Substrate Specificity of 4-OT for 1 and 2. 4-OT activity is assayed spectrophotometrically at 30 °C by following either the rate of disappearance of substrate (**1**) at 295 nm ($\epsilon = 24.1 \times 10^3$ M^{-1} cm^{-1}) or the rate of appearance of product (**3**) at 236 nm ($\epsilon = 6.58 \times 10^3$ M^{-1} cm^{-1}). All initial velocities reported in the nonequilibrium and equilibrium experiments are determined by the latter assay. In a nonequilibrium experiment, the assay mixture contains 20 mM Na_2HPO_4 buffer (1.0 mL, pH 7.3) and an aliquot (1 μL) of sufficiently dilute enzyme to obtain a linear rate. The assay is initiated by the addition of a quantity (1–10 μL) of **1** from either a 20 or a 50 mM stock solution made up in ethanol. In an equilibrium experiment, the assay mixture contains 20 mM Na_2HPO_4 buffer (1.0 mL, pH 7.3) and a quantity of substrate (1–10 μL) from the same stock solution. After 4 min, an aliquot (1 μL) of the same dilute enzyme is added to the mixture. The cuvettes are mixed by inversion. A cuvette containing only buffer is used as a blank in both experiments. At each substrate concentration, a nonequilibrium velocity is obtained immediately following the determination of an equilibrium velocity. No lag time in the production of **3** is observed in either experiment. The initial velocities are measured from linear portion of the first 5–10 s of the tracing except when very low concentrations of substrate are used. In this case, the initial velocity is measured from the first 15–20 s of the tracing. The stock solutions of **1** are made up just prior to the start of the experiment. Detectable decomposition of the solution is not observed during the lifetime of an experiment. Dilutions of enzyme are made 2 h prior to the start of an experiment. Inconsistent results are obtained otherwise. No significant inhibition of the enzyme by ethanol is observed at ethanol concentrations below 2.5% (v/v). All results are reproducible in multiple runs.

Analysis of Enzyme Kinetic Data. The data from the nonequilibrium and equilibrium experiments are fitted by using a statistical program known as MacEnzkin run on a Macintosh II computer. The program fits the initial velocity data as a function of substrate concentration to the best rectangular hyperbola by an iterative nonlinear least-squares method. It is a kind gift from Professor J. Westley and J. D. Ozeran (The University of Chicago).

For a mechanism in which two substrates A and B compete for the active site of the enzyme, the rate of product formation is described by eq 2,[9b] where A is the concentration of **1** at 4 min, B is the concentration

$$v = (V/K)_A(A) + (V/K)_B(B)/1 + (A)/K_A + (B)/K_B \quad (2)$$

of **2** at 4 min, K_A and K_B are the Michaelis constants for **1** and **2**, and V is the maximal velocity measured when the enzyme is fully saturated with substrate. The kinetic parameters K_B and $(V/K)_B$ in eq 2 are obtained from eqs 3 and 4, where K_{A+B} and $(V/K)_{A+B}$ are the kinetic

$$K_B = \%\ B/1/(K_{A+B}) - \%\ A/K_A \quad (3)$$

$$(V/K)_B = (V/K)_{A+B} - \%\ A(V/K)_A/\%\ B \quad (4)$$

constants observed in the equilibrium experiment, % A is the percentage of A present at 4 min, and % B is the percentage of B present at 4 min. Because the 4-OT reaction is not inhibited by the product, **3**, it is not necessary to include a product inhibition term in this analysis.

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Biosynthesis of Pyrroloquinoline Quinone. 2. Biosynthetic Assembly from Glutamate and Tyrosine

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Abstract: The biosynthesis of pyrroloquinoline quinone (PQQ), the prosthetic group of quinoproteins, was studied in the methylotrophic bacterium, *Methylobacterium extorquens* sp. AM1. Using ^{13}C-labeled precursors and NMR spectroscopy, we have elucidated the biosynthetic origin of PQQ. In an initial series of feeding experiments, *M. extorquens* AM1 was grown on [1-^{13}C]- or [2-^{13}C]ethanol, and the resulting ^{13}C enrichments in PQQ were compared to the labeling patterns in amino acids. These data revealed that PQQ is biosynthesized from two amino acids: one molecule of glutamate and one molecule of either tyrosine or phenylalanine. Direct incorporation of tyrosine was observed by using [^{13}C]tyrosine labeled in the phenol side chain and at the methylene position. Moreover, a double-labeling experiment with [^{15}N,^{13}C]tyrosine demonstrated that the pyrrole nitrogen is derived from the α-amino group of tyrosine. Therefore, carbons 2′, 2, 3, 3a, 4, 5, 5a, 9a, 1a and nitrogen 1 of PQQ are derived from tyrosine; the pyrrole ring forms via the intramolecular cyclization of the tyrosine. Carbons 7′, 7, 8, 9, 9′, and, in all probability, nitrogen 6 are derived from glutamate. Using this information, we have proposed biosynthetic pathways for the assembly of PQQ from tyrosine and glutamate.

Pyrroloquinoline quinone (PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione) is one of several *o*-quinones that serve as prosthetic groups in some redox enzymes. *o*-Quinone prosthetic groups are known in alcohol dehydrogenases,[1] amine oxidases,[2] and amine dehydrogenases.[3] These prosthetic groups can be divided into two classes based on the presence or absence of a covalent linkage between cofactor and enzyme. The covalently linked *o*-quinone cofactors are apparently the product of post-translational modification of amino acyl residues present in the parent protein. The best characterized of this class of compounds is 6-hydroxy-dopa, or topa, which has recently been identified at the active site of bovine serum amine oxidase,[4] suggesting that

(1) (a) Anthony, C.; Zatman, L. J. *Biochem. J.* **1967**, *104*, 953–959. (b) Anthony, C.; Zatman, L. *Biochem. J.* **1967**, *104*, 960–969. (c) Salisbury, S. A.; Forrest, H. S.; Cruse, W. B. T.; Kennard, O. *Nature (London)* **1979**, *280*, 843–844. (d) Duine, J. A.; Frank, Jzn. J.; Werwiel, P. E. J. *Eur. J. Biochem.* **1980**, *108*, 187–192.

