Warrington, U.K., 1979).
24. B. C. Wang, *Methods Enzymol.* **115**, 90 (1985).
25. M. A. Rould, J. J. Perona, D. Söll, T. A. Steitz, *Science* **246**, 1135 (1989).
26. T. A. Jones, *J. Appl. Cryst.* **11**, 268 (1978).
27. A. T. Brünger, J. Kuriyan, M. Karplus, *Science* **235**, 458 (1987).
28. A. T. Brünger, *X-PLOR v2.1 Manual* (Yale Univ. Press, New Haven, CT, 1990).
29. D. E. Tonrud, L. F. Ten Eyck, B. W. Matthews, *Acta Crystallogr.* **A43**, 489 (1987).
30. D. R. Smith, I. J. Jackson, D. D. Brown, *Cell* **37**, 645 (1984).
31. K. E. Vrana, M. E. A. Churchill, T. D. Tullius, D. D. Brown, *Mol. Cell. Biol.* **8**, 1684 (1988).
32. D. C. Rees, M. Lewis, W. N. Lipscomb, *J. Mol. Biol.* **168**, 367 (1983).
33. D. W. Christianson and R. S. Alexander, *J. Am. Chem. Soc.* **111**, 6412 (1989).
34. R. Schuh *et al.*, *Cell* **47**, 1025 (1986).
35. J. C. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 200 (1979).
36. J. M. Berg, *ibid.* **85**, 99 (1988).
37. ———, *J. Biol. Chem.* **265**, 6513 (1990).
38. L. Fairall, D. Rhodes, A. Klug, *J. Mol. Biol.* **192**, 577 (1986).
39. S. R. Jordan and C. O. Pabo, *Science* **242**, 893 (1988).
40. A. K. Aggarwal, D. W. Rodgers, M. Drottar, M. Ptashne, S. C. Harrison, *ibid.* p. 899.
41. Z. Otwinowski *et al.*, *Nature* **335**, 321 (1988).
42. C. R. Kissinger, B. Liu, E. Martin-Blanco, T. B. Kornberg, C. O. Pabo, *Cell* **63**, 579 (1990).
43. K. T. O'Neil, R. H. Hoess, W. F. DeGrado, *ibid.* **249**, 774 (1990).
44. J. B. Rafferty, W. S. Somers, I. Saint-Girons, S. E. V. Phillips, *Nature* **341**, 705 (1989).
45. N. C. Seeman, J. M. Rosenberg, A. Rich, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 804 (1976).
46. Supported by NIH grant GM-31471 (C.O.P.) and by the Howard Hughes Medical Institute. We thank B. Christy and D. Nathans for providing us with the cDNA clone of *zif268* and the sequence of the Zif268 binding site; P. Wright for providing us with the coordinates of the NMR structure of Xfin31; E. M. Westbrook and M. L. Westbrook for the use of the Midwest Area Detector User Facility; A. Collector and C. Wendling for synthesizing the DNA; U. Obeysekare for writing some of the graphics subroutines; the members of the Pabo laboratory, especially C. R. Kissinger, for help with the structure determination; and the National Cancer Institute for use of the Cray during molecular dynamics refinement. Coordinates are being deposited with the Brookhaven Data Bank. While these are being processed by Brookhaven and prepared for distribution, interested scientists may obtain a set of coordinates either by sending an appropriate BITNET address to us at PABO@JHUIGF or by sending a 1/2-inch tape with a mailing envelope and sufficient return postage.

13 February 1991; accepted 19 March 1991

A New Cofactor in a Prokaryotic Enzyme: Tryptophan Tryptophylquinone as the Redox Prosthetic Group in Methylamine Dehydrogenase

