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

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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 were determined via 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, their incorporation into PQQ was determined by ^{1}H- 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.

Pyrroloquinoline quinone; Amino acid incorporation; L-Tyrosine; Cofactor; (*Hyphomicrobium* X)

1. 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lysyl oxidase (EC 1.4.3.13) [4], dopamine-β-hydroxylase (EC 1.14.17.1) [5] and soybean lipoxygenase-1 (EC 1.13.11.12, unpublished). Since several of these enzymes are involved in the conversion or synthesis of mammalian bioregulators, insight into 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 Goosen, N. personal 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.

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

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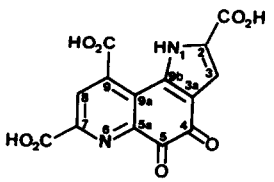

Fig.1. Structure of pyrroloquinoline quinone (PQQ).

before, namely tyrosine or phenylalanine and glutamic acid, which were used as hypothetical forerunners for a biomimetic synthesis route [7]. Therefore, studies aimed at discovering 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 ^{1}H- 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 by addition of unlabelled amino acids. After it appeared that several 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 by Unkefer and co-workers, using *Methylobacterium* AM1, have led to conclusions similar to those presented here.

2. MATERIALS AND METHODS

2.1. *Cultivation and labelling*

Hyphomicrobium X was cultivated on a mineral medium containing per l: 1.79 g K_2HPO_4, 1.38 g $NaH_2PO_4 \cdot H_2O$, 2.0 g KNO_3, and 0.2 g $MgSO_4 \cdot 7H_2O$. After autoclaving, 1 ml spore, calcium, and iron solutions according to Duine et al. [10] were added to 1 l 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 $CaCl_2 \cdot 2H_2O$/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 being 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 h at 30°C on a rotary shaker (200 rpm).

2.2. *Purification and analysis of PQQ*

Cultures of *Hyphomicrobium* X were centrifuged (15 min at 23 000 × *g*). PQQ was purified from the supernatant essentially according to [12], but with some modifications. To the supernatant 0.5–1.0 g Amberlyst A-21 ion exchanger (50–100 μM, p.A. from Serva, Heidelberg) in the chloride form was added. After incubation with stirring (2 h), the anion exchanger was packed into 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 10 times in 0.01 M HCl and applied to a Seppak C_{18}-silica cartridge (Waters). 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].

^{1}H- and ^{13}C-NMR spectra of PQQ were recorded on a Varian VXR-400 S spectrometer operating at 400 and 100.5 MHz respectively, using the pulse Fourier-transform mode. The internal reference was $(C^2H_3)_2SO$ for ^{1}H-NMR. For ^{13}C spectra, the

solvent signal of $(C^2H_3)_2SO$ was suppressed and tetramethylsilane used as an internal reference.

2.3. *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 of 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 × 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 *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. A VG 70-SE gas chromatograph-mass spectrometer equipped with a 25 m × 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.

3. RESULTS AND DISCUSSION

3.1. *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-^{13}C)-labelled fragments. In the experiment with mixture II, mass spectra of amino acids not present in this mixture yielded only (U-^{13}C)-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-^{13}C)-labelled fragments were seen (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.

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

3.2. *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). 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 (not shown).

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 to 98%, and no other unlabelled amino acids were found (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 to an extent of 94%, whereas no other amino acids were found unlabelled in the hydrolyzed protein (not shown).

In order to obtain ^{1}H- and ^{13}C-NMR spectra of [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. Whereas the ^{1}H-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

Table 1

The incorporation of administered amino acids into cellular protein of *Hyphomicrobium* X

Amino acid	Percentage incorporation		
	Mixture I (1 ml/20 ml culture medium)	Mixture I (5 ml/20 ml culture medium)	Mixture II (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

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 (C_2-COOH), 165.9 (C_7-COOH), 166.7 (C_9-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 ^{1}H- 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 ^{1}H-NMR spectrum of purified PQQ showed a singlet at 7.15 ppm, whereas the doublet at 8.64 ppm (168 Hz, 8-H) 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 (C_7-COOH) and 166.8 (C_9-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}.

Table 2

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

Amino acid	Incorporation (%)	
	Into PQQ	Into protein
–	< 5	< 1
L-Phenylalanine	< 5	98
L-Tyrosine	>95	94

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

3.3. *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 present results and those obtained by Unkefer and co-workers (personal 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

