacterium *Arthrobacter* P1 [lacking a periplasm, the quinoprotein methylamine oxidase being bound to the membrane at the cytoplasmic site (Levering *et al.*, 1981) and with PQQ covalently bound (van Iersel *et al.*, 1986)] did not excrete PQQ (Table 1).

In those cases in which quinoprotein apoenzymes are produced, it has been found indeed that exogenously added PQQ is generally able to effectively reconstitute quinoprotein apoenzymes *in vivo*: e.g. with GDH in *A. lwoffii* (van Schie *et al.*, 1984), *A. calcoaceticus* PQQ⁻-mutants (van Kleef *et al.*, 1987), and *E. coli* (Hommes *et al.*, 1984); with QDH in *A. lwoffii*, *A. calcoaceticus* and *P. aureofaciens* PQQ⁻-mutants (van Kleef and Duine, 1988); with alcohol dehydrogenase (ADH) in *P. testosteroni* (Groen *et al.*, 1986); with MDH in a PQQ⁻-mutant from *M. organophilum* XX (Biville *et al.*, 1988). The absence of feedback inhibition on PQQ synthesis with several organisms (*P. putida* biovar B, *Hyphomicrobium* X, and probably *A. calcoaceticus* too, however not *P. stutzeri*) as revealed by the experiments in which PQQ was added to the medium, suggests that there is no uptake mechanism for PQQ in these cases. Other cofactors behave differently. For instance, it has been shown (Dempsey, 1965) that after exogenous addition of either pyridoxal or pyridoxol (600 nM) to an *E. coli* B culture, *de novo* pyridoxine synthesis stops within 20 seconds. Also synthesis of the enzymes of NAD (Foster and Moat, 1980) and FAD (Brooke *et al.*, 1986) biosynthesis are subject to repression mechanisms triggered by exogenous cofactor. Further work, especially with bacteria containing cytoplasmic quinoproteins, is, however necessary to prove that PQQ has a real unique position among cofactors with respect to this aspect.

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Chapter V

Bacterial NAD(P)-independent quinate dehydrogenase is a quinoprotein

1. SUMMARY

Acinetobacter calcoaceticus LMD 79.41 produced significant amounts of pyrroloquinoline quinone (PQQ) in its culture medium when grown on quinic acid or shikimic acid. Studies with LMD 79.41 and PQQ⁻-mutants of this strain demonstrated that this organism contains an NAD(P)-independent quinate dehydrogenase (QDH) (EC 1.1.99.--), catalyzing the first degradation step of these compounds, and that the enzyme contains PQQ as a cofactor, i.e. is a quinoprotein. Synthesis of QDH was induced by protocatechuate and the enzyme appeared to be particle-bound. *Acinetobacter lwoffii* RAG-1 produced a quinoprotein QDH apoenzyme since growth on quinic acid only occurred in the presence of PQQ. The results obtained with the PQQ⁻-mutants of strain LMD 79.41 also provided some insight into the regulation of PQQ biosynthesis and assemblage of quinoprotein enzymes in the periplasmic space. Since two species of *Pseudomonas* also contained a quinoprotein QDH, it is assumed that bacterial NAD(P)-independent quinate dehydrogenase is a quinoprotein.

2. INTRODUCTION

Methylotrophic bacteria, grown on methanol, contain large amounts of quinoprotein methanol dehydrogenase [up to 20% of the protein in a cell-free extract (Anthony, 1982)] and produce PQQ in the micromolar range in their culture media (Ameyama *et al.*, 1984 and Duine *et al.*, 1985). In contrast, production of quinoprotein glucose dehydrogenase (GDH) (EC 1.1.99.17) by *Acinetobacter calcoaceticus* is low [only 0.01% of the total protein in a cell-free extract (Duine *et al.*, 1986)] and concentrations of PQQ in the spent culture medium are in the nanomolar range. Assuming that a relationship exists between quinoprotein enzyme content and PQQ production in the medium, the observation that significant amounts of PQQ were present in the medium of quinate-grown *A. calcoaceticus* cultures, seemed interesting to pursue. Quinic acid and shikimic acid are usually degraded by bacteria

via the hydroaromatic pathway to protocatechuic acid and further via the β -ketoadipate route to succinate and acetyl-CoA (Fig. 1). The regulation of these pathways has been studied in *Pseudomonas* species (Ornston, 1971), several fungi (Cain, 1969), *A. calcoaceticus* (Tresguerres *et al.*, 1970a, 1972; Ingledew *et al.*, 1971), and other organisms (Hardisson *et al.*, 1969; Johnson and Stanier, 1971).

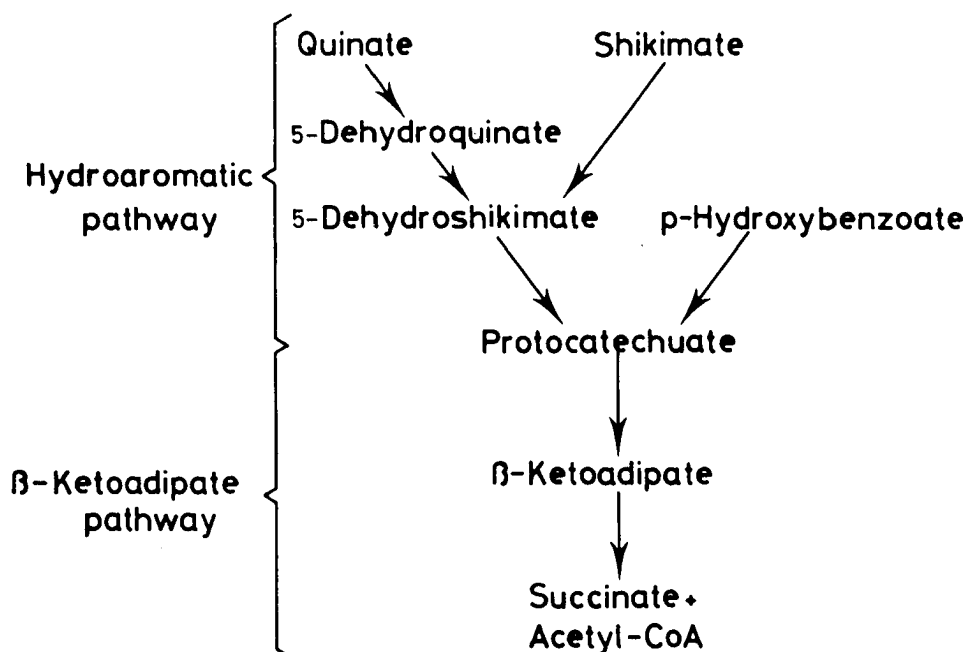


Fig. 1: Bacterial metabolism of quinic acid and shikimic acid via the hydroaromatic and β -ketoadipate pathways.

The first step in the degradation of quinic and shikimic acid is mediated by a so-called hydroaromatic dehydrogenase, being NAD-dependent (EC 1.1.1.24) in the case of *Neurospora crassa* (Ahmed and Giles, 1969), fungi (Ahmed and Giles, 1969; Cain *et al.*, 1968), *Aerobacter aerogenes* (Mitsuhashi and Davis, 1954) and *Pseudomonas aeruginosa* (Ingledew and Tai, 1972). On the other hand, a dye-linked hydroaromatic dehydrogenase has been found in *Gluconobacter oxydans* (Whiting and Coggins, 1967) and *A. calcoaceticus* (Tresguerres *et al.*, 1970a,b). Since further details about the enzyme in the

latter two organisms were lacking, especially the nature of its cofactor, it was anticipated, in view of induction of PQQ synthesis by quinate in *A. calcoaceticus*, that bacterial NAD(P)-independent quinate dehydrogenase (QDH) might be a quinoprotein. Therefore, studies were performed by comparing growth performances of PQQ⁻-mutants with wildtype strains.

3. MATERIALS AND METHODS

Cultivation

Acinetobacter calcoaceticus LMD 79.41 [originally obtained from Professor J. Hauge and identified in our laboratory with the API 20 NE system as a genuine *A. calcoaceticus* (Hauge, 1960)], PQQ⁻-mutants from this strain (LMD 82.43, M7, 1332, 1333, 1335, 1347, 1359, 1360, and 1361, (Goosen *et al.*, 1987), *A. lwoffii* LMD 73.1, and *A. lwoffii* RAG-1 [obtained from Professor D. Gutnick (Rosenberg *et al.*, 1980)] were cultivated on an inorganic medium (van Kleef *et al.*, 1987) supplemented with a carbon and energy source (0.2% w/v), as indicated.

Pseudomonas testosteroni ATCC 15667, *P. putida* biovar B LMD 84.56, *P. aureofaciens* ATCC 15926 and mutant ACN of this strain [obtained from Professor F. Lingens (Salcher and Lingens, 1980a)] were cultivated on an inorganic medium (Duine *et al.*, 1978) supplemented with a carbon and energy source (0.2% w/v) as indicated. All organisms were grown at 30°C on a rotary shaker (200 rev min⁻¹). Special precautions were taken to avoid PQQ contaminations (van Kleef *et al.*, 1987).

Enzyme assays

GDH activities were determined as described previously (Duine *et al.*, 1979), measuring the reduction rate of Wurster's Blue in 0.1M Tris/HCl buffer, pH 7.0.

QDH was assayed in a reaction mixture containing (total volume 2 ml): 0.1M Tris/HCl, pH 7.5, 80 µM Wurster's Blue, 10 mM sodium D-quinatate and cell-free extract. All measurements were performed at room temperature. One unit of enzyme activity was defined as the amount of enzyme required to convert one micromole of substrate per minute under the assay conditions.

PQQ assay

PQQ was assayed enzymically using PQQ-dependent alcohol dehydrogenase apoenzyme from *P. testosteroni* (Groen *et al.*, 1986).

Methods

Cell-free extracts were prepared as reported elsewhere (Duine *et al.*, 1979).

Electrophoresis of native proteins was performed on polyacrylamide gradient gels (PAA 4/30) at pH 8.8 by the method described by Pharmacia (PhastsystemTM). For enzyme activity staining, the gels were immersed in a solution containing 0.1M Tris/HCl buffer, pH 7.5, 10 mM sodium quinate, 600 μ M Wurster's Blue and 20 μ M PQQ. Enzyme activity was visible as a white band on a dark-blue background.

4. RESULTS

PQQ production

When *Acinetobacter calcoaceticus* LMD 79.41 is grown with acetate or succinate as a growth substrate, less than 5 nM PQQ is excreted into the culture medium. On the other hand, substrates that are degraded via protocatechuate gave rise to significant production of PQQ (50-100 nM), whereas on growth on substrates that are degraded by the β -ketoadipate pathway, but not via protocatechuate (i.e. benzoic acid, tryptophane), again less than 5 nM PQQ is excreted. It should be noted that protocatechuate is the inducer of all the enzymes of the β -ketoadipate and hydroaromatic pathways (Tresguerres *et al.*, 1970), the latter including quinate dehydrogenase as catalyst for the first step.

Growth on quinic acid and shikimic acid

PQQ⁻-mutants of *A. calcoaceticus* LMD 79.41, cultivated on quinic acid or shikimic acid under "normal" growth conditions, did not show large differences in growth rate as compared to the wildtype strain. Only when

special precautions were taken to avoid PQQ contamination (van Kleef *et al.*, 1987), differences became manifest (Table 1). Except for mutants 1333 and 1360, none of the cultures of the PQQ⁻-mutants showed growth after 16 h. On the other hand, growth occurred after 24-40 h with mutants 1332, 1335, 1359 and 1361. Mutants LMD 82.43, M7 and 1347 showed no growth at all, not even after prolonged incubation for 7 days. On addition of PQQ (1 μ M) to the growth medium, all the mutants recovered to wild type growth rates.

