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Factors relevant in the reaction of pyroloquinoline quinone with amino acids

Analytical and mechanistic implications

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In order to reveal the stability of pyrroloquinoline quinone (PQQ) in complex samples, its reaction on incubation with amino acids was followed spectrophotometrically by monitoring oxygen consumption, and with a biological assay. For several α-amino acids, the formation of a yellow coloured compound (λ_{max} = 420 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 concomitant formation of $PQQH_2$, suggesting that PQQ is able to oxidize the presumed oxazoline to the oxazole. Besides the condensation reaction, there is also a catalytic cycle in which an aldimine adduct of PQQ and the amino acid is converted into the aminophenol form of the cofactor and an aldehyde resulting from oxidative decarboxylation of the amino acid. Addition of NH_4^+ salts, as well as that of certain divalent cations, greatly stimulated both the cyclic and the linear reaction. With basic amino acids, oxazole formation scarcely occurred. However, as oxygen consumption was observed (provided that certain divalent cations were present), conversion of these compounds took place. A reaction scheme is proposed accounting for the products formed and the effects observed. Since NH_4^+ ions activate several quinoproteins (PQQ-containing enzymes) and divalent cations (Ca^{2+}, Fe^{2+}, and Cu^{2+}) are additional (co)factors in certain metallo quinoproteins, the effects of metal ions observed here could be related to the mechanistic features of these enzymes. Although all oxazoles were converted to PQQ by acid hydrolysis, PQQ was not detected when hydrolysis was carried out in the presence of tryptophan, a compound which appeared to have a deleterious effect on the cofactor under this condition. The results here described explain why analysis methods for free PQQ in complex samples fail in certain cases, or are not quantitative.

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, they fail in cases where the compound does not occur in its free form, but in an unknown derivatized form. As PQQ reacts with a variety of nucleophiles [1] it is to be expected that problems are met in its analysis 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 to the relatively stable 5-hydrazone before proteolysis is carried out [2,3]. This hydrazone derivate can then be isolated from the hydrolysate and identified and quantified by chromatographic procedures. Although this method has been successfully applied on several of these quinoproteins (PQQ-containing enzymes) [2–4], the procedure relies on the initial formation of a hydrazine adduct. Therefore, this approach may not be applicable to all quinoproteins, e.g. 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 synthesis of free PQQ in these organisms have failed so far but the apoenzymes become functional on addition of PQQ. 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 observation of adduct formation 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, for this aspect it is also very important to know the factors which are relevant for reaction of PQQ. In addition, if the products are known, it could be imagined that it is possible to convert them to PQQ. This knowledge could provide insight into the mechanism of transport and storage of PQQ *in*

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Abbreviations. PQQ, pyrroloquinoline quinone.

Enzymes. Alcohol dehydrogenase (EC 1.1.99.–); glucose dehydrogenase (EC 1.1.99.17); lipoxygenase (EC 1.13.11.12); methanol dehydrogenase (EC 1.1.99.8); methylamine dehydrogenase (EC 1.4.99.3); quinate dehydrogenase (EC 1.1.99.–); amine oxidase (EC 1.4.3.6); galactose oxidase (EC 1.1.3.9); DOPA decarboxylase, aromatic-L-amino-acid decarboxylase (EC 4.1.1.28).

vivo as well as providing clues for its analysis in complex biological systems.

Reaction of PQQ with some amines [7–9], amino acids [10, 11] and thiols [12] has been described recently. In view of the omnipresence of amino acids, the reaction of these compounds with PQQ seemed most interesting. Oshiro and coworkers [10] reported decarboxylation of amino acids to the aldehyde with PQQ as a catalyst. On the other hand, Bruice and coworkers [9] found that 7,9-didecarboxy derivate of PQQ reacted with glycine to yield an oxazole. In view of these controversial findings and their significance for the aims indicated above, it was decided to investigate the reaction of PQQ with several amino acids, to determine the effect of a number of obvious compounds on this reaction, and to search for methods to convert the presumed oxazole adducts into PQQ.