WILLIAM S. MCINTIRE,* DAVID E. WEMMER, ANDREI CHISTOSERDOV, MARY E. LIDSTROM

Methylamine dehydrogenase (MADH), an $\alpha_2\beta_2$ enzyme from numerous methylotrophic soil bacteria, contains a novel quinonoid redox prosthetic group that is covalently bound to its small β subunit through two amino acyl residues. A comparison of the amino acid sequence deduced from the gene sequence of the small subunit for the enzyme from *Methylobacterium extorquens* AM1 with the published amino acid sequence obtained by the Edman degradation method, allowed the identification of the amino acyl constituents of the cofactor as two tryptophyl residues. This information was crucial for interpreting ^1H and ^{13}C nuclear magnetic reasonance, and mass spectral data collected for the semicarbazide- and carboxymethyl-derivatized bis(tripeptidyl)-cofactor of MADH from bacterium W3A1. The cofactor is composed of two cross-linked tryptophyl residues. Although there are many possible isomers, only one is consistent with all the data: The first tryptophyl residue in the peptide sequence exists as an indole-6,7-dione, and is attached at its 4 position to the 2 position of the second, otherwise unmodified, indole side group. Contrary to earlier reports, the cofactor of MADH is not 2,7,9-tricarboxypyrroloquinoline quinone (PQQ), a derivative thereof, or pro-PQQ. This appears to be the only example of two cross-linked, modified amino acyl residues having a functional role in the active site of an enzyme, in the absence of other cofactors or metal ions.

SINCE THE ELUCIDATION OF THE STRUCTURE OF THE REDOX cofactor of methanol dehydrogenase from *Pseudomonas* TP1 as 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]-quinoline-4,5-dione (Fig. 1, **1**) (*1*), this quinone has been shown to be the noncovalently bound redox cofactor of several other bacterial enzymes (*2*). This prosthetic group was originally given the common name methoxatin, but, the more descriptive name pyrroloquinoline quinone (PQQ) has come into favor. More properly, this form should be called 2,7,9-tricarboxy-PQQ to distinguish it from other derivatives that may exist in nature.

A number of enzymes have been proposed to contain covalently bound PQQ or a PQQ derivative. In this group are the copper-containing amine oxidases: plasma amine oxidase, kidney, and placental diamine oxidase, lysyl oxidase, plant diamine oxidase, fungal amine oxidase, and methylamine oxidase from the soil

W. S. McIntire is in the Molecular Biology Division, Department of Veterans Affairs Medical Center, San Francisco, CA 94121, and in the Department of Biochemistry and Biophysics and the Department of Anesthesia, University of California, San Francisco, CA 94143. D. E. Wemmer is in the Department of Chemistry, University of California, Berkeley, CA 94720. A. Chistoserdov and M. E. Lidstrom are with the W. M. Keck Laboratories, California Institute of Technology, Pasadena, CA 91125.

*To whom correspondence should be addressed.

bacterium *Arthrobacter* P1 (*2, 3*). The design of PQQ, an orthoquinone, is appropriate for catalyzing the oxidation of alkylamines (*4*), and several research groups have offered circumstantial evidence that stable derivatives of 2,7,9-tricarboxy-PQQ could be released from several of these oxidases (*5–9*). However, Janes *et al.* recentlyreported that the covalently bound redox prosthetic group of bovine plasma amine oxidase is not PQQ, or a derivative thereof, but is unambiguously 6-hydroxydopa quinone (Fig. 1, **2**), otherwise known as topa quinone (*10*). It is likely that all of the copper-containing amine oxidases also have topa quinone as their catalytic prosthetic group.

Galactose oxidase from *Dactylium dendroides* is another enzyme reputed to contain covalently bound PQQ (*2*). Recently, Knowles and colleagues have obtained irrefutable evidence showing that the organic cofactor is not a PQQ derivative (*11*). In fact, it is not a quinone at all, but a cofactor constructed by the cross-linking of a Cys and a Tyr residue (Fig. 1, **3**) at the active site of galactose oxidase.

Other enzymes, including dopamine β-hydroxylase, tryptophan decarboxylase, DOPA decarboxylase, soy bean lipoxygenase-1, methylamine dehydrogenase (*2*), fungal laccase (*12*), and several amino acid decarboxylases (*13*), have been said to contain covalently bound PQQ or a PQQ derivative, on the basis of similar methods to those used for the amine oxidases and galactose oxidase. In light of the new findings for bovine plasma amine oxidase and galactose oxidase, the nature of the covalently bound cofactor must be reevaluated in these enzymes. In fact, the claims that bovine medulla dopamine β-hydroxylase and soy bean lipoxygenase-1 contain any organic cofactor, let alone PQQ, have already been refuted (*14–16*).