Table 1: Growth of *Acinetobacter calcoaceticus* LMD 79.41 and PQQ⁻-mutants on quinic acid and shikimic acid (a).

Strain	Growth substrate	Growth(b)		Growth substrate	Growth(b)	
		16h	24h		16h	40h
79.41W	Quinic acid	+		Shikimic acid	+	
82.43		-	-		-	-
M7		-	-		-	-
1332		-	+		-	+
1333		+			w	+
1335		-	+		-	w
1347		-	-		-	-
1359		-	+		-	-
1360		+			+	
1361		-	+		-	+

(a) *Acinetobacter* strains were grown in a PQQ-free mineral medium as indicated under Materials and Methods.

(b) - = no growth (OD600 <0.1); w = weak growth (0.1 <OD600 <1.0); + = good growth (OD600 >1.0).

QDH and GDH activities in cell-free extracts

A. calcoaceticus wildtype and PQQ⁻-mutants were grown in a PQQ-free medium on three different types of growth substrate: (1) succinate, unable to induce QDH, (2) p-hydroxybenzoate, a gratuitous inducer of QDH since it is

degraded via protocatechuate, and (3) quinate and shikimate, inducers of QDH which is needed for growth.

Table 2: GDH and QDH activities in cell-free extracts of *A. calcoaceticus* strains(a).

Strain	Growth substrate	QDH activity			GDH activity		
		(U/g wet cells)			(U/g wet cells)		
		-PQQ	+PQQ	%(b)	-PQQ	+PQQ	%(b)
79.41W	Succinate	0	0	-	n.p.	n.p.	-
79.41W	Quinate	5.1	5.8	88	31.6	34.6	91
82.43	Succinate	n.p.	n.p.	-	0.3	37.6	0.8
1332	Succinate	n.p.	n.p.	-	0.1	12.3	1.0
1332	Shikimate	1.8	4.6	39	1.5	18.3	7.9
1332	p-OH-benzoate	0.1	6.5	1.4	0.1	18.6	0.3
1359	Quinate	3.7	7.9	47	1.3	16.9	7.9
1360	Quinate	1.9	4.6	43	2.3	22.3	10.3
1360	p-OH-benzoate	0.1	6.7	1.9	0.1	17.8	0.8

n.p. = not performed

(a) *Acinetobacter* strains were grown in a PQQ-free mineral medium.

Cell-free extracts were prepared and QDH and GDH activities were measured in the presence or absence of 20 μ M PQQ.

(b) Percentage holoenzyme in the absence of PQQ.

From Table 2 it appears that:

a. Cell-free extracts of wildtype *A. calcoaceticus* contain a dye-linked QDH after growth on quinic acid but not an NAD(P)-dependent activity.

b. PQQ⁻-mutants grown on p-hydroxybenzoic acid show negligible QDH activity in their cell-free extracts. On addition of PQQ (20 μ M) to the cell-free extract, the QDH activity was restored completely as the level became comparable to that of the wildtype. GDH activities in the presence or absence of PQQ were comparable with former results for this organism, using succinate as a growth substrate (van Kleef *et al.*, 1987) (see also Table 3).

c. Growth of PQQ⁻-mutants on quinic acid or shikimic acid resulted in significant activities of holoenzyme QDH and GDH in the cell-free extracts,

but only on addition of PQQ (20 μ M), the levels of QDH and GDH became comparable to that of the wildtype.

Properties of QDH from *Acinetobacter* species

After centrifugation of a cell-free extract for 20 min at 48,000 x g most QDH activity is present in the supernatant (approx. 75%). However, the QDH activity was found in the stacking gel after electrophoresis and attempts to bind the enzyme to CM- or DEAE-Sephadex at different pH values were not successful. These results apply to both the holoenzyme and apoenzyme form and suggest that QDH is still bound to small membrane particles. No attempts were made to solubilize and/or purify the enzyme.

With Wurster's Blue as electron acceptor, a pH of 7.5 was found to be optimal for enzyme activity. DCPIP was also a good electron acceptor with an optimum at pH 6.0. QDH apoenzyme activity was inhibited (97%) by EDTA (5 mM), but only when EDTA was added before recombination of apoenzyme with PQQ. The activity of QDH holoenzyme (or apoenzyme after recombination with PQQ) was not affected at all by EDTA. This behaviour is reminiscent to that of quinoxaemoprotein alcohol dehydrogenase apoenzyme from *P. testosteroni* (Groen *et al.*, 1986), and GDH apoenzyme from *A. calcoaceticus* (P. Dokter, unpublished results). Since both examples require divalent metal ions for reconstitution, QDH probably also has some metal ion requirement.

Two strains of *A. lwoffii* were screened for growth on quinic acid. One of them (RAG-1) could grow on this substrate, but only in the presence of PQQ (Table 3). A cell free extract of this strain grown on nutrient broth + quinic acid in the absence of PQQ contained a low but detectable level of quinate dehydrogenase apoenzyme activity. Strain LMD 73.1 did not grow on quinic acid in the presence of PQQ, and QDH was not induced when it was grown on nutrient broth + quinic acid.

QDH in *Pseudomonas* species

P. testosteroni ATCC 15667, known to contain quinoxaemoprotein alcohol dehydrogenase apoenzyme when grown on alcohols (Groen *et al.*, 1986), was tested for growth on quinic acid in the presence or absence of PQQ. No growth was observed in either case (Table 3).

Table 3: Growth on quinic acid and QDH activities in cell-free extracts of several bacteria(a).

Organism	Carbon source	Growth		QDH activity (U/g wet cells)	
		-PQQ	+PQQ	-PQQ	+PQQ
<i>A. lwoffii</i> LMD 73.1	Quinate	-	-	n.p.	n.p.
	NB + quinate (a)	+	+	0	0
<i>A. lwoffii</i> RAG-1	Quinate	-	+	n.p.	n.p.
	NB + quinate	+	+	0	0.27
<i>P. testosteroni</i>	Quinate	-	-	n.p.	n.p.
<i>P. putida</i> biovar B	Quinate	+	n.p.	1.4	1.7
<i>P. aureofaciens</i>	Quinate	+	n.p.	3.7	3.3
<i>P. aureofaciens</i> ACN	Quinate	+	+	0.05	4.3

n.p. = not performed

(a) 0.8% Nutrient broth + 0.08% quinic acid

P. putida biovar B, LMD 84.56 was able to grow on quinic acid and its cell-free extract contained a dye-linked QDH (Table 3). No NAD(P)-linked activity could be demonstrated.

P. aureofaciens ATCC 15926 and its mutant strain ACN (this is a PQQ⁻ mutant, unpublished results) were tested. The wildtype strain was able to grow on quinic acid in the absence of PQQ and contained both an NAD-dependent (0.91 U/g wet cells) and a dye-linked QDH (Table 3). Presumably due to the NAD-linked activity, mutant ACN was able to grow on quinic acid and contained besides the NAD-dependent QDH (1.28 U/g wet cells) also quinoprotein QDH apoenzyme (Table 3).

5. DISCUSSION

Acinetobacter calcoaceticus LMD 79.41 was able to use quinic acid and shikimic acid as a carbon and energy source. The first step in the degradation of these compounds is probably mediated by a dye-linked hydroaromatic dehydrogenase, since growth on these substrates, but also on

protocatechuate, gave cell-free extracts with QDH activity, whereas NAD(P)-dependent QDH activity was absent. These observations are in agreement with those of Canovas *et al.*, obtained with *A. calcoaceticus* ATCC 23393 (Tresguerres *et al.*, 1970 a,b; Ingledew *et al.*, 1971). The results of the studies with the PQQ⁻-mutants clearly showed that the dye-linked dehydrogenase is a quinoprotein.

The behaviour of PQQ⁻-mutants grown on hydroaromatics deserves some further attention. With quinic acid or shikimic acid as the carbon source, some PQQ⁻-mutants showed (retarded) growth on these substrates, indicating that they are leaky with respect to PQQ biosynthesis. In fact, most PQQ⁻-mutants tested (more than 20, results not all shown) were more or less leaky, with exception of LMD 82.43(M2) and M7 (and M3,M8 and M11, results not shown), which are supposed to be deletion mutants, and 1347 which is an insertion mutant obtained by special techniques (Goosen *et al.*, 1987). Cell-free extracts of the leaky mutants contained a significant amount of QDH and GDH holoenzyme activity. The results obtained with p-hydroxybenzoate illustrate that the observed holoenzyme activity can not be attributed to contamination with PQQ. With this growth substrate, a gratuitous inducer of QDH, the activity of QDH in cell-free extracts of the PQQ⁻-mutants was negligible in the absence of PQQ. However, QDH apoenzyme was induced since addition of PQQ restored the activity to the wild type level. Similar results were obtained with GDH. From this, it appears that the precautions taken to avoid PQQ contamination were effective.

Growth of this organism on L-arabinose takes place via quinoprotein GDH (Goosen *et al.*, 1987). None of the leaky PQQ⁻-mutants, however, could grow on L-arabinose, not even after prolonged incubation for 7 days, whereas the wildtype strain grew readily on this substrate (results not shown). Quinate on the other hand was (partly) able to induce holoenzyme QDH in the PQQ⁻-mutants. Regulation of the biosynthesis of PQQ, therefore, seems to be different with respect to these enzymes.

GDH is a periplasmic enzyme (Dokter *et al.*, 1985). Assuming that QDH is also a periplasmic enzyme, the presence of GDH holoenzyme, besides QDH holoenzyme, in cell-free extracts of quinate grown PQQ⁻-mutants suggests that the assemblage of PQQ with quinoprotein apoenzyme in the periplasmic space is a non-selective process.

The finding of quinoprotein QDH in *A. calcoaceticus* prompted us to test other micro-organisms for the presence of this enzyme. *A. lwoffii* synthesizes

quinoprotein GDH apoenzyme but not the cofactor (van Schie *et al.*, 1984). Therefore, the presence of QDH apoenzyme was tested in two strains. One of them, RAG-1, indeed contained this enzyme, the other one, LMD 73.1, did not.

Pseudomonas testosteroni ATCC 15667 contains quinoprotein alcohol dehydrogenase apoenzyme (Groen *et al.*, 1986). However, growth on quinic acid in the presence of PQQ could not be demonstrated. It should be noted that p-hydroxybenzoic acid is a growth substrate for all *P. testosteroni* strains, indicating that the β -ketoadipate route is present. Obviously, a hydroaromatic pathway is absent in this organism.

P. aureofaciens ATCC 15926 appeared to contain both NAD-dependent as well as quinoprotein QDH activity. Strain ACN, a PQQ⁻-mutant of this organism (unpublished results), synthesized quinoprotein QDH apoenzyme. Strain ACN is an overproducer of the antifungal antibiotic pyrrolnitrin (Salcher and Lingens, 1980 a,b). It is unable to synthesize 2-hydroxyphenazine-1-carboxylic acid, the yellow orange pigment of *P. aureofaciens* and accumulates anthranilic acid during growth on L-tryptophan. Since several of our PQQ⁻-deletion mutants (M2,M7,M8,M11) accumulate anthranilic acid as well (unpublished results), the gene for anthranilate hydroxylase is probably located close to the PQQ genes.