(2) (a) Hartmann, C.; Klinman, J. P. *J. Biol. Chem.* **1987**, *262*, 962–965. (b) Hartmann, C.; Klinman, J. P. *Biofactors* **1988**, *1*, 41–49.

(3) (a) De Beer, R.; Duine, J. A.; Frank, Jzn. J.; Large, P. J. *Biochem. Biophys. Acta* **1980**, *622*, 370–374. (b) MacIntire, W. S.; Stults, J. T. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 562–568.

(4) Janes, S. M.; Mu, D.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990**, *248*, 981–987.

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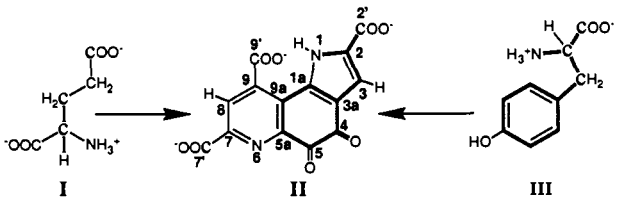
Scheme I



I II III

topa is the product of oxidation of an active-site tyrosyl residue. The *o*-quinone PQQ (II), the cofactor of methanol dehydrogenase (MDH)[1a,b,5] and glucose dehydrogenase,[6] is not covalently linked to these bacterial enzymes. Although tightly bound to MDH, PQQ can be removed by nonhydrolytic enzyme denaturation.[7] Recently, mutants of *Methylobacterium extorquens* AM1[8] and related organisms[9] were isolated that require exogenous PQQ for the expression of MDH activity. Glucose dehydrogenase is produced by *Escherichia coli* as an apoenzyme and is active only when the organism is cultured in the presence of PQQ.[10] Moreover, activity is restored to the apo form of glucose dehydrogenase in vitro by incubation with PQQ[10b,11] and the structural analogue 4,7-phenanthroline-5,6-dione.[12] Clearly, PQQ is not produced by posttranslational modification of amino acyl residues in these bacterial dehydrogenases.

While PQQ was first characterized in methylotrophic bacteria, it is now known to be widely distributed in bacteria[13] and stimulates the growth of many bacterial species.[14] Moreover, rigorous exclusion of PQQ from the diet of mice causes lathyrism, indicating that PQQ may be a nutritional requirement in mice.[15] Clearly, PQQ plays a diverse and important role in nature; however, little has been revealed about its biosynthesis. While some of the bacterial genes for the biosynthesis of PQQ have been cloned from *Acinetobacter calcoaceticus* and expressed in *E. coli*, until recently none of the biosynthetic precursors or intermediates had been identified. In a preliminary communication, we reported that glutamate (I) and tyrosine (II) are the biosynthetic precursors of PQQ;[16] this result was based on feeding experiments in which [1-^{13}C]- and [2-^{13}C]ethanol were used as growth substrates for the facultative methylotroph *M. extorquens* AM1 (Scheme I). Van Kleef and Duine provided further evidence that tyrosine and not phenylalanine is a precursor of PQQ in another Gram-negative methylotroph, *Hyphomicrobium* X.[17] In this article, we provide labeling evidence for the direct incorporation of tyrosine into PQQ where an intramolecular cyclization of the tyrosyl backbone yields the fused *o*-quinone and pyrrole rings; addition of glutamic acid completes the quinoline skeleton.

Experimental Procedures

Chemicals and Labeled Compounds. Authentic PQQ (Fluka Chemical Corp.), [$^2H_6$]DMSO (Aldrich Chemical Co.), and [$^{12}C_2$,$^2H_6$]DMSO (MSD Isotopes) were obtained from commercial sources. ^{13}C-Labeled ethanols were prepared by the rhenium-catalyzed hydrogenation of [1-^{13}C]- (92 atom %) and [2-^{13}C]acetate (98 atom %).[18a] The [1-^{13}C]- and [2-^{13}C]acetate were synthesized from [^{13}C]carbon monoxide and methanol, or carbon monoxide and [^{13}C]methanol,[18b] respectively. L-[3′,5′-$^{13}C_2$]Tyrosine was prepared from L-serine and [2,6-$^{13}C_2$]phenol by using tyrosine phenol-lyase as described by Walker and co-workers.[19a] Similarly, L-[α-^{15}N, 2′,6′-$^{13}C_2$]tyrosine was synthesized from L-[α-^{15}N]serine and [3,5-$^{13}C_2$]phenol; L-[β-^{13}C]tyrosine was synthesized from L-[3-^{13}C]serine and phenol. The ^{13}C-labeled phenols were prepared from *p*-nitrophenol via the condensation of [1,3-$^{13}C_2$]acetone with nitromalonaldehyde.[19] L-[3-^{13}C]Serine was provided by the Los Alamos National Stable Isotopes Resource; it was produced by microbial transformation of [^{13}C]CH_3OH and glycine using *M. extorquens* AM1.