MATERIALS AND METHODS

Oxazole formation

Oxazole formation was monitored by measuring the rate of increase of absorption at 420 nm in a standard mixture consisting of the following components (total volume 3 ml): 0.033 M Tris/HCl, pH 8.3; 0.3 M NH_4Cl; 0.4% (mass/vol.) amino acid; 10 μM EDTA, 50 μM PQQ. To this mixture Ca^{2+} was added to a concentration of 110 μM, unless otherwise indicated. EDTA was added to minimize interference of metal ions which bind more strongly to EDTA than Ca^{2+}. With basic amino acids, Cu^{2+} was used as metal ion, and with amino acid esters, no divalent cation was added. The reaction was started by addition of amino acid. The type of oxazole was determined from the HPLC retention time and absorption spectrum. HPLC was performed on a Waters RCM 100 module containing a 10 μM C_{18} 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% (by vol.) concentrated H_3PO_4. The eluate was scanned (at 250 nm) with a Hewlett Packard 1040A photodiode-array detector, taking spectra at the apex of the peaks. Yields of oxazoles were calculated on the basis of a molar extinction coefficient at 420 nm of 15000 $M^{-1} \cdot cm^{-1}$.

Oxygen consumption

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

Biological assay

Samples were analyzed with a biological assay, measuring the extent of reconstitution of the quinohaemoprotein 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 reference compounds

(a) From glycine, L-serine and L-threonine (oxazole 1). 100 mg PQQ · 1 H_2O was dissolved in 100 ml of the standard mixture, and incubated with stirring at room temperature while leading a stream of O_2 through the solution. During the reaction, the pH was kept at 8.2 with 0.1 M NaOH. After 2 h, the pH was brought to 1.5 and the solution stored overnight at 4 °C. The precipitate was filtered off, dissolved in water (pH 4.0) and the solution heated to 90 °C. After acidification to pH 1.5 and storage at 4 °C, the precipitate was collected by filtration. This procedure was repeated once, and the product was dried *in vacuo* over P_2O_5 to give about 110 mg of an orange-brown powder (in all cases). NMR spectroscopy was performed on a sample crystallized from hot dimethylsulfoxide.

(b) From methyl glycinate (oxazole 3). 100 mg PQQ · 1 H_2O was dissolved in 110 ml of the standard mixture for amino acid esters, and incubated with stirring for 2 h. The solution was acidified to pH 1.0 and, after storage at 4 °C, the precipitate filtered off and dissolved in 5 ml dimethylformamide at 50 °C. After two days standing at room temperature, the precipitate was collected and crystallized from dimethylformamide. The product (10–20 mg) was washed with ether and dried in air.

Hydrolysis of oxazoles

Alkaline hydrolysis was performed by dissolving the oxazole in 2 M NaOH and incubating with 1% glycine at 100 °C under exclusion of O_2 for at least 8 h (the inclusion of glycine improved the yields, probably because it keeps the cofactor in its reduced form under the applied conditions). Acid hydrolysis was performed by dissolving the oxazole in 2 M HCl and incubating at 100 °C for 2 h.

Samples were neutralized either with concentrated HCl or NaOH solutions, and diluted before carrying out the biological assay. To study oxazole formation and decomposition in a protein hydrolysate, a synthetic mixture of amino acids [13] was used.

NMR spectroscopy

^{1}H-NMR spectra were recorded on a Varian EM 360 spectrometer operating at 60 MHz, using tetramethylsilane as an internal reference. The compounds were dissolved in deuterated dimethylsulfoxide.

RESULTS

Oxazole formation

The reaction of PQQ with glycine in the standard mixture resulted in the formation of a yellow product (λ_{max} = 420 nm) with concomitant O_2 uptake and disappearance of biological activity (Fig. 1). Since the product formed was not biologically active, it could be calculated from Fig. 1 that 3.2 mol O_2 were consumed/mol of PQQ converted. As only 0.5 mol O_2 is required to convert PQQ to the oxazole (see below), the remaining O_2 consumption can be attributed to the catalytic degradation of the amino acids [7].