P. putida biovar B LMD 84.56 possesses a dye-linked QDH activity. Although no experiments with PQQ⁻-mutants of this organism were possible, from the similarity in properties of the dye-linked QDH activity in its cell-free extract, it is concluded that the enzyme is a quinoprotein.

Gluconobacter oxydans strains produce PQQ (Duine *et al.*, 1979) and contain quinoprotein GDH when grown on glucose or gluconic acid (Ameyama *et al.*, 1981). They are not able to grow on quinic acid or shikimic acid, but some of them are able to convert these compounds to their 5-dehydro forms (Whiting and Coggins, 1967). The oxidation step is catalyzed by a constitutive, particle-bound dehydrogenase (Whiting and Coggins, 1967). Since the enzyme is neither NAD(P)- nor FAD-linked (Whiting and Coggins, 1967), it is probably a quinoprotein too. Therefore, the incomplete oxidation of quinate by this organism may be comparable to the incomplete oxidation of glucose to gluconic acid by *A. calcoaceticus*. The latter organism is able to derive useful energy from this oxidation step (Müller and Babel, 1986, van Schie *et al.*, 1987), by transferring electrons from GDH to the electron transport chain via a special cytochrome b (Dokter *et al.*, in preparation). In this context, it may be interesting to note that growth

of *G. oxydans* in the presence of quinate also induced a special cytochrome b (Whiting and Coggins, 1967).

Quinic acid and shikimic acid occur in many higher plants (Böhm, 1965), often in appreciable concentrations. Various studies have concluded that both shikimic acid and quinic acid are actively metabolized during growth of the plant, but, since attempts to detect a NAD(P)-dependent QDH (EC 1.1.1.24) have failed so far, their way of metabolism is unknown (Haslam, 1974). The possibility exists that a dye linked enzyme analogous to that in bacteria is involved (Haslam, 1974). The occurrence of a quinoprotein QDH (apo)enzyme in higher plants thus remains to be verified.

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Chapter VI

A search for intermediates in the biosynthesis of PQQ

1. SUMMARY

Studies on the biosynthesis of pyrroloquinoline quinone (PQQ) were performed with *Acinetobacter calcoaceticus* PQQ⁻-mutants belonging to genetically different complementation groups. All mutants were unable to grow on L-arabinose, the conversion of this substrate by the organism only occurring via quinoprotein (PQQ-containing) glucose dehydrogenase (EC 1.1.99.17). In general, the same observation and conclusion applied to shikimate and quinate, requiring active quinoprotein quinate dehydrogenase (EC 1.1.99.--), although some mutants appeared to be leaky with respect to PQQ biosynthesis under this condition. A number of mutants appeared to be unable to grow on anthranilate and accumulated this compound when the growth medium was supplemented with L-kynurenine. Combined with other observations, it strongly suggests that these are deletion mutants, missing a gene for synthesis of anthranilate hydroxylase (EC 1.14.12.1) as well as nearby located genes for the biosynthesis of PQQ. Supplementation of the growth media with amino acids did not result in stimulation of PQQ biosynthesis. Also cross-feeding experiments, using normal and permeabilized cells with extensive variation in combinations and conditions, did neither result in stimulation nor reconstitution of PQQ synthesis. Under conditions optimal for PQQ production in the wild type strain, as well as under stress conditions using a limiting amount of added cofactor, excretion of intermediates by PQQ⁻-mutants could not be detected. Similar results were obtained with PQQ⁻-mutants from *Methylobacterium organophilum* and *Pseudomonas aureofaciens*. A tentative explanation, accounting for the absence of detectable intermediates in the route of biosynthesis, is given.

2. INTRODUCTION

Pyrroloquinoline quinone (PQQ) appears to be the cofactor in several enzymes, from prokaryotic as well as eukaryotic origin (Duine *et al.*, 1986).

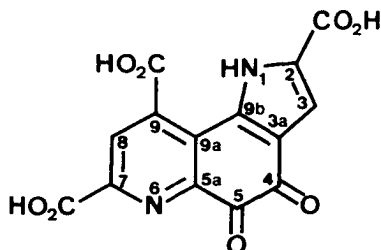


Fig. 1 : Structure of pyrroloquinoline quinone (PQQ).

Since its structure (Fig. 1) is unfamiliar with that of well known cofactors, analogous reasoning helpful in the prediction of a role as vitamin in mammalian organisms is not possible. Although a surprising number of routes for chemical synthesis has been published (Corey *et al.*, 1981; Gainor and Weinreb, 1981; MacKenzie *et al.*, 1983, 1986; Hendrickson and de Vries, 1985; Buchi *et al.*, 1985), so far nothing is known about its biosynthesis. At first sight, phenylalanine, tyrosine, alanine or glutamic acid come to question as precursors for biosynthesis (these compounds have already attracted the attention to develop a biomimetric route for chemical synthesis (Buchi *et al.*, 1985)). In principle two possibilities exist to investigate biosynthesis, namely by analyzing the effect of labeled precursors on the product or by carrying out a search for intermediates. This paper deals with the latter approach. The establishment of precursors of PQQ biosynthesis will be reported elsewhere.

Genetic studies on the biosynthesis of PQQ have already been reported (Goosen *et al.*, 1987). Screening mutants from *Acinetobacter calcoaceticus* LMD 79.41, genetic complementation experiments revealed four different classes of PQQ⁻mutants. Since the genes came to expression (Dr. N.Goosen, personal communication) in *Escherichia coli* and *Acinetobacter lwoffii* (non-producers, see Hommes *et al.*, 1984; van Schie *et al.*, 1984), this could be interpreted that bacterial PQQ biosynthesis starts with a frequently occurring metabolite and is completed in four steps. So far two quinoproteins (PQQ-containing enzymes) have been detected in this organism: glucose dehydrogenase [GDH, two quite different types are known and both appear to exist in this bacterial strain (Cleton-Jansen *et al.*, 1988)] and quinate dehydrogenase (QDH, van Kleef and Duine, 1988). To stimulate synthesis of intermediates and as a further check on the inability of the

mutants to synthesize PQQ, growth experiments were performed on substrates which are, or are expected to be, dissimilated via these enzymes. To develop an assay for intermediates, cross-feeding experiments were performed with members of all classes of mutants under a variety of conditions. To exclude negative results due to transport problems, similar experiments were carried out with permeabilized cells.

Production of PQQ by *A. calcoaceticus* is rather poor compared with that of methylotrophs or alcohol-grown pseudomonads (Anthony, 1982; Duine *et al.* 1985b). In principle, using a combination of chromatography and absorption spectroscopy for analysis of the growth media of these bacteria, it should be possible to detect intermediates in the latter cases. Therefore, to increase the chance for detection, it seemed worthwhile to investigate also representatives of these groups, together with their PQQ⁻-mutants. Although mutants unable to dissimilate C₁-compounds have been obtained from several methylotrophic bacteria (Nunn and Lidstrom, 1986; Machlin *et al.*, 1988; Harms *et al.*, 1985), the only PQQ⁻-mutant found so far is *Methylobacterium organophilum* MTM1 (Biville *et al.*, 1988). As will be shown in this paper, several PQQ⁻-mutants from *A. calcoaceticus* accumulate anthranilate when grown in the presence of L-kynurenine. Since *Pseudomonas aureofaciens* mutant ACN (unable to synthesize pyrrolnitrin) might be a PQQ⁻-mutant, as it accumulates anthranilate (Salcher and Lingens, 1980), this strain was investigated too.

3. MATERIALS AND METHODS

Bacterial strains

Bacterial strains utilized in this study are listed in Table 1.

Culture conditions

A. calcoaceticus strains were grown on a mineral medium as described (van Kleef *et al.*, 1987), supplemented with a carbon source (0.2%) as indicated. PP 1347 was grown in the presence of kanamycin (20 µg/ml) (Goosen *et al.*, 1987). *Ps. aureofaciens* strains were cultivated on an inorganic medium

Table 1: Bacterial strains

Strains	Phenotype	Mutant class	Source or reference

<i>Acinetobacter</i>			
<i>calcoaceticus</i>			
LMD 79.41	Wild type	-	Hauge, (1960)
PP 1332,1361	PQQ ⁻	I	Goosen <i>et al.</i> , (1987)
PP 1333,1351,1360	PQQ ⁻	II	"
PP 1353,1359	PQQ ⁻	III	"
PP 1347	PQQ ⁻	IV	"
M1,M2,M3,M6,M7,M8,M11	PQQ ⁻	deletion	"
			this study
<i>Pseudomonas</i>			
<i>aureofaciens</i>			
ATCC 15926	Wild type	-	Salcher and Lingens (1980)
ACN	PQQ ⁻	not known	van Kleef and Duine (1988)
			Salcher and Lingens (1980)
<i>Methylobacterium</i>			
<i>organophilum</i>			
XX	Wild type	-	Patt <i>et al.</i> (1976)
MTM 1	PQQ ⁻	not known	Biville <i>et al.</i> (1988)

(Duine *et al.*, 1978) with a carbon and energy source (0.2%) as indicated. *M. organophilum* strains were cultivated on an inorganic medium (Biville *et al.*, 1988) with 0.3% methanol as carbon and energy source. All organisms were grown at 30°C with shaking (200 rpm).

In fed-batch experiments, *A. calcoaceticus* strains were precultured on a 10 mM potassium phosphate mineral medium (20 ml) with a carbon source (0.2%) as indicated. When good growth was obtained ($OD_{610} > 1.0$), the culture was diluted ten times with the same mineral medium and feeding started with the carbon source (0.8%) in mineral medium at a flow rate of 10 ml/hr.

In all experiments, special precautions were taken to avoid PQQ contaminations (van Kleef *et al.*, 1987).

Mutagenic treatment

A. calcoaceticus LMD 79.41 was grown on mineral medium supplemented with succinate (0.2%) to a density of $2-3 \times 10^8$ cells per ml. The cells were washed, and the pellet was suspended in mineral medium (10 ml). Ethyl methanesulphonate was added to a concentration of 50-250 $\mu\text{g/ml}$. After incubation at 30°C for 60 min, the cells were washed, diluted, and plated on Nutrient agar (Difco, 0.8%, with phenol red (0.008%) as pH indicator) containing 1.0% glucose. Colonies with cells containing active GDH turn yellow (gluconic acid formation) during overnight incubation, those with non-active GDH remain red. PQQ⁻-mutants were selected from the red colonies by checking these with the same procedure in the presence of PQQ (2 μM). The seven mutants obtained in this way are designated as "M"-mutants in Table 1.

Cross-feeding between *Acinetobacter* PQQ⁻-mutants

In the experiments described below, at least two PQQ⁻-mutants from each different class were tested, with the exception of class IV, since only one mutant was available. In every experiment, all possible combinations of mutants were tested.

a. On agar plates.

A. calcoaceticus wild type and mutant strains were precultured on succinate. Combinations of two PQQ⁻-mutants or wild type plus one PQQ⁻-mutant were streaked on Nutrient Agar (Difco, 0.8%) with Phenol red (0.008%) or McConkey agar, both containing 1.0% glucose.

b. In shaken cultures.

Cross-feeding was tested in two ways. PQQ⁻-mutants were precultured on p-hydroxybenzoic acid. In one experiment, quinate medium was inoculated (1 ml/50 ml) with two different PQQ⁻-mutants. In another experiment, quinate medium (20 ml) was inoculated with one PQQ⁻-mutant (1 ml) to which 5 ml of the spent culture medium produced by another PQQ⁻-mutant, sterilized by filtration, was added. Growth rates were determined by measuring OD₆₁₀ during incubation.

c. After toluene treatment.