Culture Conditions. *M. extorquens* AM1 (*Pseudomonas* AM1, ATCC 14718) was cultured on methanol (0.5%) or ethanol (0.5%) in a standard mineral medium,[20] with the following modifications (g/L): $(NH_4)_2SO_4$ (0.2), NH_4Cl (1.6), KH_2PO_4 (2.72), and citric acid (0.006). The organism was cultured in a stirred tank fermentor (13 L, 10% inoculum) until the methanol (or ethanol) was exhausted (24–48 h, ~4.0 OD at 560 nm). Labeled carbon sources were added to sterile culture medium as follows: [^{13}C]methanol, 60 g/16 L; [1-^{13}C]ethanol, 40 g/10 L; and [2-^{13}C]ethanol, 40 g/10 L. Labeled tyrosines ([3′,5′-$^{13}C_2$]tyrosine, [α-^{15}N, 2′,6′-$^{13}C_2$]tyrosine, [β-^{13}C]tyrosine) were filter sterilized and added (0.5 mM, 225 mg/2.5 L) to growth medium containing unlabeled methanol (4 g/L) as the carbon source. In all cases labeled precursors were added to the medium immediately prior to inoculation.

Chromatography and Isolation Procedures. The culture medium of *M. extorquens* AM1 was the source of biosynthetic PQQ in these studies. The isolation of PQQ from 1 L of liquid culture was based on published procedures[14a,21] and was carried out as follows: after centrifugation (10000*g* for 10 min), the culture broth was passed over DEAE Sephadex-G25 (10 mL) and discarded. A step gradient of NaCl in 5 mM potassium phosphate buffer (pH 7) was used to elute PQQ (20 mL each of 0.1, 0.2, 0.5, and 1.0 M NaCl). Fractions containing PQQ (λ_{max} 248 nm) were pooled and passed over a reversed-phase column (0.5 g, Baker spe C_{18}). The spent solution was adjusted to pH 2 with HCl and loaded on a second C_{18} column, which was then washed with 10 mL of 5 mM HCl. PQQ was eluted with 2 mL of 50% aqueous methanol and dried in vacuo. The yield of PQQ was typically 1 mg/L of culture broth. Amino acids were obtained from protein hydrolysates[22] and separated by ion-exchange chromatography.[23]

NMR Spectroscopy. ^{13}C and ^{1}H NMR spectra were acquired with Bruker AM-200 WB or AF-250 spectrometers. ^{13}C NMR spectra of PQQ were obtained in [$^2H_6$]DMSO at 298 K by using a 45° pulse, with 32K data points (0.3 Hz/point) and with the ^{1}H decoupler gated off for 10 s to minimize NOE effects. Biosynthetic samples of PQQ were composed of 10 mg/3.0 mL [$^2H_6$]DMSO in a 10-mm tube ([^{13}C]ethanol experiments), or 2 mg/0.4 mL [$^{12}C_2$,$^2H_6$]DMSO in a 5-mm tube (labeled tyrosine experiments). The assignments for the 14 ^{13}C NMR signals of PQQ were determined from the ^{1}H–^{13}C coupling patterns ($^1J_{C-H}$ and $^3J_{C-H}$) and carbon–carbon correlations. Carbon–carbon couplings were observed with a sample of [U-^{13}C]PQQ (90+% ^{13}C) isolated from cultures grown on [^{13}C]methanol (99.7%). Unambiguous assignments were reported previously[16] and were achieved by selecting for one-bond ^{13}C coupling interactions ($^1J_{C-H}$ = 55 Hz) in ^{13}C COSY experiments.[24] ^{13}C enrichments in PQQ derived from [^{13}C]ethanol were determined from the relative integrals of ^{13}C NMR resonances; the integrals were determined by Lorentzian line-shape analysis and normalized to the enrichment at C-8, which was determined by ^{1}H NMR analysis ($^1J_{C-H}$ = 170 Hz) to be 12% from [1-^{13}C]ethanol and 61% from [2-^{13}C]ethanol. The