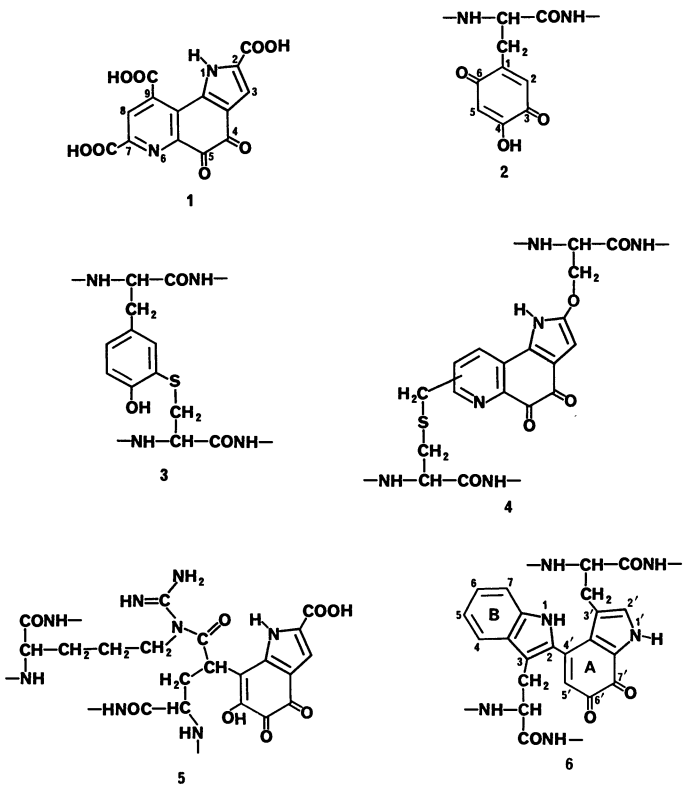


Fig. 1. Structures of: 2,7,9-tricarboxy-PQQ, **1**; 6-hydroxydopaquinone or topa quinone of bovine plasma amine oxidase, **2** (*10*); the galactose oxidase cofactor (composed of Cys^{228} and Tyr^{272} in the polypeptide), **3** (*11*); the MADH cofactor previously proposed **4** (*24*); pro-PQQ (shown with Glu^{57} attached to the quinonoid cofactor, which is cross-linked to Arg^{107} in *Thiobacillus versutus* MADH), **5** (*23*); and the MADH cofactor proposed herein, **6**.

Methylamine dehydrogenase (MADH, E.C. 1.4.99.3) is one of the enzymes in which the cofactor structure must be reexamined. This enzyme has been isolated from several methylotrophic bacteria including bacterium W3A1 [a member of the taxon *Methylophilus*, which is an unrecognized genus (*17*)], *Methylophilus methylotrophus*, *Paracoccus denitrificans*, *Methylobacterium extorquens* AM1 (formerly *Pseudomonas* sp., strain AM1 and *Methylobacterium* sp., strain AM1), *Methylomonas* sp., strain J (formerly *Pseudomonas* sp., strain J), and *Thiobacillus versutus* (formerly *Thiobacillus* A2). All these dehydrogenases have several factors in common, for example, basic redox properties, and basic ultraviolet (UV) visible and resonance Raman spectra (*18–22*). They all almost certainly contain the same covalently bound redox cofactor. All have $\alpha_2\beta_2$ structures with the cofactor covalently attached in each small β subunit at two widely separated sites on the polypeptide chain (*22–24*). The sequences around these sites have been determined for the polypeptide from *M. extorquens* AM1 (*22, 25*), bacterium W3A1 (*24*), and *T. versutus* (*23*) (Fig. 2). In each case, the two amino acyl residues that bind the cofactor were not identified.

Periplasmic MADH converts methylamine to ammonia and formaldehyde. The electrons captured by the enzyme in this process are transferred to the copper protein amicyanin, although c-type cytochromes have also been implicated in this role (*26*). Although early spectral evidence implicated PQQ as the prosthetic group of MADH (*9, 27*), more recent studies cast doubt on this interpretation.