A. calcoaceticus PQQ⁻-mutants were precultured (20 ml) on succinate and subcultured (50 ml) to the end of the exponential growth phase on the same carbon source. Cells were harvested by centrifugation (15 min at 23,000 x g), washed with mineral medium and resuspended in mineral medium (50 ml). Toluene was added at a concentration of 5 µl/ml and the suspension was agitated vigorously (by vortex mixing) for 1 min. Suspensions from two PQQ⁻-mutants (2 x 5 ml) were incubated on a rotary shaker at 30°C after addition of glucose to a concentration of 10 mM. To establish background as well as maximal attainable GDH holoenzyme activity, suspensions of every single PQQ⁻-mutant (10 ml) were incubated in the same medium with and without PQQ (20 µM). Glucose consumption was determined during incubation.

d. After Triton X-100 treatment.

A. calcoaceticus strains were precultured on succinate (20 ml) and subcultured on heptadecane (0.5%, v/v) plus Triton X-100 (0.04%, v/v) for 4 days (Dokter et al., 1985). The presence of quinoprotein GDH holo- or apoenzyme in the culture fluid was checked and the cells harvested by centrifugation (15 min at 23,000 x g). Supernatants were sterilized by filtration and added (5 ml) to 15 ml of fresh heptadecane/Triton X-100 medium, inoculated with a PQQ⁻-mutant (precultured on succinate). Incubation was continued for 3 days. GDH holo- and apoenzyme activities were measured in the culture fluids, and cultures were divided into two equal portions with or without 20 µM PQQ, to which glucose at a concentration of 10 mM was added, and these incubated at 30°C on a rotary shaker. Glucose consumption was determined during incubation.

Screening for intermediates in culture media

A. calcoaceticus PQQ⁻-mutant strains were precultured on quinate plus 2 nM PQQ (a limiting concentration), and subcultured on quinate medium without PQQ. *Methylobacterium organophilum* MTM 1 was cultured on 0.3% methanol supplemented with various concentrations of PQQ.

Cells were harvested by centrifugation (15 min at 23,000 x g) and the supernatant was directly applied to an Amberlyst A-21 anion exchanger (50-100 μ m, p.A. from SERVA, Heidelberg, FRG) in the chloride form (0.7 x 2 cm) and successively eluted (in several fractions) with 50% MeOH, 90% MeOH, 50% MeOH + 0.3M NaCl, 50% MeOH + 1.25 M NaCl and 50% MeOH + 3 M NaCl. Fractions were analyzed for the presence of compounds having fluorescence properties or absorption spectra not present in the culture fluid of the wild type strain. Screening for fluorescence occurred by direct irradiation with a 350 nm UV lamp. HPLC analysis of the fractions was performed on a Waters RCM 100 module containing a 10 μ m C₁₈ RCM reversed phase cartridge. The eluent (flowrate 1.5 ml/min) consisted of a linear gradient (20 min) of 10-90% MeOH with 0.4% (v/v) H₃PO₄. The eluate was monitored with a Hewlett-Packard 1040A photodiode-array detector, taking absorption spectra of the eluted compounds.

Assays

GDH activities were determined as reported elsewhere (Duine *et al.*, 1979). The glucose consumption rate of whole cells was determined by a glucose assay (Boehringer GOD-PAP method) at marked time intervals. PQQ was determined by measuring the reconstitution of quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni* (Groen *et al.*, 1986).

4. RESULTS

Behaviour of PQQ⁻-mutants on different carbon sources

Acinetobacter calcoaceticus LMD 79.41 wild type and PQQ⁻-mutants were grown in batch cultures supplemented with a carbon source. With the large majority of carbon sources, no difference in growth rate was observed between wild type and mutant strains. Among the carbon sources tested (0.2%) were succinate, acetate, ethanol, DL-malic acid, L-leucine, L-glutamic acid, benzoic acid (0.1%), p-hydroxy benzoic acid, kynurenic acid, protocatechuic acid, L-tyrosine, n-heptadecane, putrescine, L-tryptophan, catechol. Carbon sources on which growth rates differed are shown in Table 2.

Table 2: Growth of *Acinetobacter calcoaceticus* LMD 79.41 wild type and PQQ⁻-mutants on different carbon sources (0.2%).

Carbon source	Growth ¹						
	W	M3,M6	M1,M2,M7	Class	Class	Class	Class
			M8,M11	I	II	III	IV
L-arabinose	+	-	-	-	-	-	-
Quinate/Shikimate	++	-	-	+/-	+/-	+/-	-
L-kynurenine	+	+	w	+	+	+	+
Anthranilate (0.1%)	+	+	-	+	+	+	+

¹ - = no growth; w = weak growth; + = good growth; ++ = excellent growth.

The failure of PQQ⁻-mutants to grow on L-arabinose and quinate/shikimate can be attributed to the absence of active quinoprotein GDH and QDH, respectively. The observation that some mutants, after a delay, are able to grow on quinate/shikimate can be explained only by assuming that these mutants are leaky with respect to PQQ biosynthesis, since appreciable amounts of QDH and GDH holoenzyme activities were found in cell-free extracts under these conditions, and all deletion mutants were unable to grow on these compounds (van Kleef and Duine, 1988).

Some of the deletion mutants (M1, M2, M7, M8, and M11) were not able to grow on anthranilate and showed poor growth on L-kynurenine. Under the latter condition, anthranilate was accumulated. *A. calcoaceticus* dissimilates L-tryptophan via N-formylkynurenine, L-kynurenine, anthranilate and catechol, and subsequently via the β -ketoacid route (Wheelis, 1972). Apparently, these mutants are not able to convert anthranilate to catechol, a reaction catalyzed by anthranilate hydroxylase. Since (i) addition of exogenous PQQ did not restore growth on anthranilate, (ii) no stimulation in PQQ biosynthesis (as measured by reconstituted GDH activity) was found upon addition of catechol, and (iii) none of the mutants affected in only one gene of PQQ biosynthesis accumulated anthranilate (results not shown), it is assumed that the mutants which are not able to grow on anthranilic acid are deleted not only in one or more genes of PQQ biosynthesis, but also in the

gene coding for anthranilate hydroxylase. Probably these genes are located close together on the chromosome.

The same conclusions apply for *P. aureofaciens* ACN. This strain is an overproducer of the antifungal antibiotic pyrrolnitrin (Salcher and Lingens, 1980). It is unable to synthesize 2-hydroxyphenazine-1-carboxylic acid, the yellow orange pigment of *P. aureofaciens* and accumulates anthranilic acid when grown on L-tryptophan. Since this strain appeared to synthesize quinoprotein apo-GDH, which could be reconstituted on addition of PQQ (results not shown), strain ACN is a PQQ⁻-mutant. In view of its properties, it is probably also a deletion mutant with the genes for PQQ biosynthesis and anthranilate hydroxylase close together.

The observation that all *A. calcoaceticus* mutants are able to grow on L-tryptophan (a carbon source which is assumed to be dissimilated only via the aromatic pathway (Wheelis, 1972)), without accumulation of anthranilic acid, implies that this view is incorrect, and L-tryptophan is also dissimilated via other routes, for instance via the quinolinic pathway as used by *Pseudomonas acidovorans* (Behrman, 1962). On the other hand, *P. aureofaciens* appears to have only one route for the dissimilation of L-tryptophan, since it accumulates anthranilic acid on growth on this substrate. The weak growth on this substrate can be ascribed to the degradation of the alanine moiety, formed in the route from L-tryptophan to anthranilic acid (Salcher and Lingens, 1980).

Cross-feeding between *Acinetobacter* PQQ⁻-mutants

a. On agar plates

Since *A. calcoaceticus* LMD 79.41 wild type possesses quinoprotein GDH, but no other enzymes for glucose dissimilation (Juni, 1972), PQQ production can be measured as gluconic acid production from glucose on acid-base indicator plates. The wild type was able to feed all PQQ⁻-mutants, showing that reconstitution is possible under this condition. However, no PQQ⁻-mutant was found to feed other ones from different classes.

b. In cultures

A. calcoaceticus strains were precultured on p-hydroxybenzoic acid to induce quinoprotein QDH and PQQ synthesis (van Kleef and Duine, 1988). Since some PQQ⁻-mutants show delayed growth on quinate owing to leakyness in PQQ biosynthesis, growth rates on quinate of single PQQ⁻-mutants were compared either with growth rates of two mutants or one mutant with sterile spent culture medium from another mutant. In neither case the growth rate on quinate was enhanced.

c. After breakdown of membrane permeability

The failure of PQQ⁻-mutants to show cross-feeding under normal conditions (a,b) can be explained by assuming that precursors are not excreted and/or taken up by the organism. To overcome this problem, cells were treated in order to remove the cell's permeability barrier and make it accessible for low molecular weight compounds.

c1. After toluene treatment

Following toluene treatment, less than 0.3% of the cells were viable. Since all PQQ⁻-mutants were able to convert 10 mM glucose in less than one hour in the presence of PQQ, it was evident that the organism still had an effectively operating electron transport chain. No glucose consumption could be detected in the samples without PQQ after 3 and 8 hours incubation, indicating that cross-feeding did not occur under this condition.

c2. After triton X-100 treatment

A. calcoaceticus strains able to grow on higher alkanes show a marked stimulation in growth rate on addition of low concentrations (0.005%) of Triton X-100 (Dokter et al., 1985). At higher Triton X-100 concentration (0.04%) periplasmic and cytoplasmic enzymes appear in the culture medium. Probably the detergent causes a partbreakdown of the permeability barrier of the membranes (Dokter et al., 1985). This unique feature of *A. calcoaceticus* strains was used in another attempt to demonstrate cross-feeding between PQQ⁻-mutants. However, after growth of a PQQ⁻-mutant on

heptadecane/Triton X-100 in the presence of sterilized spent culture medium from another mutant grown under this condition, no GDH holoenzyme activity could be demonstrated in the culture fluids, nor could glucose be converted by these cultures when PQQ was absent. In the presence of PQQ, glucose was readily oxidized.

Production of PQQ with *Acinetobacter calcoaceticus* LMD 79.41

It has been shown that the production of nicotinic acid in *Escherichia coli* is enhanced on addition of L-aspartate (Ortega and Brown, 1960). It was therefore tried to establish if there were substances that stimulate the production of PQQ in growing cultures of *A. calcoaceticus* LMD 79.41, when added to the growth medium. Since the major part of PQQ produced by a micro-organism is excreted in its growth medium (Ameyama *et al.*, 1985; Duine *et al.*, 1985b), PQQ production was established by measuring its concentration in the culture fluid. To obtain measurable basal levels of PQQ in the supernatant, *A. calcoaceticus* was grown on quinate. All amino acids were tested (0.01%) as co-substrates, to check their suitability as precursor. No stimulating effects were seen.