Reaction of PQQ with L-serine or L-threonine also yielded yellow products which appeared to be identical to that of glycine as judged from HPLC (retention time of 17.5 min), absorption spectra (Fig. 2), and ^{1}H-NMR analysis (signals at 7.30 ppm 8.36 ppm and 9.29 ppm). In view of the NMR signals, the most likely structure is the one indicated in Fig. 3, analogous to that proposed by Bruice et al. [9] for the product of 7,9-didecarboxy derivative of PQQ, ethyl ester and glycine,

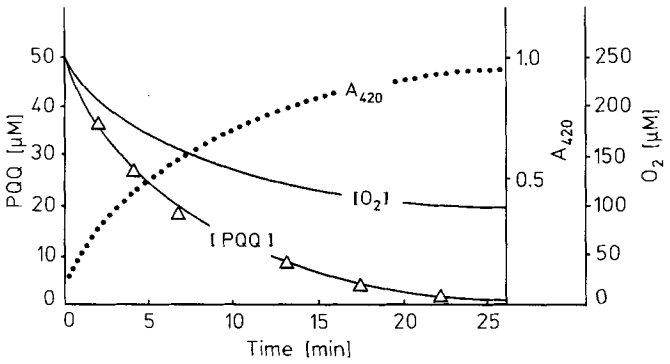

Fig. 1. *Aerobic oxidation of glycine with PQQ.* A_{420} is indicative for the amount of oxazole 1 formed. Oxygen consumption and PQQ concentration were measured as described in Materials and Methods

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 ultraviolet detection at 250 nm. Spectra were taken with the photodiode array detector at the apex of the peaks. Yields were calculated using the molar extinction coefficient at 420 nm of the glycine oxazole (ε = 15000 $M^{-1} \cdot cm^{-1}$) as a standard. Structures of the oxazole types are presented in Fig. 3

Amino acid	Retention time t_R	Yield	Oxazole type
	min	%	
Glycine	17.7	100	1
L-Serine	17.6	75	1
L-Threonine	17.7	86	1
L-Tryptophan	17.5	70	1
L-Tyrosine	18.3	9	1
L-Alanine	17.5	5	1
	15.4	8	2
L-Valine	18.0	6	2
L-Leucine	17.7	10	2
L-Isoleucine	18.7	10	2
L-Phenylalanine	19.8	24	2
L-Methionine	18.0	40	2
L-Glutamate	15.8	13	2
L-Glutamine	15.5	32	2
L-Aspartate	15.9	22	2
L-Asparagine	15.6	30	2
L-Histidine	14.9	9	2
Methyl glycinate	16.9	54	3
Ethyl glycinate	17.7	56	3

the signals at 7.30 ppm and 8.36 ppm arising from the C3H and C8H of PQQ, respectively [14], and the signal at 9.29 ppm arising from the methenyl hydrogen. Most probably, the product formed from L-tryptophan and L-tyrosine has the same structure (indicated as oxazole 1), in view of the similar 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 these data, it is assumed that the products have the structure as indicated in Fig. 3 (oxazole 2), with the residue (R) being the R of the amino acid used [R-CH(NH_2)-COOH]. L-Alanine behaved exceptionally since both oxazoles 1 and 2 were found.

No yellow compounds were detected for L-proline, L-lysine and L-ornithine. PQQ became reduced to $PQQH_2$ (as revealed

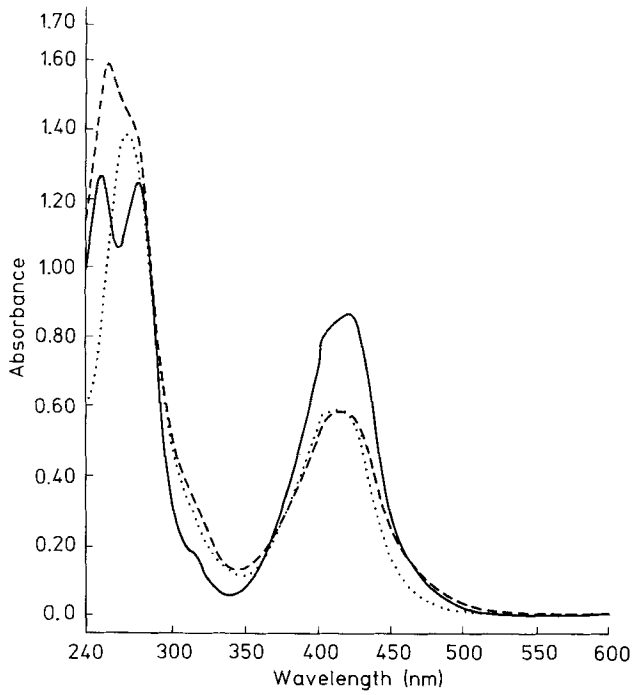

Fig. 2. *Absorption spectra of different types of oxazoles.* Spectra were taken after virtually complete conversion of PQQ into oxazole in the standard mixture, containing either glycine (——), L-methionine (– – –) or methyl glycinate (·····)