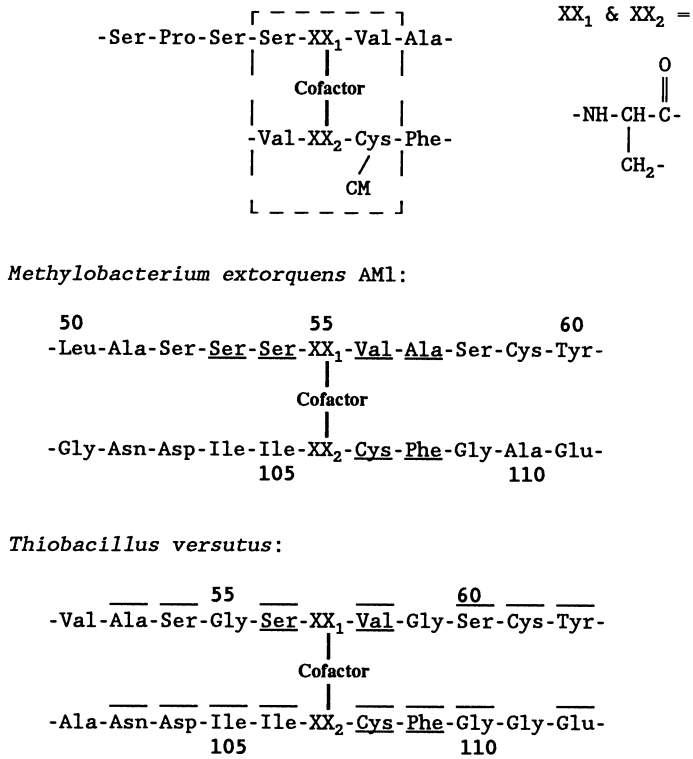


Fig. 2. The complete amino acid sequences shown for bacterium W3A1 bispeptidyl-cofactor represents the material isolated from a pronase digest of the semicarbazide-derivatized, carboxymethylated (CM) cofactor-containing subunit (*24*). XX_1 and XX_2 represent the amino acyl portions of the cofactor. The boxed portion represents the remaining bis(tripeptidyl)-cofactor after sequential treatment of this fragment with aminopeptidase-M and carboxypeptidase-Y. Sequences are shown for the analogous regions of MADH from *M. extorquens* AM1 (*22, 25*) and *T. versutus* (*23*). Underlining indicates identical residues when compared to the W3A1 sequence, and overlining indicates identical residues in the *M. extorquens* AM1 and *T. versutus* sequences.

In 1986, McIntire and Stults proposed that the cofactor of MADH from bacterium W3A1 is not 2,7,9-tricarboxy-PQQ, but a decarboxylated form of PQQ bound to the polypeptide through a Ser oxygen ether and a Cys sulfur ether (Fig. 1, **4**) (*24*). Mass spectral data, together with the other evidence available, suggested a PQQ-like compound.

Recently, Duine and co-workers reported that PQQ derivatives could be released from MADH of *T. versutus* (*7, 8*). However, the methods used in these investigations were those that erroneously indicated 2,7,9-tricarboxy-PQQ as cofactor for the copper-containing amine oxidases, galactose oxidase, soy bean lipoxygenase-1, and dopamine β-hydroxylase.

The 2.25 Å structure of the *T. versutus* MADH has been published, and the pro-PQQ structure (Fig. 1, **5**) was proposed. It was concluded that during isolation of the cofactor, treatment with Pronase or acid or both cleaves the putative Glu^{57} amido bond to the Arg^{107} guanidino group of pro-PQQ. The Glu^{57} peptide bonds are also cleaved in the process. This allows the α-amino group of Glu^{57} to attack the quinonoid portion of the indole ring for ring closure, and would result in the formation of 2,7,9-tricarboxy-PQQ-5-phenylhydrazone free of bound amino acyl groups. Curiously, weak electron density was observed in the region of the x-ray map that should contain the 2-carboxy group of pro-PQQ (*23*).