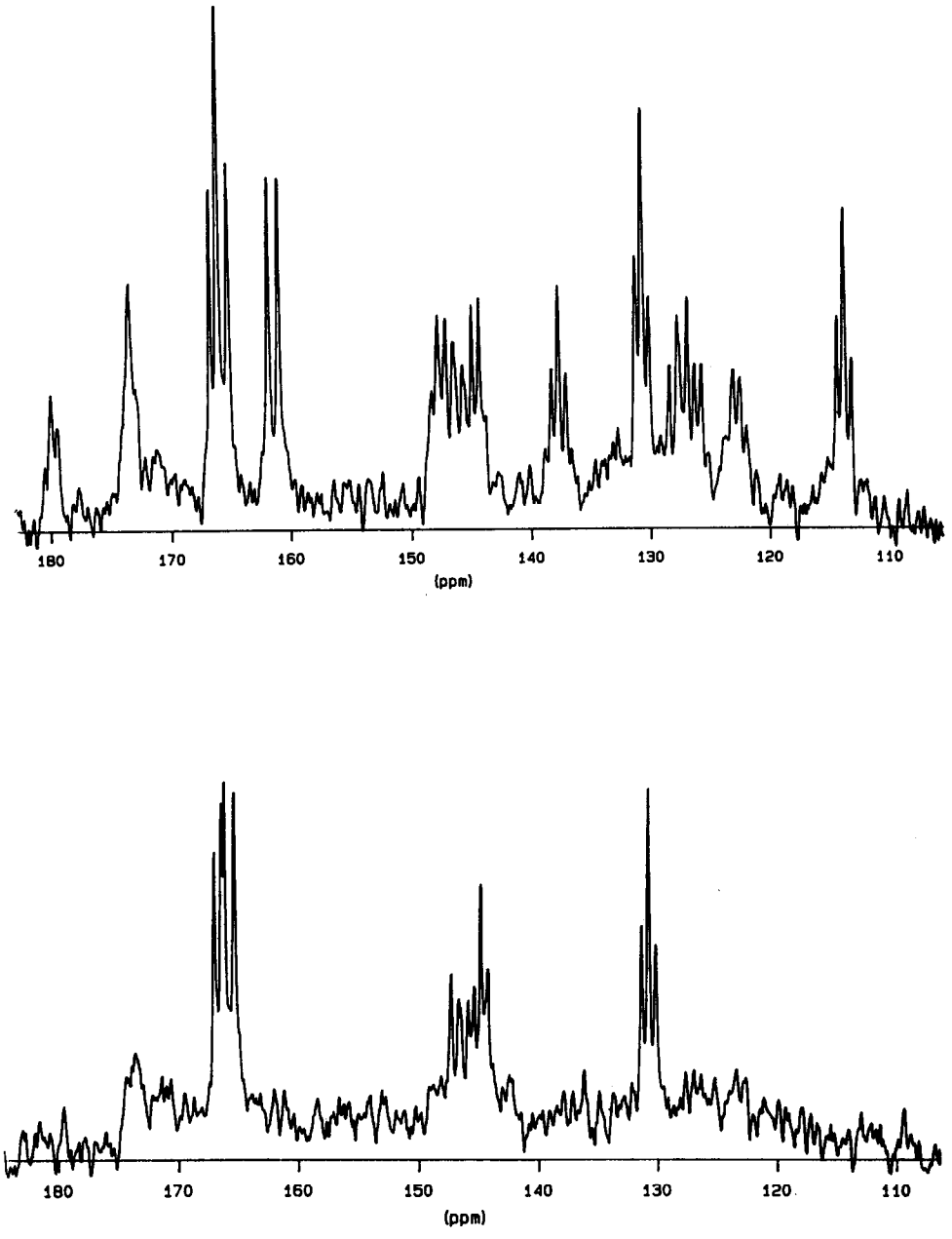

Fig.2. ^{13}C-NMR spectra of [U-^{13}C]PQQ (top) and PQQ purified from a culture of *Hyphomicrobium* X grown on [U-^{13}C]methanol in the presence of L-tyrosine (bottom).

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-dihydroxyphenylalanine (DOPA). Since the genes for PQQ

biosynthesis in *Acinetobacter calcoaceticus* have been sequenced (Goosen, N. unpublished) 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 (Goosen, N. 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 are sparse [20]. 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 allowing a desaturation and a hydroxylation step. It should be mentioned here that so far no intermediates in the biosynthesis of PQQ have been found, either using PQQ^- mutants from different complementation groups, or from different organisms (Van Kleef, M.A.G. and Duine, J.A. 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 phospho*enol*pyruvate 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 C_1- or C_2-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 (e.g. L-alanine, L-glutamate, and L-aspartate), however, did not result in an increase of cell dry weight, either in batch or 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 we know, this paper is the first report demonstrating that certain amino acids are not incorporated, while others become incorporated into the cellular protein of a methylotrophic bacterium without degradation.

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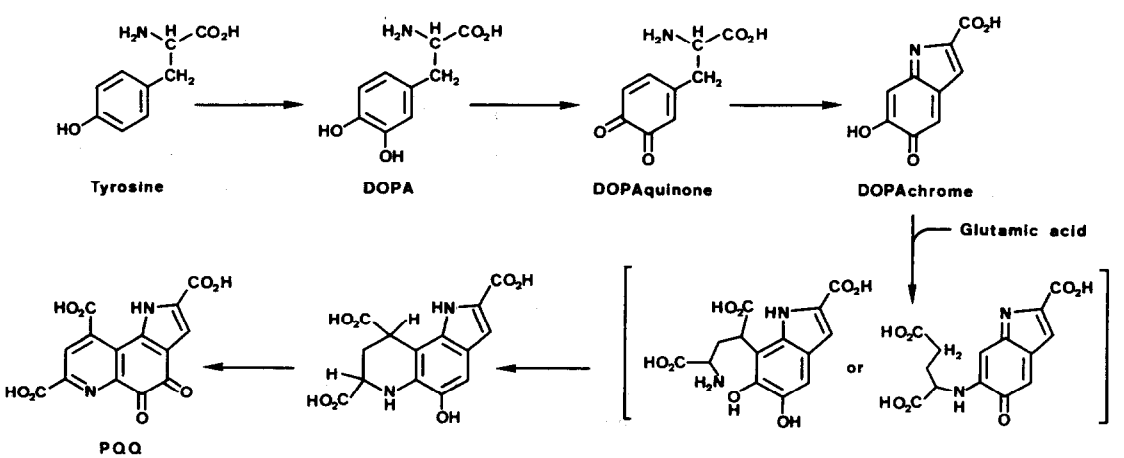

Fig.3. Proposed route for PQQ biosynthesis.

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