(5) Frank, J., Jr.; van Krimpen, S. H.; Verwiel, P. E. J.; Jongejan, J. A.; Mulder, A. C.; Duine, J. A. *Eur. J. Biochem.* **1989**, *184*, 187–195.
(6) (a) Neiderpruem, D. J.; Doudoroff, M. *J. Bacteriol.* **1965**, *89*, 697–705. (b) Duine, J. A.; Frank, Jzn. J.; Zeeland, J. K. *FEBS Lett.* **1979**, *108*, 443–446.
(7) Duine, J. A.; Frank, J., Jr. *Biochem. J.* **1980**, *187*, 221–226.
(8) Lidstrom, M. E. *FEMS Microbiol. Rev.* **1990**, *87*, 431–436.
(9) (a) Biville, F.; Turlin, E.; Gasser, F. *J. Gen. Microbiol.* **1989**, *135*, 2917–2929. (b) Goosen, N.; Vermaas, D. A. M.; Van de Putte, P. *J. Bacteriol.* **1987**, *169*, 303–307.
(10) (a) Hommes, R. W. J.; Postma, P. W.; Neijssel, O. M.; Tempest, D. W.; Dokter, P.; Duine, J. A. *FEMS Microbiol. Lett.* **1984**, *24*, 329–333. (b) Ameyama, M.; Nonobe, M.; Shinagawa, E.; Matsushita, K.; Takimoto, K.; Adachi, O. *Agric. Biol. Chem.* **1986**, *50*, 49–57. (c) van Kleef, M. A. G.; Dokter, P.; Mulder, A. C.; Duine, J. A. *Anal. Biochem.* **1987**, *162*, 143–149.
(11) Kilty, C. G.; Maruyama, K.; Forrest, H. S. *Arch. Biochem. Biophys.* **1982**, *218*, 623–625.
(12) Conlin, M.; Forrest, H. S.; Bruice, T. C. *Biochem. Biophys. Res. Commun.* **1985**, *131*, 564–566.
(13) Duine, J. A.; Jongejan, J. A. *Annu. Rev. Biochem.* **1989**, *58*, 403–426.
(14) (a) Ameyama, M.; Shinagawa, E.; Matsushita, K.; Adachi, O. *Agric. Biol. Chem.* **1984**, *48*, 2909–2911. (b) Ameyama, M.; Shinagawa, E.; Matsushita, K.; Adachi, O. *Agric. Biol. Chem.* **1985**, *49*, 699–709. (c) Ameyama, M.; Shinagawa, E.; Matsushita, K.; Takimoto, K.; Nakashima, K.; Adachi, O. *Agric. Biol. Chem.* **1985**, *49*, 3623–3626.
(15) Killgore, J.; Smidt, C.; Duich, L.; Romero-Chapman, N.; Tinker, D.; Reiser, K.; Melko, M.; Hyde, D.; Rucker, R. B. *Science* **1989**, *245*, 850–852.
(16) (a) Houck, D. R.; Hanners, J. L.; Unkefer, C. J. *J. Am. Chem. Soc.* **1988**, *110*, 6920–6921. (b) Some of these data were reported at the First International Symposium on PQQ and Quinoproteins; Delft, The Netherlands. Houck, D. R.; Hanners, J. L.; Unkefer, C. J.; van Kleef, M. A. G.; Duine, J. A. *Antonie van Leeuwenhoek* **1989**, *56*, 93–101.
(17) van Kleef, M. A. G.; Duine, J. A. *FEBS Lett.* **1988**, *237*, 91–97.
(18) (a) Ott, D. G. *Synthesis with Stable Isotopes of Carbon, Nitrogen, and Oxygen*; J. Wiley: New York, 1981; p 29. (b) Ott, D. G. *Ibid.* p 106.
(19) (a) Walker, T. E.; Matheny, C.; Storm, C. N.; Hayden, H. *J. Org. Chem.* **1986**, *51*, 1175–1179. (b) Viswanatha, V.; Hruby, V. J. *J. Org. Chem.* **1979**, *44*, 2892–2896.
(20) Beardsmore, A. J.; Aperghis, P. N. G.; Quayle, J. R. *J. Gen. Microbiol.* **1982**, *128*, 1423–1439.
(21) Geiger, O.; Gorish, H. *Anal. Biochem.* **1987**, *164*, 418–423.
(22) Putter, I.; Barreto, A.; Markley, J. L.; Jardetzkey, O. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *64*, 1396–1403.
(23) Hirs, C. H. W.; Moore, S.; Stein, W. H. *J. Am. Chem. Soc.* **1954**, *76*, 6063–6065.
(24) (a) Bax, A.; Freeman, R. *J. Magn. Reson.* **1981**, *42*, 164–168. (b) Bax, A.; Freeman, R. *J. Magn. Reson.* **1981**, *44*, 542–561.

Table I. Labeling of Amino Acids in *M. extorquens* AM1 by [2-^{13}C]Ethanol

amino acid	^{13}C enrichments,[a] atom % ^{13}C				
	C-1	C-2	C-3	C-4	C-5
alanine	17	69	70		
aspartate	25	71	71	25	
glutamate	20	65	60	77	3

[a] ^{13}C NMR spectra were obtained with the ^{1}H decoupler gated off (60–300 s, 45 °C pulse). ^{13}C Enrichments were determined as described in Experimental Procedures and normalized to the enrichment at the α-carbons as determined by ^{1}H NMR.

^{13}C enrichments in PQQ derived from ^{13}C-labeled tyrosine were determined by analogous procedures: C-9a from ^{1}H NMR of H-8 ($^{3}J_{C-H}$ = 6 Hz), C-5 calculated relative to C-9a, C-1a from ^{1}H NMR of H-3 ($^{3}J_{C-H}$ = 7 Hz), C-4 calculated relative to C-1a, and C-3 from ^{1}H NMR of H-3 ($^{1}J_{C-H}$ = 178 Hz). NMR spectra of amino acids were acquired in D_2O at 298 K with the ^{1}H decoupler gated off (60–300 s, 45° pulse); ^{13}C enrichments, determined from relative integrals, were normalized to the enrichment at the α-carbons as determined by ^{1}H NMR. Data were obtained by using relatively long relaxation delays and were not corrected for partial T_1 saturation effects. The Lorentzian line-shape analysis was carried out on a MicroVax II using a modified Levenberg–Marquart algorithm implemented by the NMR1 software package supplied by the National Institutes of Health Resource for NMR Data Analysis (Syracuse, NY).

Results and Discussion

Evidence for Glutamate as a Biosynthetic Precursor to PQQ. Labeling with [^{13}C]Ethanol. The initial evidence that glutamate and tyrosine were the biosynthetic precursors of PQQ was obtained from feeding experiments with [1-^{13}C]- and [2-^{13}C]ethanol.[16] *M. extorquens* AM1, a facultative methylotroph, assimilates ethanol as an acetate unit.[25] The assimilatory pathway begins with a two-electron oxidation of ethanol in a reaction catalyzed by the PQQ-dependent methanol dehydrogenase, the same enzyme required for growth on methanol. It follows that PQQ biosynthesis is requisite for growth on ethanol. In fact, we observed that during growth on ethanol, *M. extorquens* AM1 excretes PQQ, up to 1.5 mg/L, into the liquid culture medium. Therefore, the first experimental approach to this biosynthesis problem was to grow *M. extorquens* AM1 on ^{13}C-labeled ethanol, isolate the excreted PQQ, and use ^{13}C NMR to determine the ^{13}C enrichments at each carbon in PQQ; the labeling patterns were then interpreted in terms of the operative pathways for constructing carbon skeletons from [1-^{13}C]- and [2-^{13}C]acetate. As an internal control, the ^{13}C-labeling patterns in amino acids, obtained from protein hydrolysates, were rigorously analyzed by NMR and compared to the labeling of PQQ in both experiments. The ^{13}C enrichments in PQQ and amino acids from the feeding experiments with [1-^{13}C]ethanol were published in a preliminary report.[16] A detailed interpretation of the data from [2-^{13}C]ethanol presented here reveals that PQQ is constructed partially from glutamate.