Growth of PQQ⁻-mutants under PQQ-limitation

When a mutant is grown under conditions where only a limiting amount of the required growth factor is available, enzymes involved in the synthesis of that growth factor are often derepressed, resulting in an increased production of intermediates (Chandler and Gholson, 1972). To enhance the level of intermediates in *A. calcoaceticus* PQQ⁻-mutants, strains were precultured on quinate plus PQQ, and subcultured on quinate without added cofactor. The growth behaviour is shown in Figure 2. A linear growth rate was observed, depending on the inoculum size (thus the concentration of PQQ in the inoculation medium), indicating that the limiting factor was PQQ. When good growth was obtained ($OD_{610} > 1.0$), cells were harvested by centrifugation (15 min at 23,000 x g) and the culture fluid was concentrated and chromatographed on an Amberlyst A21 ion-exchanger. Eluent fractions of different classes of PQQ⁻-mutants were compared with each other and with those of the wild type strain grown on quinate. No differences, indicating

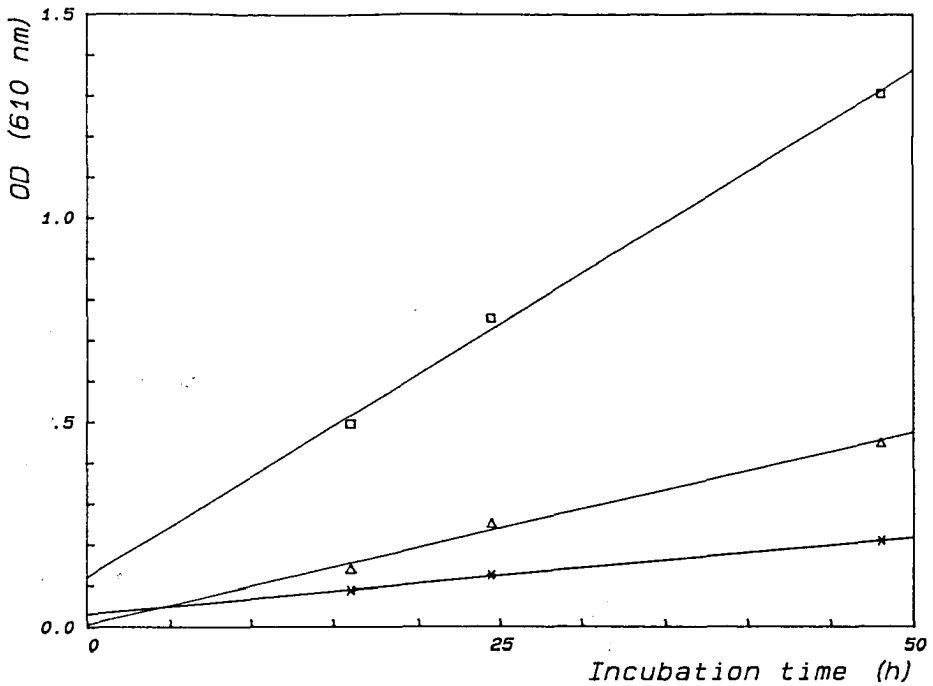


Fig. 2 : Growth rate of *Acinetobacter calcoaceticus* PQQ⁻-mutants under PQQ-limiting conditions.

PP 1353 was precultured on quinate (0.2%) plus 5 nM PQQ, and subcultured on quinate without PQQ (50 ml). Inoculum volumes were 0.1 ml (*), 0.2 ml (Δ), and 0.8 ml (□).

the production of any intermediate were found.

In another experiment, MTM1, a PQQ⁻-mutant from *Methylobacterium organophilum* XX (Biville *et al.*, 1988) was grown on methanol under PQQ-limiting conditions. Culture fluids were checked with HPLC analysis. Again, no intermediate could be detected.

Fed-batch cultivation of *A. calcoaceticus* PQQ⁻-mutants on quinate

Since the failure to detect any intermediates of PQQ in culture fluids of *A. calcoaceticus* might be ascribed to the low concentration of PQQ found in

this strain when grown on mineral media (50-100 nM), a fed-batch method of culturing was applied since it was found that PQQ production was relatively high under this condition (a five- to tenfold increase). After incubation for 20-48 h, culture fluids were cleaned from debris, concentrated and chromatographed on Amberlyst A21 ion-exchanger, and analyzed with HPLC. The presence of intermediates was not detected.

5. DISCUSSION

The results obtained with *Acinetobacter calcoaceticus* LMD 79.41 wild type and PQQ⁻-mutants can be summarized as follows:

1. No differences in growth rate between wild type and mutant strains is observed on carbon sources which are not degraded via quinoproteins (GDH or QDH).
2. PQQ production in *A. calcoaceticus* is not enhanced on addition of amino acids.
3. Cross-feeding between *A. calcoaceticus* PQQ⁻-mutants could not be demonstrated, neither on agar plates nor in shaken cultures. Treatments causing permeability breakdown had no effect. Only the wild type is able to feed the PQQ⁻-mutants, obviously as it excretes PQQ into its culture fluid which allows reconstitution of GDH apoenzyme, located in the periplasm of the PQQ⁻-mutants.
4. No intermediates could be detected when PQQ⁻-mutants were grown under stress conditions (PQQ-limitation), or under fed-batch conditions, optimal for PQQ production in the wild type strain.

To explain these observations, several hypotheses can be postulated:

- a. Intermediates are excreted, but their concentration is too low to be detected.

As nothing is known yet about the regulation of PQQ biosynthesis, it is difficult to present arguments against this hypothesis. It is however somewhat difficult to envisage that precursors are found neither with *M. organophilum* nor with *A. calcoaceticus* PQQ⁻-mutants under conditions by which the wild type strains excrete substantial amounts of PQQ (μM level), since this would imply that excretion of intermediates by the PQQ⁻-mutants is much lower than PQQ excretion of the wild type strains under this condition.

b. Intermediates are excreted, but cannot be taken up by the cells.

NadC mutants of *Escherichia coli* excrete significant amounts of quinolinic acid, a precursor of NAD, into their culture media (Chandler and Gholson, 1972). Quinolinic acid, however, cannot be taken up by other nad-mutants, probably because it is unable to pass the bacterial membrane (Kerr and Tritz, 1973). Since PQQ itself is a highly polar compound under physiological conditions so that problems may exist for transport to the cytoplasm (assuming that no uptake systems are present), it is highly probable that the same applies to the intermediates. The experiments causing permeability breakdown contradict this hypothesis, however. Triton X-100 treatment affects the membrane structure, allowing the passage of whole enzymes. It has to be admitted, however, that it is not known whether strongly polar compounds can pass the cytoplasmic membrane under this condition. Toluene treatment normally makes the membrane completely permeable to low molecular weight compounds. On the other hand, as PQQ biosynthesis in the wild type strain after the toluene treatment was difficult to check (GDH holoenzyme is already present before the treatment), this negative result could also be due to blocking of the PQQ biosynthesis under this condition. Despite this, hypothesis b seems very unlikely.

c. Intermediates are excreted, but unstable or reactive.

This hypothesis is very unlikely since neither intermediates, nor degradation products from intermediates could be detected for any PQQ⁻ mutant.

d. Intermediates are not excreted, but remain in the cells.

Since (polar) compounds not of any use to the cell, are normally excreted (Weiss and Edwards, 1980), this behaviour would be highly unusual. In addition, this should indicate that permeabilization of cells is not effective for excretion of intermediates.

e. There are no (low molecular weight) intermediates.

For the time being, this is the most attractive explanation, since several observations fit in this hypothesis. Genetic studies on the biosynthesis of PQQ (Goosen *et al.*, 1987, and Dr. N. Goosen, personal communication) have revealed that only four genes are involved. Gene I and II probably code for one enzyme consisting of two subunits with M_r 29,700 and M_r 10,800. Gene III codes for an enzyme containing a subunit of M_r 37,400 or 43,600. Gene IV appears to be very small, equivalent to a protein of 23 amino acids. The function of this small DNA fragment is not clear at the moment, that is

whether it functions on RNA or protein level. An interesting hypothesis would be that this peptide is the template for PQQ biosynthesis, and the whole process proceeds on a complex preventing escape of the peptide (in fact, L-tyrosine and most probably L-glutamic acid have very recently been established as the precursors of PQQ biosynthesis, to be published elsewhere). Synthesis could occur by modification of the amino acids, followed by cyclisation steps, and finally excision of PQQ at the appropriate site. Since free PQQ is very reactive towards nucleophiles, such a mechanism could avoid degradation of PQQ. Further genetic studies, and identification of the gene products will be necessary to prove this hypothesis.

Since many bacteria are unable to provide their quinoprotein apoenzymes with PQQ, (Hommes *et al.*, 1984; van Schie *et al.*, 1984; Shimao *et al.*, 1984; Groen *et al.*, 1986), and PQQ⁻-mutants isolated from different bacteria show normal growth characteristics on substrates not degraded via quinoproteins, the conclusion seems to be justified at first sight that free PQQ has no vital function in the metabolism of the bacteria studied. However, since it cannot be excluded that the biosynthesis of PQQ in an enzyme where it is covalently bound occurs in a different way, this conclusion may be premature.

ACKNOWLEDGMENT

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Chapter VII

L-tyrosine is the precursor of PQQ biosynthesis in *Hyphomicrobium* X

1. SUMMARY

A method was developed to study amino acids as possible precursors of PQQ biosynthesis. Cultures of *Hyphomicrobium* X, growing on ^{13}C -methanol, were supplemented with unlabelled amino acids. Uptake and participation in metabolism was determined with gas chromatography/mass spectrometry of derivatized amino acids, obtained from hydrolyzed cellular protein, by measuring their ^{12}C content. Several amino acids appeared to be incorporated into the protein to a significant extent, without degradation or conversion. Among these were the aromatic amino acids, L-tyrosine and L-phenylalanine. Using the same replacement approach, incorporation of them into PQQ was determined with ^1H - and ^{13}C -NMR spectroscopy of purified PQQ obtained from the culture medium. It appeared that the complete carbon skeleton of tyrosine was present, forming the o-quinone and pyrrole-2-carboxylic acid moieties in PQQ, while phenylalanine was not incorporated at all. Starting with L-tyrosine, possible biosynthetic routes to PQQ are discussed.

2. INTRODUCTION

PQQ is a novel cofactor initially discovered in several bacterial dehydrogenases [1]. Recent studies indicate that its presence has been overlooked in several well-known metallo-enzymes in higher organisms: bovine serum amine oxidase (EC 1.4.3.6)[2], porcine kidney diamine oxidase (EC 1.4.3.6)[3], human placental lysyl oxidase (EC 1.4.3.13)[4], dopamine- β -hydroxylase (EC 1.14.17.1) [5], and soybean lipoxygenase-1 (EC 1.13.11.12, unpublished results). Since several of these enzymes are involved in the conversion or synthesis of mammalian bioregulators, insight in how they are provided with this cofactor is of utmost importance to shed light on a possible role of PQQ or related substances as vitamin. In addition, this information could provide clues for the design of drugs with PQQ or its route of biosynthesis as a target. Although the (bacterial) genes for biosynthesis of the cofactor have been cloned and brought to expression in

bacteria unable to provide their apo-enzymes with PQQ ([6], and Dr. N. Goosen, private communications), nothing is known so far with respect to intermediates and precursors. The latter aspect is the topic of this paper while the search for intermediates will be described elsewhere.

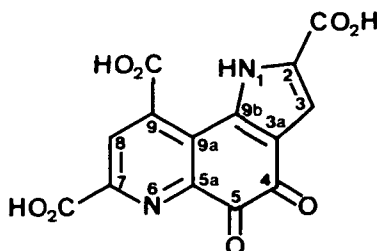


Fig. 1: Structure of pyrroloquinoline quinone (PQQ).