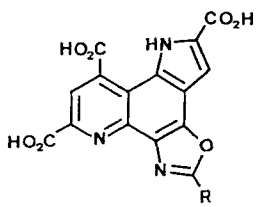

Fig. 3. *Structure of oxazole derivatives of PQQ.* Oxazole 1, R = H; oxazole 2, R = R group of amino acid [R-CH(NH_2)-COOH]; oxazole 3, R = carboxylate ester or carboxamide moiety of glycine ester or glycinamide, respectively ($COOC_nH_{2n+1}$ or $CONH_2$)

by the absorption spectrum) on incubation with L-cysteine. In addition, a precipitate was formed, most probably L-cystine.

Glycinamide and methyl glycinate gave yellow products with absorption spectra and retention times different from the product of glycine (Fig. 2, Table 1). ^{1}H-NMR analysis of the product formed from the methyl ester revealed that the ester group was retained in the product since, in addition to the signals of the aromatic protons (at 7.43 ppm and 8.55 ppm), a singlet was found at low field (3.78 ppm), originating from the $-OCH_3$ moiety (oxazole 3, Fig. 3). Amino acids with a modified amino group (*N*-methyl-serine) did not react (Table 2).

Oxidation of α-amino acids

Glycine was used as a model compound to determine the optimal conditions and kinetics of the aerobic oxidation of α-amino acids with PQQ. The addition of NH_4^+ salts (0.3 M) increased the rate of oxazole formation and oxygen consumption at least 30-fold. The optimum pH was 8.3 (Tris/HCl buffer). A tenfold decrease in glycine concentration in the

Table 2. *Oxygen consumption and oxazole formation rates for several amino acids and related compounds*
Ca^{2+} or Cu^{2+} were added to the standard mixture, as indicated. Compounds showing no activity in the standard mixture were further tested with both divalent cations. The ratio mol O_2 consumed/mol oxazole formed was calculated for an incubation time sufficient for conversion of more than 90% of PQQ into oxazole. Not determined, nd; no addition, –

Amino acid	Cations added	Initial rate of O_2 consumption	Initial rate of oxazole formation	O_2 consumed/oxazole formed
		$nmol \cdot l^{-1} \cdot s^{-1}$		mol/mol
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	nd
L-Leucine	Ca^{2+}	8	2.5	nd
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	–	nd	80	nd
Methyl glycinate	–	21	95	0.67
Ethyl glycinate	–	25	60	0.74

Table 3. *The effect of divalent cations on O_2 consumption rates*
The salts of the cations were added to the standard mixture in a concentration of 100 μM

Addition	Initial rate of oxygen consumption with					
	Lys	Orn	Gln	Asn	Arg	Gly
	$nmol \cdot l^{-1} \cdot s^{-1}$					
None	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

standard mixture 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, the influence of metal ions on oxygen consumption and oxazole formation was investigated. Only Ca^{2+} and Cu^{2+}, and to a slight extent M^{2+} ions, stimulated the reaction (Table 3), whereas Ba^{2+}, monovalent or trivalent cations did not. With Mn^{2+}, oxygen consumption was very low and a different yellow coloured compound was formed (λ_{max} = 383 nm). In the presence of Fe^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} both oxazole 1 and the 383-nm compound were observed while oxygen consumption rates were low. These metal ions even inhibited O_2 consumption in the standard mixture (in the presence of Ca^{2+}) since, on addition of increasing amounts of e.g. Mn^{2+}, O_2 consumption linearly decreased. Complete abolishment required the addition of 1 mol Mn^{2+}/2 mol PQQ.

The 383-nm compound was also the main product when Ca^{2+} or Cu^{2+} were present in high (1 mM) concentrations. It was converted to oxazole 1 after acidification of the mixture to pH 4. Addition of excess EDTA to the 383-nm compound resulted in the reappearance of PQQ.

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

Oxidation of basic amino acids and ω-amides of acid amino acids

For studies with basic amino acids, the α,ω-diamino acids, lysine and ornithine were used. In the standard mixture, O_2 uptake was very low compared with glycine. However, replacement of Ca^{2+} by Cu^{2+}, Mn^{2+} or Zn^{2+} resulted in high O_2 consumptions, while replacement by Ni^{2+} inhibited the reaction completely (Table 3). The presence 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. ω-Aminocarboxylic acids, like δ-aminopentanoic acid and ε-aminohexanoic acid, were unreactive. On the other hand, the ω-amide forms of the 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 higher than with the other basic amino acids (Table 3), and oxazole formation occurred (results not shown).