First of all, it is necessary to review the pathway of ethanol assimilation in *M. extorquens* AM1.[25] As mentioned above, this facultative methylotroph assimilates ethanol via acetate. Thus, acetyl-CoA serves as the primary substrate for the construction of carbon skeletons; four-carbon compounds are assembled by malate synthase activity, and three-carbon compounds are derived from decarboxylation of oxalacetate. Therefore, compounds derived from malate (e.g., aspartate) will be labeled at C-2 and C-3 by [2-^{13}C]ethanol as would three-carbon compounds such as pyruvate and alanine. These predicted labeling patterns were confirmed by determining the ^{13}C enrichments in the amino acids biosynthesized from [2-^{13}C]ethanol (Table I). These amino acids were isolated from protein hydrolysates of cells harvested at the end of log phase; therefore, this is essentially an end-point analysis. The results demonstrate that aspartate was essentially derived (via malate) from a "tail to tail" joining of two acetate units; alanine

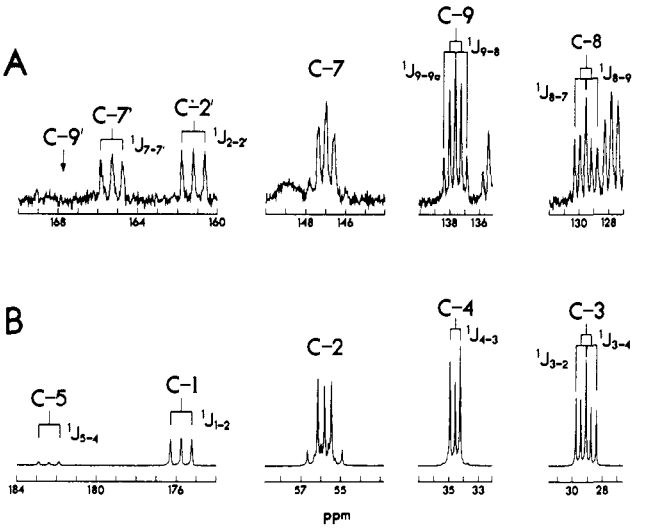


Figure 1. Portions of the ^{13}C NMR spectra of PQQ (A) and glutamate (B) that were derived biosynthetically from [2-^{13}C]ethanol. Spectra were obtained by using the parameters described in Experimental Procedures. See Table II for a quantitative summary of the labeling patterns.

Table II. ^{13}C–^{13}C Coupling in L-Glutamate and PQQ Derived from [2-^{13}C]Ethanol

glutamate				PQQ			
obsd C	^{13}C, atom %	couplg partner	% couplg	obsd C	^{13}C, atom %	couplg partner	% couplg
1	20	2	65	7′	24	7	62
2	65	1	21	7	64	7′	15
2		3	68	7		8	61
3	60	2	66	8	61	7	66
3		4	100	8		9	100
4	77	3	75	9	76	8	80
4		5	<2	9		9′	ND[a]
5	3	4	71	9′	ND[a]	9	ND[a]

[a] ND, C-9 in PQQ was not enriched with ^{13}C above natural abundance; a resonance for C-9 was not detected above the noise in the ^{13}C NMR spectrum (Figure 1a).

was produced (via pyruvate) by β-decarboxylation of oxalacetate. With the exception of glutamate C-5, all the amino acids exhibited some background scrambling (17–25%) of label from C-2 of ethanol into positions ostensibly derived from C-1 of ethanol. Glutamate is a special case because acetate units are incorporated directly into glutamate C-4 and C-5 via the first few reactions of the tricarboxylic acid cycle and are not subjected to the scrambling observed in all other positions. Therefore, the labeling probabilities of glutamate C-4 and C-5 are strongly correlated with the labeling probabilities in the precursor ethanol. This phenomenon is demonstrated in the ^{13}C NMR spectrum of glutamate (Figure 1) derived from [2-^{13}C]ethanol (90% ^{13}C at C-2, 1.1% ^{13}C at C-1). The highest level of ^{13}C enrichment, 77-fold over natural abundance, is found in glutamate C-4; its neighbor, C-5 derived from the same acetate unit, carries the lowest enrichment of ^{13}C (3-fold natural abundance) of any carbon in the amino acids tested. The labeling pattern of glutamate C-1 and C-2 is characteristic of the α-amino acids; [2-^{13}C]ethanol labels C-2 more than 3 times as much as C-1. The opposite labeling pattern was observed in glutamate biosynthesized from [1-^{13}C]-ethanol.[16]

Analysis of the ^{13}C NMR spectrum of PQQ (Figure 1A) biosynthesized from [2-^{13}C]ethanol reveals that the labeling pattern of the five-carbon unit C-7′, C-7, C-8, C-9, and C-9′ is essentially identical with the labeling of glutamate produced in the same feeding experiment (Figure 1B). The C-9′ carboxylate of PQQ did not yield an observable resonance in this experiment and is assumed to contain essentially no ^{13}C above natural abundance. Carbon-9 contained the greatest amount of ^{13}C label in PQQ, 76 atom %. Thus the labeling probabilities of PQQ C-9

(25) (a) Dunstan, P. M.; Anthony, C.; Drabble, W. T. *Biochem. J.* **1972**, *128*, 99–106. (b) Dunstan, P. M.; Anthony, C.; Drabble, W. T. *Biochem. J.* **1972**, *128*, 107–115.