PQQ has an unfamiliar structure when compared with known cofactors or vitamins. Although there is a slight resemblance with compounds like kynurenic acid, xanthurenic acid, and pseudanes, it is not easy to see how these compounds can be processed by common biochemical reactions to PQQ (e.g. the replacement of hydroxyl by carboxylic acid groups). On the other hand, from plain paper chemistry several couples of amino acids can be indicated which might be precursors in the biosynthesis. Such a couple has been mentioned already before, namely tyrosine or phenylalanine and glutamic acid, which were used as hypothetical forerunners for a biomimetic synthesis route [7]. Therefore, studies aimed to discover amino acid incorporation into PQQ seemed a logical approach.

In view of the many connections between biochemical pathways, studies on amino acid incorporation in general are hampered by the fact that normal organisms tend to scramble these compounds on administration. For that reason, specialists like the obligate methylotrophic bacteria seem the organisms of choice since by definition, amino acids cannot be carbon and energy sources as several of the common pathways are absent or incomplete. *Hyphomicrobium* X seemed an attractive candidate since it excretes substantial amounts of PQQ (in the range of 2-5 μM) into the culture medium during growth on methanol [8] (in fact this was the source of material used in our structure elucidation of the cofactor [9]). Thus, it was reasoned that labelling experiments, followed by ^1H - and/or ^{13}C -NMR analysis of PQQ

would be feasible and might reveal replacement of amino acids (synthesized via normal biosynthesis) by supplemented amino acids. Unfortunately, however, data on the uptake of amino acids or on degradation due to their possible use as nitrogen source, are not available. Therefore, it was first necessary to obtain information on these aspects. Since ^{13}C -labelled amino acids are expensive, the organism was grown on the rather cheap ^{13}C -methanol and replacement attempted to achieve by addition of unlabelled amino acids. After it appeared that several of them are taken up and incorporated in unchanged form into the cellular protein, studies could be started on a role in the biosynthesis of PQQ. During this work we learned that independent studies of Dr. Unkefer and co-workers, using *Methylobacterium* AML, have led to similar conclusions as presented here.

3. MATERIALS AND METHODS

Cultivation and labelling

Hyphomicrobium X was cultivated on a mineral medium containing per liter: 1.79 g K_2HPO_4 , 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.0 g KNO_3 , and 0.2 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$. After autoclaving, 1 ml of spore, calcium, and iron solutions according to Duine *et al.* [10] were added to 1 l of mineral medium (it should be noted here that the concentration of the calcium solution mentioned in that paper is incorrect and should be 25 mg $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}/\text{l}$). ^{13}C -methanol (>99%, Sigma) was added as a carbon and energy source, as indicated. Cultures were pregrown on ^{13}C -methanol (20 ml) to the stationary phase and subsequently transferred to the same medium to which unlabelled amino acids were added. To establish incorporation of amino acids into protein of *Hyphomicrobium* X, two synthetic mixtures of amino acids were used, the composition based on the amino acid profile of *H. methylovorum* [11]. Mixture I was used to obtain general information on amino acid uptake and participation in metabolism. It consisted of (g/l): L-lysine (0.80), L-histidine (0.41), L-arginine (0.78), L-aspartate (1.05), L-threonine (0.51), L-serine (0.39), L-glutamate (1.26), L-proline (0.50), glycine (0.63), L-alanine (0.89), L-valine (0.71), L-isoleucine (0.52), L-leucine (0.86), L-cysteine (0.13), L-methionine (0.21), L-tyrosine (0.27), L-phenylalanine (0.51) and L-tryptophan (0.30). L-asparagine and L-glutamine were not included, since *Hyphomicrobium* strains

are able to use these compounds as nitrogen sources [24], and their presence cannot be detected in a protein hydrolysate, due to their conversion to L-aspartic acid and L-glutamic acid, respectively. Mixture II consisted of the most probable candidates for PQQ biosynthesis. It contained (g/l): L-glutamate (1.26), L-tyrosine (0.27), L-phenylalanine (0.51), and L-tryptophan (0.30). To establish incorporation of L-phenylalanine and L-tyrosine into PQQ and protein, these amino acids were tested separately at a final concentration of 165 and 181 mg/l, respectively. Cultures were incubated for 40 hrs at 30°C on a rotary shaker (200 rev min⁻¹).

Purification and analysis of PQQ

Cultures of *Hyphomicrobium* X were centrifuged (15 min at 23,000 x g). PQQ was purified from the supernatant essentially according to [12], but with some modifications. To the supernatant 0.5-1.0 g of Amberlyst A-21 ion exchanger (50-100 µM, p.A. from SERVA, Heidelberg, FRG) in the chloride form was added. After incubation with stirring (2 h), the anion exchanger was packed to a column, which was successively washed with methanol/water (1 : 1, v/v), methanol/ water (9 : 1, v/v), and 0.3 M NaCl in methanol/water (1 : 1, v/v). Elution of PQQ was performed with 1.25 M NaCl in methanol/water (1 : 1, v/v). Fractions containing PQQ were diluted ten times in 0.01 M HCl and applied to a Seppak C₁₈-silica cartridge (Waters Ass.). After washing with 0.01 M HCl, PQQ was eluted with methanol/water (7 : 3, v/v), and the eluate evaporated to dryness.

PQQ concentrations were determined by a biological assay using quinoprotein alcohol dehydrogenase apoenzyme from *P. testosteroni* [13].

¹H- and ¹³C-NMR spectra of PQQ were recorded on a Varian VXR-400 S spectrometer operating at 400 MHz and 100.5 MHz respectively, using pulse Fourier-transform mode. The internal reference was the solvent (C²H₃)₂SO for ¹H-NMR. For ¹³C-spectra, the solvent signal of (C²H₃)₂SO was suppressed and tetramethylsilane used as an internal reference.

Incorporation of amino acids into cellular protein

Amino acids from cellular protein were obtained and derivatized essentially according to [14]. 50 mg wet cells were washed with 10 ml 0.1 M HCl, suspended in an excess of 6 M HCl in an ampoule, sealed under vacuum,

and hydrolyzed at 110°C for 24 h. The hydrolysate was centrifuged to remove insoluble material, evaporated to dryness, redissolved in 1-2 ml water and brought to pH 2.0-2.5 with 1 M NaOH. The sample was applied to a small column (5 x 30 mm) of Dowex 50W-X8 (H⁺) 100-200 mesh. After washing with 5 column volumes of water, the amino acids were eluted with 3 M ammonia. After evaporation to dryness, the residue was dissolved in 3 ml of n-butanol, acidified with gaseous HCl (to 3 M), in an ampoule (sealed under vacuum), and heated for 3 h at 110°C. After evaporation of the solvent, the resulting butyl esters were converted to their N,O-trifluoroacetates by treating them with 50% trifluoroacetic anhydride in methylene chloride for 16 h at room temperature. The solvent was evaporated, and the residue taken up in methylene chloride for gas chromatography-mass spectroscopy analysis. An VG 70-SE gas chromatograph-mass spectrometer equipped with a 25 m x 0.25 mm CP-Sil-5 CB column was used. The amino acids were separated by temperature programming from 40 to 200°C at 6°C/min and from 200 to 300°C at 10°C/min. All spectra were recorded at 70 eV with a source temperature of 250°C. The level of incorporation of unlabelled amino acids was calculated from the peak heights in the mass spectra obtained by scanning of the appropriate peaks as they eluted from the gas chromatograph.

4. RESULTS AND DISCUSSION

Incorporation of amino acids into cellular protein

Mass spectra of amino acids, obtained from cells grown in the presence of mixture I, only showed unlabelled and/or U-¹³C labelled fragments. In the experiment with mixture II, mass spectra of amino acids not present in this mixture yielded only U-¹³C labelled fragments, indicating that no replacement due to conversion of supplemented amino acids occurs (Table 1, Mixture II), whereas in mass spectra of the amino acids constituting mixture II again unlabelled and U-¹³C labelled fragments were seen (results not shown). These results demonstrate that amino acids are not degraded to any extent in *Hyphomicrobium* X. From Table 1, it appears that only leucine, isoleucine, valine, methionine and phenylalanine are incorporated into the protein to a measurable extent. L-lysine and L-alanine were incorporated only to a very low extent, while the remaining amino acids seemed not to be

incorporated at all. Tyrosine showed low incorporation with mixture I, but sufficient incorporation with mixture II. Tryptophan, cysteine, asparagine and glutamine were destroyed during the acid hydrolysis, so that their effect could not be measured.

Table 1: The incorporation of administrated amino acids into cellular protein of *Hyphomicrobium X*.

Amino acid	Percentage incorporation (%)		
	Mixture I	Mixture I	Mixture II
	(1 ml/ 20 ml culture medium)	(5 ml/ 20 ml culture medium)	(5 ml/ 20 ml culture medium)
Glycine	< 10	< 10	n.p.
L-alanine	15	16	2
L-valine	37	59	< 1
L-leucine	40	72	< 1
L-isoleucine	45	83	< 1
L-serine	< 7	< 7	n.p.
L-threonine	< 10	< 10	n.p.
L-methionine	23	36	n.p.
L-proline	2	10	2
L-aspartate	< 3	< 3	< 3
L-glutamate	< 2	< 2	< 2
L-phenylalanine	49	97	>99
L-tyrosine	6	< 1	35
L-lysine	9	24	< 1
L-histidine	< 1	< 1	n.p.
L-arginine	< 1	< 1	n.p.
n.p. = not performed			

Since incorporation of alanine and glutamate was too low, only the aromatic amino acids L-tyrosine and L-phenylalanine were used in further studies.

Incorporation of L-phenylalanine and L-tyrosine into PQQ and cellular protein.

Although *Hyphomicrobium* X excretes a sufficient amount of PQQ when grown on methanol, this cofactor is degraded in the presence of amino acids ([15], unpublished results). Since it was found that ammonium salts were strong activators for the reaction of PQQ with most of the amino acids, KNO_3 was used instead of NH_4Cl . With nitrate as a nitrogen source, PQQ concentrations in the culture medium appeared to be unaffected by the presence of amino acids (results not shown).

Table 2: The incorporation of L-phenylalanine and L-tyrosine into PQQ and protein.

Incorporation into PQQ was calculated from the appropriate peak areas of the ^1H -NMR spectra.

Amino acid	Incorporation (%)	
	into PQQ	into protein
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--	< 5	< 1
L-phenylalanine	< 5	98
L-tyrosine	> 95	94
-----	-----	-----

The incorporation of L-phenylalanine and L-tyrosine into protein of *Hyphomicrobium* X is shown in Table 2. On administration of L-phenylalanine, this amino acid was incorporated for 98%, and no other unlabelled amino acids were found (results not shown), indicating that *Hyphomicrobium* X is not able to synthesize L-tyrosine directly from L-phenylalanine by the action of L-phenylalanine 4-monooxygenase (EC 1.14.16.1). Most probably, these amino acids are both synthesized from a common precursor, namely prephenic acid [16]. On administration of L-tyrosine, this amino acid was incorporated for 94%, whereas no other amino acids were found unlabelled in the hydrolyzed protein (results not shown).

In order to obtain ^1H - and ^{13}C -NMR spectra of uniformly labelled U- ^{13}C -PQQ, this compound was purified from a culture of *Hyphomicrobium* X, grown solely in the presence of ^{13}C -methanol (0.25%) as carbon and energy source.