Oxidation of glycinamide and methyl glycinate

To obtain an insight into the mechanism of the oxidative decarboxylation, methyl glycinate and glycinamide were investigated. Rates of oxazole 3 formation were comparable to those for glycine under standard conditions. Interestingly, this required the absence of divalent cations while low amounts of oxygen were consumed (less than 1 mol O_2/mol oxazole formed). In the presence of certain divalent cations (Ca^{2+}, Cu^{2+} and Mn^{2+}) a different yellow coloured compound (λ_{max} = 307 nm and 410 nm) was formed immediately. With Mn^{2+}, the compound was stable and oxazole 3 was formed after addition of excess EDTA. With Ca^{2+} and Cu^{2+}, the compounds were not stable, as judged from the decreases in absorbance at 307 nm and 410 nm on prolonged incubation. Oxazole 3 was not formed in this conversion, but this was formed on adding an excess of EDTA.

Anaerobic experiments

HPLC analysis of the reaction products of PQQ with glycine in the standard mixture under anaerobic conditions showed that $PQQH_2$ and oxazole 1 were formed in a 1:1 molar ratio (Table 4). With lysine or ornithine, the main

Table 4. *Anaerobic conversion of amino acids in the presence of different divalent cations*
Yields of the oxazole derivatives, PQQ and $PQQH_2$ were determined from the areas of the peaks in a HPLC chromatogram. Known amounts of oxazole 1, PQQ, and $PQQH_2$ were used as standards

Amino acid	Metal ion added	PQQ	$PQQH_2$	Oxazole	
				1	3
		%			
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

Table 5. *Stability of PQQ under strongly acidic conditions in the presence of amino acids*
PQQ was incubated with amino acids in 2 M HCl for 2 h. at 100 °C. Samples were neutralized, diluted and PQQ concentrations were measured using the biological assay [6]

Amino acid	[PQQ]	Remaining activity
	μM	%
None	1 – 50	100
Glycine (0.2%)	1 – 50	100
Amino acid mixture [13]	5.0	79
	2.0	70
	1.0	45
	0.5	29
Tryptophan (0.1%)	1.0	0

product was $PQQH_2$ but low amounts of yellow coloured compounds were detected (Table 4).

Hydrolysis of PQQ-oxazoles

Reasonably good yields of PQQ (up to 50%) were obtained upon alkaline hydrolysis of oxazole 1, but only when comparatively high concentrations of oxazole were used (> 10 μM) and the solution was held semi-anaerobic by O_2 removal and addition of excess glycine. Alkaline hydrolysis is of little practical use for PQQ analysis, since degradation of PQQ can be expected under these conditions [3].

When oxazoles 1 or 2 were 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 μM. When PQQ (5 – 50 μM) was converted completely to oxazoles in nutrient broth (10 g/l) under standard conditions, however, no PQQ was detected after subsequent hydrolysis. With a synthetic mixture of amino acids, some PQQ could be detected (10 – 50% recovery depending on the PQQ concentration). Since oxazoles were obviously formed and hydrolyzed, but recovery of PQQ was variable, the stability of PQQ itself under hydrolysis conditions was checked (Table 5). These experiments showed that severe losses of PQQ occurred in the presence of amino acids in 2 M HCl. Testing the amino acids separately, L-tryptophan was found to be the only causative factor. No PQQ could be detected after incubation of a 1 μM PQQ solution with 0.1% L-tryptophan for 2 h at 100 °C in either 2 M or 6 M HCl. With the other amino (acids, no losses of PQQ were found.

DISCUSSION

Some aspects of the reaction of PQQ with amino acids have 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 condensation products, mainly the oxazole. Since the action of PQQ with amino acids and related compounds appeared to be a very complicated topic, it became beyond the scope of the study to make thorough investigations on all reactions and intermediates individually. However, from the important features which have already been established, it is possible to derive a working hypothesis, as presented in Fig. 4.