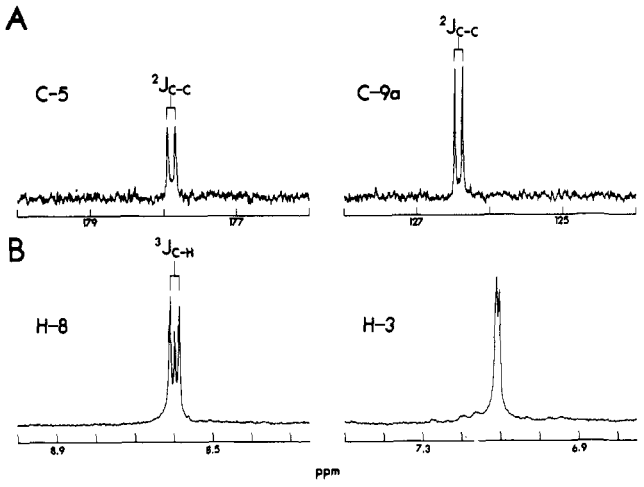


Figure 2. Portions of the ^{13}C (A) and the ^1H NMR (B) spectra of PQQ isolated from AM1 cultures that contained L-[3′,5′-$^{13}C_2$]tyrosine. C-5 and C-9a are *J*-coupled and appear as doublets ($^2J_{C-C}$ = 6.71 Hz) in the ^{13}C NMR spectrum (A). No other resonances were observed in the ^{13}C spectrum. H-3 is a doublet as a result of coupling to the pyrrole NH proton (B). H-8 is a complex multiplet as a result of coupling to the ^{13}C-enriched C-9a; the center line arises from the molecules with ^{12}C at C-9a; the outer doublet is from molecules that contain ^{13}C at C-9a.

and C-9′ are highly correlated, as was observed for glutamate C-4 and C-5. The absolute ^{13}C enrichments in C-7′, C-7, and C-8 matched the enrichments of glutamate C-1, C-2, and C-3, respectively. A detailed comparison of the ^{13}C–^{13}C coupling patterns in glutamate and PQQ provides more convincing evidence that C-7′ through C-9′ of PQQ are derived from the five carbons of glutamate (Table II). PQQ C-9 is 80% coupled to C-8, and glutamate C-4 is 75% coupled to C-3. Carbon 8 of PQQ is 100% coupled to C-9 and 66% coupled to C-7; these are the identical numbers for the coupling of glutamate C-3 with C-4 and C-2, respectively. The other ^{13}C–^{13}C coupling patterns in these two five-carbon units are also essentially identical. These data leave little doubt that the glutamate carbon skeleton is biosynthesized by the same pathway as C-7′ through C-9′ of PQQ. Thus far, efforts to label PQQ directly with glutamate have failed. We observed that glutamate added to *M. extorquens* AM1 cultures was removed rapidly from the medium. We suspect that, as is the case with succinate,[26] glutamate added to the medium induces the expression of a functional TCA cycle in *M. extorquens* AM1, and labeled glutamate is rapidly oxidized.

Direct Evidence for Tyrosine as a Biosynthetic Precursor to PQQ. Labeling with [3′5′-$^{13}C_2$]Tyrosine. Experiments with ^{13}C-labeled ethanols reported previously[16] indicated that either tyrosine or phenylalanine was a precursor to the six-membered ring containing the *o*-quinone and to the attached pyrrole-2-carboxylate moiety. This transformation would entail an intramolecular cyclization of the amino acid backbone with C-2 of the phenyl (of phenol) group. Because such a reaction is known to occur in the biosynthesis of melanin,[27] tyrosine seemed to be the most likely candidate as a direct precursor to PQQ. This hypothesis was tested by feeding ^{13}C-labeled tyrosine to *M. extorquens* AM1 and analyzing the resulting labeling pattern in PQQ. Details of the feeding experiments with tyrosine are given under the Experimental Procedures; in general the organism was grown in the presence of 0.5 mM ^{13}C-labeled tyrosine. PQQ isolated from cultures that contained L-[3′,5′-$^{13}C_2$]tyrosine was labeled only at C-5 and C-9a (Figure 2A). Furthermore, the ^{13}C resonances for C-5 and C-9a are doublets (Figure 2A) as a result of geminal ^{13}C–^{13}C coupling, demonstrating that the phenol group of tyrosine

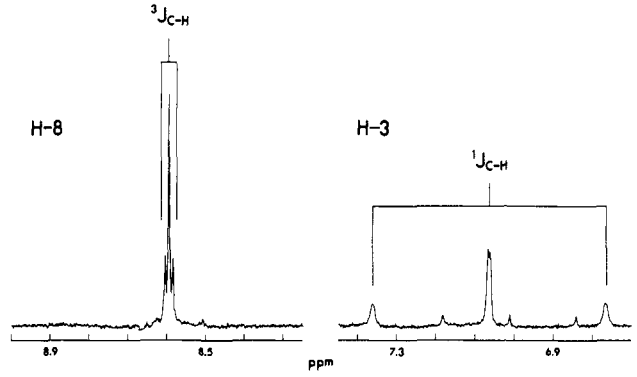


Figure 3. Portions of the ^1H NMR spectrum of PQQ isolated from cultures of AM1 that contained a 50:50 mixture of [3′,5′-$^{13}C_2$]- and [β-^{13}C]tyrosine. The ^{13}C satellites on H-8 are a result of three-bond coupling to C-9a ($^1J_{C-H}$ = 6.1 Hz). Direct coupling of C-3 to H-3 yields ^{13}C satellites split by 178.9 Hz.