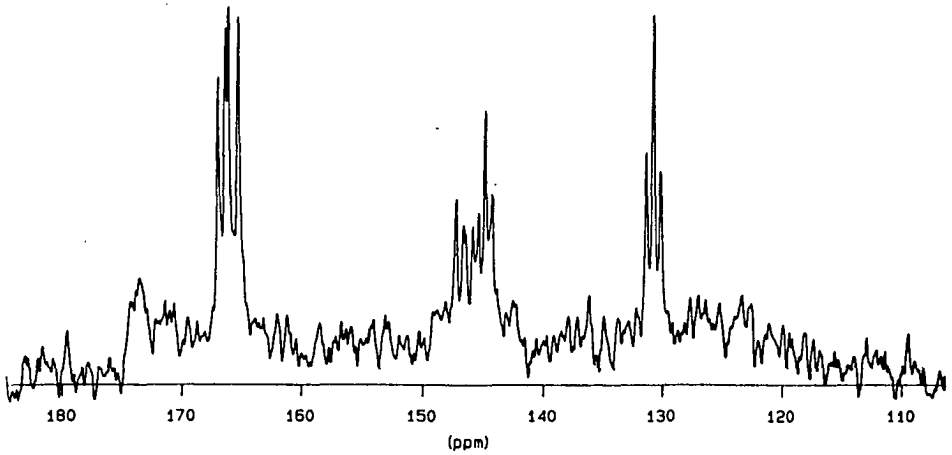
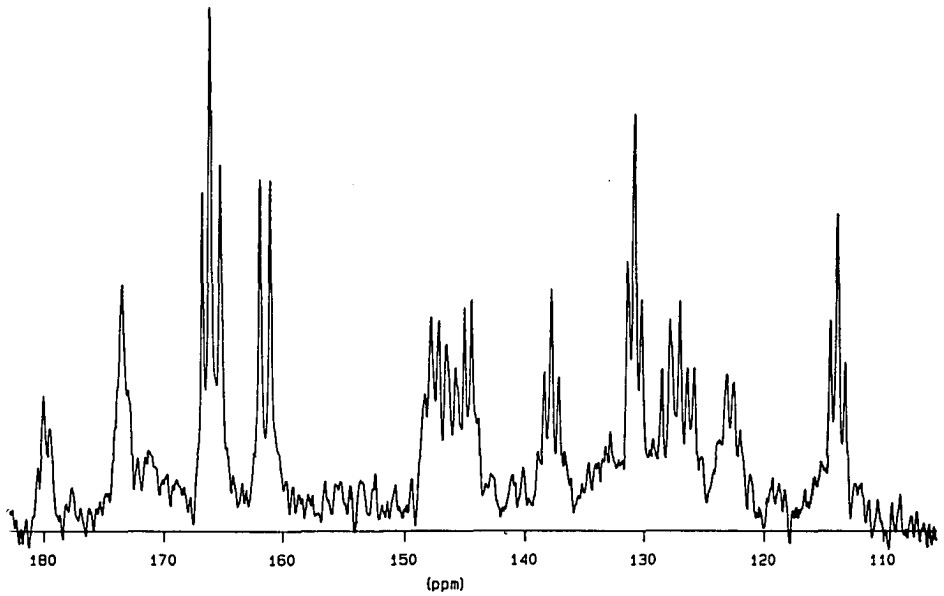


Fig. 2: ^{13}C -NMR spectra of $\text{U-}^{13}\text{C-PQQ}$ (top) and PQQ purified from a culture of *Hyphomicrobium X* grown on ^{13}C -methanol in the presence of L-tyrosine (bottom).

Whereas the ^1H -NMR spectrum of PQQ only shows signals at 7.24 ppm (doublet 2 Hz, 3-H), 8.63 ppm (8-H) and 13.3 ppm (broad, 1-H), in the spectrum of U- ^{13}C -PQQ two doublets at 7.14 ppm (180 Hz, 3-H) and 8.64 ppm (168 Hz, 8-H) are found, due to the ^{13}C ,H splitting of the aromatic protons.

The ^{13}C -NMR spectrum of U- ^{13}C -PQQ is shown in Fig. 2 (top) [17]. Signals were found at δ 113.9 (C_3), 123.0 (C_{3a}), 126.1 (C_{9a}), 127.8 (C_2), 130.7 (C_8), 137.8 (C_{9b}), 144.7 (C_9), 146.6 (C_7), 148.1 (C_{5a}), 161.6 ($\text{C}_2\text{-COOH}$), 165.9 ($\text{C}_7\text{-COOH}$), 166.7 ($\text{C}_9\text{-COOH}$), 173.5 (C_4) and 180.0 (C_5).

When PQQ was purified from the culture medium of *Hyphomicrobium* X, grown in the presence of ^{13}C -methanol (0.2%, v/v) plus L-phenylalanine, no significant changes in ^1H - and ^{13}C -NMR spectra were found as compared to the spectra of U- ^{13}C -PQQ, indicating that L-phenylalanine is not incorporated into the PQQ skeleton (Table 2). However, when L-tyrosine was present in the culture medium, the ^1H -NMR spectrum of purified PQQ showed a singlet at 7.15 ppm, whereas the doublet at 8.64 ppm (168 Hz, 8-H) had remained unaltered, indicating that 3- ^{13}C had been totally replaced by 3- ^{12}C (Table 2). Inspection of the ^{13}C -NMR spectrum (Fig. 2, bottom) shows that only signals at δ 130.7 (C_8), 144.7 (C_9), 146.5 (C_7), 165.8 ($\text{C}_7\text{-COOH}$), and 166.8 ($\text{C}_9\text{-COOH}$) are present, indicating that all carbon atoms of L-tyrosine are incorporated into PQQ. The C_9 -signal now appears as a triplet, due to the absence of coupling with C_{9a} .

Possible routes of biosynthesis

From assessment of L-tyrosine as precursor of PQQ, glutamate is the very likely precursor of the remaining part (Fig. 1). Unfortunately, since the incorporation of this amino acid is too low, this attractive hypothesis could not be tested.

The results described in this paper, and those obtained by Unkefer and co-workers (private communications), provide convincing evidence for L-tyrosine as precursor of PQQ. One can therefore speculate on the biosynthetic route of this cofactor starting with L-tyrosine. Of the various known enzymes able to convert L-tyrosine, only tyrosine 3-monooxygenase (EC 1.14.16.2) and tyrosinase (monophenol monooxygenase, EC 1.14.18.2) are relevant, since these enzymes do not affect the carbon skeleton of L-tyrosine. As the presence of tyrosine 3-monooxygenase has not been demonstrated in prokaryotes so far, this does not seem to be a very likely candidate.

Tyrosinase catalyzes the conversion of L-tyrosine to dopaquinone, via 3,4-dihydroxy phenylalanine (DOPA). Since the genes for PQQ biosynthesis in *Acinetobacter calcoaceticus* have been sequenced (Dr. N. Goosen, unpublished results), and the complete amino acid sequence of tyrosinase from *Streptomyces glaucescens* [18] and *Neurospora crassa* [19] has been reported, these data were compared. However, no sequence homology was found between the tyrosinases and any of the PQQ genes (Dr. N. Goosen, personal communication). Although the possibility exists that tyrosinase has other functions in *Acinetobacter*, precluding the isolation of mutants, lacking this enzyme, the involvement of tyrosinase in PQQ biosynthesis is questionable, since tyrosinase activity has not been demonstrated in *Acinetobacter*, and reports on tyrosinases in bacteria have been sparse [20].

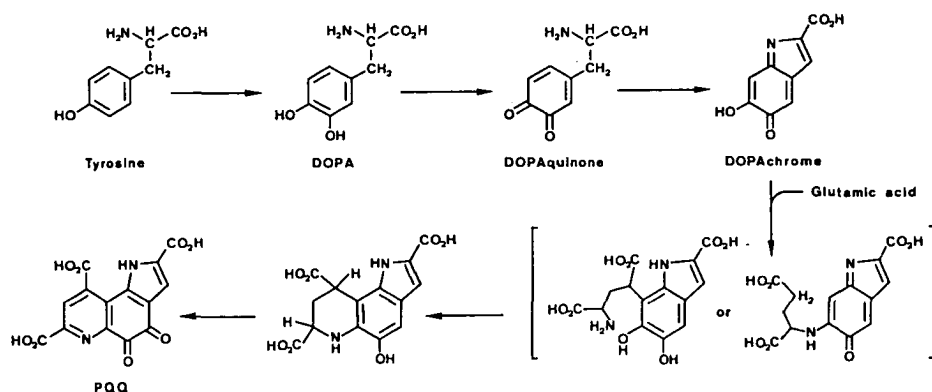


Fig. 3: Proposed route for PQQ biosynthesis.

However, in the absence of a better alternative, a tentative pathway, which includes tyrosinase in the first step, is depicted in Fig. 3. L-tyrosine is converted to dopaquinone, via DOPA, followed by ring closure to dopachrome. From here, two routes are possible to link L-glutamate to dopachrome. Finally, PQQ is formed following a desaturation and a hydroxylation step. It should be mentioned here that so far no intermediates in the biosynthesis of PQQ have been found, neither using PQQ⁻ mutants from different complementation groups, nor from different organisms (M.A.G. van Kleef and J.A. Duine, to be published elsewhere). Therefore, the whole process might proceed on a matrix, preventing the dissociation of reactive intermediates and enabling the correct positioning and ring closures.

Due to the absence of a pyruvate dehydrogenase complex, as well as phosphoenolpyruvate synthase and pyruvate decarboxylase, *Hyphomicrobium* strains grow poorly, if at all, in rich media such as nutrient broth [21,22]. When the organism was grown on C₁- or C₂- carbon and energy sources in the presence of pyruvate and succinate, these latter compounds were incorporated into new cell material [23]. Addition of these compounds, as well as that of several amino acids (a.o. L-alanine, L-glutamate, and L-aspartate), however, did not result in an increase of cell dry-weight, neither in batch nor in carbon-limited continuous cultures [24]. In the case of amino acids, this behaviour can be explained by our finding that most of the amino acids that were tested are not incorporated to any significant extent into the cellular protein of *Hyphomicrobium* X. As far as known to us, this paper is the first report demonstrating that certain amino acids are not incorporated, while other amino acids become incorporated into the cellular protein of a methylophilic bacterium without degradation.

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Samenvatting

Sommige enzymen hebben voor hun werking metaalionen of kleine organische molekulen nodig. Deze stoffen, die coenzymen of cofactoren worden genoemd, al naar gelang de mate van stevigheid waarmee ze aan het enzym gebonden zijn, en of ze in katalytische hoeveelheden nodig zijn, zijn meestal gerelateerd aan een van de vitaminen. Een stof is een vitamine voor een organisme, als deze het nodig heeft voor zijn groei, maar niet zelf kan maken, en daarom uit het voedsel moet opnemen. Vele jaren geleden zijn de meeste vitaminen/cofactoren geïsoleerd en is de structuur ervan opgehelderd. Hoewel men dacht nu alle vitaminen wel te kennen werd tien jaar geleden toch nog een nieuwe cofactor ontdekt - pyrroloquinoline quinone (PQQ) - die blijkt voor te komen in allerlei organismen, van microbe tot mens. Het eerste enzym waarin PQQ werd aangetoond was methanoldehydrogenase, wat een rol speelt in de afbraak van methanol. Nu is methanol een goedkope grondstof, en daarom commercieel interessant. Echter, de micro-organismen die in de industrie worden gebruikt kunnen in het algemeen niet op methanol groeien. Door nu de genen van de methanolafbraak naar deze organismen over te brengen (uit organismen die wel op methanol kunnen groeien) zou dit probleem kunnen worden opgelost. Aangezien hiervoor, naast de genen die voor de enzymen van de methanolafbraak coderen, ook de genen voor de biosynthese van PQQ moeten worden overgedragen, was dat reden om een onderzoek in deze richting te starten. Daar er recentelijk PQQ in amine oxidases en dopamine- β -hydroxylase is aangetoond, enzymen die de concentraties van belangrijke bioregulatoren in hogere organismen regelen, is onderzoek naar de biosynthese zelfs uitermate belangrijk geworden. Nemen deze organismen PQQ als vitamine uit hun voedsel op, of synthetiseren zij de cofactor zelf? Wellicht kunnen ook bepaalde ziektebeelden met PQQ, of intermediären (tussenprodukten) uit de biosyntheseroute, worden bestreden.