Entry into both the linear and the cyclic pathway is governed by the primary addition of the nucleophilic amino group either at C5 or at C4 . Since earlier studies [15] have revealed a substantial difference in electrophilic character of the two carbonyl groups, favoring C5 as the site of primary nucleophilic addition, it can be safely assumed that the reaction starts at the C5 position of PQQ also in this case. The fact that the rates of the linear and the cyclic pathway seem to be affected equally by ammonia and metal ions (results not shown) supports this assumption. Determination of the orientation of oxygen and nitrogen in the resulting pyrroloquinoline oxazoles should give a definite answer with respect to the site of attachment.

The oxidation rate of α-amino acids was enormously stimulated by addition of NH_4^+ salts. Since the 5-imino form of PQQ is formed on addition of ammonia to PQQ [15], this stimulation can be explained on the basis of the strong electrophilic character of the 5-imino group. Particularly, the addition of the amino group of α-amino acids will take place more readily on the 5-imino derivative of PQQ than on the parent 5-carbonyl form. A similar example of covalent catalysis has been described by Jencks [16]. The explanation given for the stimulatory effect in the case of α-amino acids is in accordance with the observation that the oxidation of amines and α,ω-diamino acids with PQQ was not dependent on NH_4^+ salts.

In the proposed scheme (Fig. 4) the quinone imine formed after addition of the amino acid to the 5-imino derivative of PQQ tautomerizes to the Schiff base. Evidence for this rearrangement has been obtained recently for the reaction of *o*-quinones with *sec*-alkyl primary amines [17]. An issue of special interest is the location of the step in which actual branching between the cyclic and linear pathways takes place. Knowledge of the chemical nature of the branch point intermediate might eventually enable one to devise conditions favoring suppression of stable adduct formation or, in the opposite case, promoting the rapid and complete conversion of PQQ to a limited set of pyrroloquinoline oxazoles. Since glycinamide and glycine esters give rise to oxazoles which

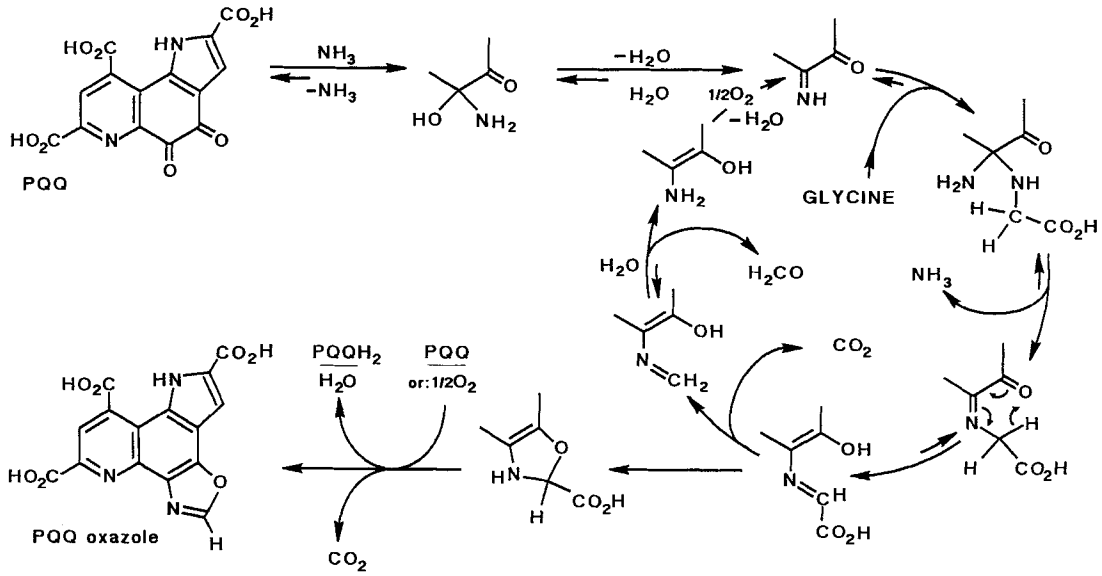

Fig. 4. *Proposed reaction mechanism for amino acid conversion and oxazole 1 formation*

have retained the amide or ester moiety, it is obvious that decarboxylation is no prerequisite for oxazole formation. Therefore, the Schiff base seems an attractive candidate as branch point intermediate. The tendency of this intermediate to lose the carboxyl group will then govern the distribution between the cyclic and the linear pathway. In this context it is also relevant to mention that DOPA decarboxylase from pig kidney is a quinoprotein [18] and that from the available information a role can be ascribed to PQQ and pyridoxal phosphate in the decarboxylation process [19].