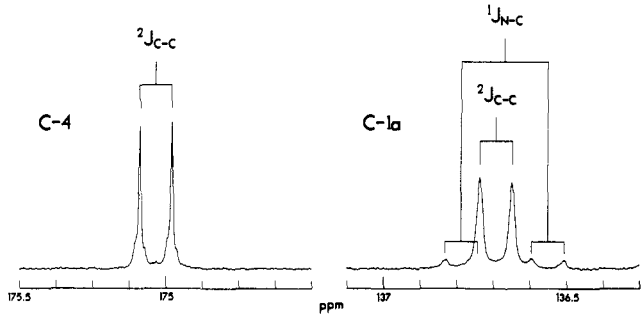


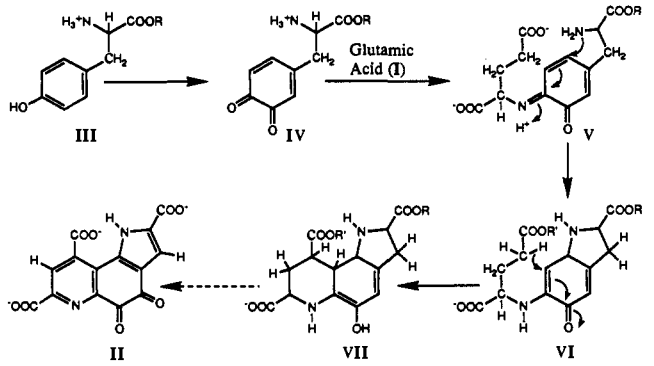
Figure 4. Portions of the ^{13}C NMR spectrum of PQQ isolated from cultures of AM1 that contained [2′,6′-$^{13}C_2$, α-^{15}N]tyrosine. As a result of their geminal coupling, C-4 and C-1a are doublets ($^2J_{C-C}$ = 5.5 Hz). The pyrrole nitrogen is enriched to 15.7% with ^{15}N, which is directly coupled to C-1a ($^1J_{N-C}$ = 5.6 Hz). Substitution of ^{15}N for ^{14}N in the pyrrole nitrogen results in a 0.025 ppm upfield shift of the C-1a ^{13}C resonance. No other resonances were detected in the spectrum.

is incorporated intact into the *o*-quinone-containing ring of PQQ. Because C-9a is vicinally coupled to H-8 ($^3J_{C-H}$ = 6 Hz; Figure 2B), ^1H NMR provided quantitative data on the ^{13}C enrichment: C-9a contained 83 atom % ^{13}C.

Labeling with [3′,5′-$^{13}C_2$] and [β-^{13}C]Tyrosine. The results outlined above demonstrate that the phenol ring of tyrosine provides the six carbons of the *o*-quinone-containing ring. To examine the possibility that internal cyclization of the tyrosyl backbone forms the pyrrole-2-carboxylic acid moiety, PQQ was labeled with an equimolar mixture of L-[3′,5′-$^{13}C_2$]- and L-[β-^{13}C]tyrosine; intact incorporation of tyrosine would yield PQQ labeled equivalently at C-3, C-5, and C-9a. On the other hand, indirect incorporation of tyrosine, involving an intermediate step such as β-elimination of phenol (i.e., phenol ammonia lyase), would dilute the label at C-3 relative to that at C-9a and C-5. PQQ isolated from cultures fed L-[3′,5′-$^{13}C_2$]tyrosine and L-[β-^{13}C]-tyrosine contained only three resonances corresponding to C-5 (179.2 ppm), C-9a (126.1 ppm), and C-3 (113.8 ppm). ^{13}C enrichments at C-9a ($^3J_{C-H}$) and C-3 ($^1J_{C-H}$) were determined from the fractional intensity of the ^{13}C satellites observed in the ^1H NMR spectrum (Figure 3). The labeling of C-9a (41%) and C-3 (42%) was identical, demonstrating that the carbon skeleton of tyrosine is incorporated as an intact unit. These data are consistent with a biosynthetic pathway in which the tyrosyl backbone is cyclized to form the pyrrole-2-carboxylic acid moiety in PQQ; the reactions being similar to those that occur in melanin biosynthesis.[27] In addition, these data rule out more complicated biosynthetic routes to the pyrrole ring such as that used in lincomycin biosynthesis to produce the pyrrolidine ring from tyrosine.[28]

(26) Taylor, I. J.; Anthony, C. *J. Gen. Microbiol.* **1976**, *93*, 99–106.
(27) (a) Raper, H. S. *Physiol. Rev.* **1928**, *8*, 245–282. (b) Mason, H. S. *J. Biol. Chem.* **1948**, *172*, 83–99. (c) Lerner, A. B.; Fitzpatrick, T. B.; Calkins, E.; Summerson, W. H. *J. Biol. Chem.* **1949**, *178*, 185–195. (d) Canovas, F. G.; Garcia-Carmona, F.; Sanchez, J. V.; Pastor, J. L. I.; Teruel, J. A. L. *J. Biol. Chem.* **1982**, *257*, 8738–8744.
(28) Brahme, N. M.; Gonzales, J. E.; Rolls, J. P.; Hessler, E. J.; Mizsak, S.; Hurley, L. H. *J. Am. Chem. Soc.* **1984**, *106*, 7873–7878.