Het onderzoek naar de biosynthese van PQQ is, in samenwerking met de RU Leiden, ongeveer vijf jaar geleden van start gegaan. In Leiden is naar de genetica van dit proces gekeken, waar men inmiddels heeft ontdekt dat er (vier) genen betrokken zijn bij de biosynthese van PQQ. Bovendien is men erin geslaagd deze genen over te dragen, en ook te laten functioneren in andere organismen die zelf geen PQQ kunnen maken, en is de basenvolgorde van het DNA vastgesteld, waaruit de aminozuurvolgorde van de enzymen voor de biosynthese kan worden vertaald.

In Delft is getracht de precursors (uitgangsstoffen) en intermediären in de biosyntheseroute te ontdekken. Dit is enerzijds geprobeerd door de mutanten te gebruiken die geen PQQ meer kunnen synthetiseren. Deze mutanten zijn vergeleken met het oorspronkelijke wilde type, door ze bijvoorbeeld op verschillende koolstofbronnen te groeien. Verder is gekeken of deze mutanten in staat zijn elkaar te voeden, doordat de ene mutant de door de andere uitgescheiden intermediären opneemt (en daarmee zijn PQQ synthese kan herstellen), en tevens of de uitgescheiden intermediären konden worden geïsoleerd en de structuur ervan bepaald. Anderszijds is getracht met behulp van labelings-experimenten met stabiele isotopen vast te stellen of bepaalde precursors in het PQQ molecuul worden ingebouwd.

Acinetobacter calcoaceticus is een bacterie die wel glucose kan omzetten in gluconzuur, namelijk met behulp van het PQQ-afhankelijke enzym glucose dehydrogenase (GDH), maar niet in staat is om op een van deze twee verbindingen te groeien. Door nu een bacteriekweek met een mutagene stof te behandelen, en te kijken of er bacteriën zijn ontstaan die in aanwezigheid van glucose geen zuur meer kunnen produceren, konden gemakkelijk zogenaamde PQQ⁻-mutanten worden verkregen, aangezien deze alleen na toevoegen van extern PQQ gluconzuur produceren. Dit komt omdat deze mutanten nog wel het eiwitgedeelte van het glucose dehydrogenase kunnen maken, wat met extern PQQ opnieuw actief holoenzym (enzym met cofactor) vormt. Het bleek al spoedig dat alle geïsoleerde mutanten een wisselende GDH activiteit vertoonden, die 1-25% van de activiteit van het wilde type kon bedragen. Dit leek erop te wijzen dat de mutanten toch nog in staat waren een kleine hoeveelheid PQQ te synthetiseren. Het verschijnsel werd nader bestudeerd, en er werd ontdekt dat zeer kleine hoeveelheden PQQ, die als verontreiniging aan het glaswerk of in kweekmedia kunnen voorkomen, verantwoordelijk waren voor de gemeten activiteit. Door het nemen van speciale voorzorgsmaatregelen kon deze verontreiniging met PQQ worden voorkomen, en o.a. worden vastgesteld dat de bacterie *Escherichia coli* geen PQQ synthetiseert, dit in tegenspraak met de bevindingen van een Japanse groep. Met de PQQ⁻-mutanten van *A. calcoaceticus* is bovendien een zeer gevoelige test opgezet, waarmee concentraties van 30 nanogram PQQ per liter nog gemeten konden worden (Hoofdstuk II).

De groei van de geïsoleerde mutanten (er waren mutanten in elk van de vier genen beschikbaar) op een groot aantal koolstofbronnen werd vergeleken met die van het wilde type. In tegenstelling tot het wilde type bleek geen van de mutanten meer te groeien op L-arabinose, een suiker die wordt afgebroken

via het al eerder genoemde glucose dehydrogenase. De ontdekking dat het wilde type bij groei op quinaat en shikimaat ongebruikelijk hoge hoeveelheden PQQ in het kweekmedium uitscheidde was een aanwijzing dat een PQQ afhankelijk enzym een rol zou kunnen spelen in de afbraak van deze stoffen. Een aantal mutanten bleek inderdaad niet meer in staat op deze koolstofbronnen te groeien, terwijl andere mutanten dit wel konden, maar langzamer groeiden dan het wilde type. In alle mutanten kon een PQQ afhankelijk quinaat dehydrogenase (QDH) worden aangetoond. Ook in andere bacteriën bleek dit enzym aanwezig te zijn. Het feit dat sommige mutanten toch in staat waren om, zij het wat langzamer, op quinaat te groeien, bewijst dat onder deze omstandigheden deze mutanten wel degelijk in staat zijn minimale hoeveelheden PQQ te synthetiseren. In deze mutanten kon naast apoenzym (enzym zonder cofactor) dan ook katalytisch actief (holoenzym) aangetoond worden (Hoofdstuk V).

Enkele mutanten bleken niet meer in staat op anthranilzuur te groeien, waarschijnlijk omdat het enzym anthranilaat hydroxylase bij deze mutanten niet meer actief was. Omdat geen verband met de biosynthese van PQQ kon worden aangetoond, en deze mutanten alle deletiemutanten bleken te zijn (bij dit type mutanten zijn vaak meerdere genen niet meer werkzaam), is aangenomen dat de genen voor anthranilaat hydroxylase en de biosynthese van PQQ zeer dicht bij elkaar op het chromosoom gelegen zijn, en de desbetreffende mutanten in beide genen gestoord zijn.

Een probleem bij het vaststellen of er mutanten waren die intermediairen ophoopten en uitscheidde in het medium was, dat het wilde type erg weinig PQQ synthetiseerde, zodat wellicht de concentratie van intermediairen te laag was om te kunnen worden aangetoond. Daarom werd onderzoek verricht om tot een hogere PQQ productie in dit organisme te komen. Het kweken op quinaat, zoals hierboven al vermeld, alsmede het kweken onder "fed-batch" omstandigheden (hierbij wordt de koolstofbron gedurende de groei druppelsgewijze toegevoegd, waardoor de bacteriën langzaam groeien en veel tijd krijgen om PQQ te synthetiseren) leidde tot veel hogere PQQ concentraties in het kweekmedium bij het wilde type. Ook onder deze omstandigheden kon echter geen ophoping van intermediairen worden vastgesteld in de mutanten. Tevens bleek dat zij elkaar onder geen enkele omstandigheid konden helpen wat betreft PQQ vorming. Een model dat de afwezigheid van intermediairen kan verklaren is de aanname dat de PQQ biosynthese verloopt op een matrijs, bijvoorbeeld een klein stukje eiwit. De tussenproducten

blijven gebonden aan deze matrijs, zodat er geen vrije intermediairen aan te tonen zijn (Hoofdstuk VI).

Aangezien men in het PQQ molecuul de structuur van bepaalde aminozuren, waaronder fenylalanine, tyrosine, glutaminezuur en alanine, kan terugvinden, werden labelings-experimenten uitgevoerd waarbij naar inbouw van deze aminozuren in PQQ werd gekeken.

PQQ blijkt echter in het kweekmedium te reageren met aminozuren. Daarom werd eerst onderzoek verricht naar de aard van de ontstane produkten en de omstandigheden waaronder ze gevormd worden. De resultaten hiervan zijn beschreven in Hoofdstuk III. Met behulp van deze resultaten zijn de kweekcondities zodanig aangepast dat geen reactie van PQQ met aminozuren meer optrad.

Daar de aanpak van de biosynthese via labelings-experimenten met radioactieve isotopen chemische afbraak van PQQ vereist om vast te kunnen stellen waar radioactieve atomen in het PQQ molecuul zijn ingebouwd, terwijl het PQQ molecuul zeer moeilijk afbreekbaar is, werd besloten de inbouw met behulp van stabiele isotopen vast te stellen, aan de hand van massaspectrometrie of NMR analyse. Met deze technieken is het namelijk mogelijk direkt vast te stellen waar het gelabelde atoom is ingebouwd, dus zonder dat PQQ afgebroken behoeft te worden. Door de lage PQQ produktie destijds kwam *Acinetobacter* niet in aanmerking als organisme, daar voor de analyse minimaal 0,5 milligram PQQ benodigd is. Daarom werd gezocht naar andere organismen die wel voldoende PQQ produceerden. Aanvankelijk werden de experimenten uitgevoerd met *Pseudomonas* stammen, die bij groei op alcoholen voldoende PQQ uitscheiden. Door nu te kweken in aanwezigheid van ongelabelde aminozuren en met gelabeld ^{15}N -ammoniumchloride als stikstofbron, was het de bedoeling dat de inbouw van aminozuren werd vastgesteld uit de verdringing van ^{15}N door ^{14}N (van de aminozuren) in het PQQ-molecuul. Het bleek echter dat de *Pseudomonas* stammen de toegevoegde aminozuren prefereerden als koolstofbron boven het toegevoegde alcohol, met als gevolg dat de aminozuren niet direct werden ingebouwd, maar eerst afgebroken, waardoor de gelabelde en ongelabelde stikstofatomen niet specifiek, maar geheel willekeurig werden ingebouwd. Om dit te voorkomen, werd voor volgende experimenten gekozen voor een (PQQ producerende) bacterie, *Hyphomicrobium* X, die volgens de literatuur niet in staat is aminozuren af te breken. Door moeilijk te vermijden verontreinigingen leverde massaspectrometrie te weinig informatie op om exact vast te kunnen stellen op welke plaats de gelabelde atomen in PQQ

werden ingebouwd, maar met behulp van ^{13}C -NMR bleek dit wel mogelijk. Eerst moest echter worden uitgezocht of de toegevoegde aminozuren wel in het eiwit van *Hyphomicrobium* X werden ingebouwd, en dat zonder dat ze eerst worden afgebroken. Deze bacterie werd daarom op ^{13}C -gelabeld methanol gekweekt in aanwezigheid van ongelabelde (^{12}C) aminozuren. Een aantal van hen, waaronder fenylalanine en tyrosine, bleek intact in het eiwit van *Hyphomicrobium* te worden ingebouwd. Van deze aminozuren werd nagegaan of dat niet alleen voor het eiwit, maar ook voor PQQ gold. Uit de experimenten kon worden geconcludeerd dat alle koolstofatomen van tyrosine werden ingebouwd in PQQ, en geen enkel koolstofatoom van fenylalanine. Het ontbrekende stuk in het PQQ molecuul is nu glutaminezuur, wat helaas niet als precursor kon worden getest, omdat dit aminozuur niet in het eiwit wordt ingebouwd. Met tyrosine als precursor is een mogelijke biosyntheseroute opgesteld (Hoofdstuk VII).

Daar gedurende het onderzoek bij veel organismen onderzoek is gedaan naar de produktie van PQQ, kunnen ook hierover een aantal uitspraken worden gedaan, welke zijn beschreven in Hoofdstuk IV.