Decarboxylation leads to an aldimine derivative of PQQ which is hydrolyzed to formaldehyde (in the case of glycine) and an aminophenol derivative of PQQ. Ring closure of the Schiff base leads to an oxazoline derivative of PQQ. It has been shown that oxazole formation from quinones and quinone imines and amines proceeds via an oxazoline [20]. Another molecule of the quinone was assumed to function as oxidant in the final dehydrogenation step [20]. The results of experiments with PQQ and glycine 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 by assuming that PQQ is the hydrogen acceptor in the final dehydrogenation step of the oxazoline form to the oxazole derivatives of PQQ. The failure to detect any aminophenol derivative of PQQ in the anaerobic mixture is rather surprising. Since under aerobic conditions the cyclic pathway operates effectively (in the case of glycine, 3.2 mol O_2 were consumed/mol PQQ converted), one would expect the aminophenol derivative of PQQ 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 the aldimine and/or aminophenol derivatives. Finally, under aerobic conditions, the reduced products from the cyclic (aminophenol form of PQQ) and linear ($PQQH_2$) pathway are regenerated by oxygen.

HPLC and ^{1}H-NMR analysis showed that differently substituted oxazoles can be formed, depending on the amino acid used. The appearance of unsubstituted oxazole (oxazole 1) with serine, threonine, tryptophan and tyrosine illustrates that amino acids with side chains having even a very modest leaving group character will eventually give rise to the branch-point intermediate depicted in Fig. 4 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 withdrawing group [21].

Reaction rates of PQQ with 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 these compounds. Furthermore, the ring closure as well as the decarboxylation reaction could be hampered by stericfactors.

No oxazole formation is observed with lysine and related α,ω-diamino acids. Since oxidation occurs, presumably the ω-amino group is converted into an aldehyde group, just as in the case of primary amines.

The presence of certain metal ions resulted in large stimulatory or 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 a depolarizing effect on the charged carboxyl group. Whether they play a role in facilitating (stable) adduct formation between PQQ and the amino acid can not be conclusively answered at the moment, but the inhibitory effect of special cations, notably Mn^{2+}, may well be the result of a particularly tight complex involving ligands provided by 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 idea 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 in the mechanism mentioned above [21].

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 has been shown to be activated by NH_4^+ salts *in vitro* [22]. In addition, the activity of quinoprotein methylamine dehydrogenase is stimulated by NH_4^+ salts [23]. Quinoprotein glucose dehydrogenase from various bacteria, and quinate dehydrogenase from *Acinetobacter calco-aceticus* require divalent cations (Mg^{2+} or Ca^{2+}) for cofactor binding [24–26], whereas

quinohaemoprotein alcohol dehydrogenase from *Pseudomonas testosteroni* is strictly dependent on Ca^{2+} ions for anchoring of the cofactor [6]. Lipoxygenase contains a complex of Fe^{+} and PQQ [27], while Cu^{2+} occurs in copper-containing amine oxidases and galactose oxidase [28]. Therefore, the strong effects of metal ions observed on the reaction of PQQ with amino acids make it very likely that further studies can provide relevant information for the catalytic mechanism of these enzymes.

Experiments with PQQ derivatives (results not shown) have demonstrated 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 inactivity may also result from the absence of a difference in reactivity of the two carbonyl groups in the first compound (leading to occupation of both carbonyl groups).

All model oxazole derivatives of PQQ tested were easily and nearly quantitatively converted into PQQ with 2 M HCl at 100 °C. In contrast, no PQQ was recovered after hydrolysis of oxazoles made in nutrient broth, due to the reactivity of PQQ towards L-tryptophan during acid hydrolysis. Since this phenomenon will also occur during hydrolysis of quinoproteins with 6 M HCl, analysis procedures comprising such a step cannot be reliable. Summarizing, the results imply that identification and quantitation of free PQQ in complex samples (containing amino acids) will fail for samples at physiological conditions (formation of oxazoles) as well as for acid hydrolyzed samples (reaction with tryptophan). The reaction product(s) formed from PQQ and L-tryptophan under acidic conditions is (are) presently under investigation.

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