Scheme II



Labeling with [α-^{15}N,2′,6′-$^{13}C_2$]Tyrosine. To determine if the pyrrole nitrogen was derived from the α-amino group of tyrosine, L-[α-^{15}N,2′,6′-$^{13}C_2$]tyrosine was fed to *M. extorquens* AM1 cultures. Intramolecular cyclization of this compound would yield PQQ with a ^{15}N (N-1) directly bonded to ^{13}C (C-1a) and would result in a direct ^{13}C–^{15}N spin–spin coupling network. The spectrum of PQQ isolated from this culture was labeled with ^{13}C at C-4 and C-1a; the ^{13}C enrichment, estimated from the ^{1}H NMR of signal of H-3, is 50.7%. From integration of the ^{13}C NMR signal of C-1a (Figure 4), it is estimated that 15.7% of the molecules labeled with ^{13}C also contain ^{15}N ($^1J_{C\text{-}1a\text{-}N}$ = 14.8 Hz). The overall ^{15}N enrichment was determined to be 10.6% from integration of ^{1}H NMR signal of the N–H resonance, which is directly coupled to N-1 ($^1J_{N\text{-}H}$ = 97 Hz). As expected, there is significant dilution of the ^{15}N label due to transamination; however, the incorporation of ^{15}N into PQQ is 30-fold greater than that expected if the tyrosine nitrogen equilibrated with the free ammonium pool. In addition, there is a strong correlation between ^{13}C and ^{15}N labeling. That is, of the PQQ molecules labeled with ^{15}N in the pyrrole nitrogen, a greater fraction contains ^{13}C at C-1a (75%) than contains ^{12}C at C-1a (25%). Clearly the α-nitrogen of tyrosine is not randomized in PQQ, demonstrating that tyrosine, and not its requisite α-keto acid, is the precursor for PQQ biosynthesis.

Conclusion

The data presented here demonstrate that PQQ is biosynthesized from the amino acids tyrosine and glutamate. In contrast to the biosynthetic pathways leading to other cofactors such as riboflavin or folic acid, the biochemical transformation that leads to PQQ is remarkably efficient in the sense that all the carbons and probably both nitrogens of the precursors are conserved in the product. Because the process involves the loss of 12 electrons in the conversion of tyrosine and glutamate to product, PQQ biosynthesis involves primarily oxidative reactions. Oxidation of the phenol side chain must be an early step in the pathway because it is requisite for the formation of the pyrrole ring by cyclization of the tyrosine backbone. Given our data, we have outlined a possible route for PQQ biosynthesis (Scheme II). In this route, tyrosine (III) or some derivative of tyrosine is oxidized to dopaquinone (IV) in a reaction catalyzed by a monophenol monooxygenase like enzyme (tyrosinase, EC 1.14.18.1). Glutamate (I) could form a Schiff base with dopaquinone. The cyclization of the tyrosine backbone to form the pyrrole ring could occur by a Michael-type addition analogous to the known nonenzymatic cyclization of dopaquinone to form dopachrome.[27] Alternatively, the biosynthesis of PQQ may involve dopachrome as an intermediate to which glutamate is added in a subsequent reaction. To date, we have been unable to detect tyrosinase activity in crude extracts of *M. extorquens* AM1. In addition, no sequence homology exists between tyrosinase genes from *Streptomyces glaucescens*[29] or *Neurospora crassa*[30] and the PQQ biosynthesis genes examined to date from *Acinetobacter calcoaceticus*.[31] The absence of tyrosinase activity may indicate that a derivatization of tyrosine precedes oxidation of its phenol side chain. Alternatively, the enzymatic oxidation of tyrosine may donate electrons to an intermediate redox cofactor rather than directly to O_2.

Acknowledgment. This work was supported by the DOE, OBES, Division of Biological Energy Research and by the National Stable Isotopes Resource NIH (RR 02231).

(29) Huber, M.; Hintermann, G.; Lerch, K. *Biochemistry* **1985**, *24*, 6038–6044.
(30) Lerch, K. *J. Biol. Chem.* **1982**, *257*, 6414–6419.
(31) Goosen, N.; Horsman, H. P. A.; Huinen, R. G. M.; Van de Putte, P. *J. Bacteriol.* **1989**, *171*, 447–455.

How Can the Solvent Affect Enzyme Enantioselectivity?

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Abstract: Enantioselectivity of the protease subtilisin Carlsberg in the transesterification between the chiral alcohol *sec*-phenethyl alcohol and vinyl butyrate was found to be greatly affected by the solvent. For example, the $(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$ ratio varies from 3 in anhydrous acetonitrile to 61 in anhydrous dioxane. A mechanistic model is proposed that explains these findings. This model is supported by the experimental data obtained concerning the dependence of subtilisin's enantioselectivity on the structure of the chiral alcohol, on physicochemical characteristics of the solvent (systematic correlations were found with the dielectric constant and the dipole moment), and on such additives as water and the water mimic formamide. Similar dependencies (although of a smaller magnitude) were observed for the related enzyme subtilisin BPN′.

To organic chemists, enantioselectivity is the most valuable feature of enzymes.[1] A major obstacle to a wider synthetic exploitation of enzyme enantioselectivity is its relative inflexibility: under each set of conditions, the stereochemical outcome of a given enzyme–substrate reaction is predetermined. If one wishes to alter it, two options are available—to change the reactants or to change the reaction conditions. The former, although frequently used, requires either an (time-consuming and empirical) enzyme screening or a (otherwise unnecessary and often undesirable) deviation from the initial substrate structure.[1] Thus, in principle, the latter option is preferable; there are a few recent illustrations of it, e.g., the temperature dependence of enantioselectivity of some

(1) Simon, H.; Bader, J.; Gunther, H.; Neumann, S.; Thanos, J. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 539–553. Whitesides, G. M.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 617–638. Jones, J. B. *Tetrahedron* **1986**, *42*, 3351–3403. Yamada, H.; Shimizu, S. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 622–624. Crout, D. H. G.; Christen, M. In *Modern Synthetic Methods 1989*; Scheffold, R., Ed.; Springer Verlag: Berlin, 1989; pp 1–114.

0002-7863/91/1513-3166$02.50/0