A PQQ derivative or pro-PQQ as the covalently bound cofactor of MADH is inconsistent with other data for MADH from bacterium W3A1 (*24*) and *M. extorquens* AM1 (*28*): (i) An extremely stable semicarbazide-derivatized bispeptidyl-cofactor was isolated in 47 percent yield from the bacterium W3A1 enzyme with the use of methods similar to those of Duine's group. If the putative guanidino-amido linkage between Glu^{57} and Arg^{107} of the *T. versutus* enzyme had remained, it should have been very labile, and produced an amino acid upon acid hydrolysis that would not have been detected during peptide sequencing. This was not the case. (ii) A pro-PQQ structure for the semicarbazide-derivatized bispeptidyl-cofactor of bacterium W3A1, would have a mass of 1012, while the measured mass is 939. (iii) The fast atom bombardment (FAB) mass spectral (MS) data for the W3A1 semicarbazide-derivatized bispeptidyl-cofactor show that the prosthetic group does not have aromatic carboxyl groups either as –COOH, –COOR–, or –CONHR–, which is inconsistent with either PQQ or pro-PQQ. (iv) In *M. extorquens* AM1, 83 mutants that require PQQ for growth on methanol have been shown to fall into seven genetic complementation classes. None of the *pqq* genes are required for growth of *M. extorquens* AM1 on methylamine, which rules out a common pathway for generation of the cofactor or a common precursor. These data suggest that the two cofactors do not share biosynthetic steps.

Several new studies have shown that the covalently bound cofactor of MADH is not PQQ, pro-PQQ, a derivative of these, or topa quinone: (i) resonance Raman spectral studies of various phenylhydrazine derivatives of MADH, its small subunit, and the bispeptidyl-cofactor (*29*); (ii) ^1H and ^{13}C nuclear magnetic resonance (NMR) studies; (iii) the DNA sequence-deduced amino acid sequence of the small subunit of MADH from *M. extorquens* AM1 (*25*). We propose that the MADH cofactor is a derivative of two cross-linked Trp residues (Fig. 1, **6**). Support for this hypothesis is outlined below.

Identification of the amino acyl constituents of the covalently bound cofactor of MADH. Identification of the amino acyl residues that bind the cofactor in MADH was crucial for deciphering the cofactor structure. This information was provided by DNA sequencing of the cloned gene from *M. extorquens* AM1 (*25*). Two oligonucleotide probes were synthesized on the basis of the known amino acid sequence of the small, cofactor-containing subunit of MADH from this organism (*22*). One probe was for the NH_2-terminal portion, and the other was for a COOH-terminal segment of the polypeptide. These probes were used in hybridizations with genomic clone banks containing *M. extorquens* AM1 DNA, and several clones were isolated. These clones contained a common 5.2-kb Bam HI–Hind III fragment that hybridized to both probes. A portion of this fragment was sequenced, and the open reading

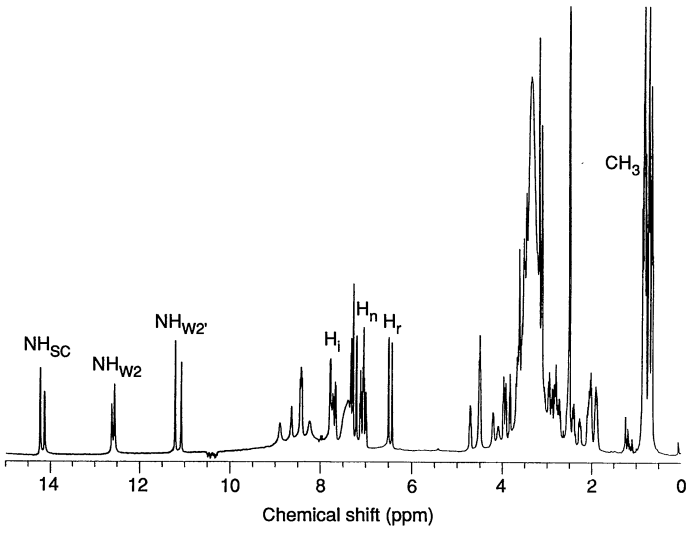


Fig. 3. The ^1H NMR spectrum of the semicarbazide- and carboxymethyl-derivatized bis(tripeptidyl)-cofactor (depicted in the boxed part of Fig. 2). The spectrum was taken in DMSO-d_6 solution at 20°C at 500 MHz. Peaks of protons from the semicarbazone portion (NH_{SC}), the two Trp indole nitrogen-linked protons (NH_{W2} and $NH_{W2'}$), aromatic protons from the cofactor (H_i, H_n, and H_r; see Fig. 6), and the Val methyl groups (CH_3) are labeled.

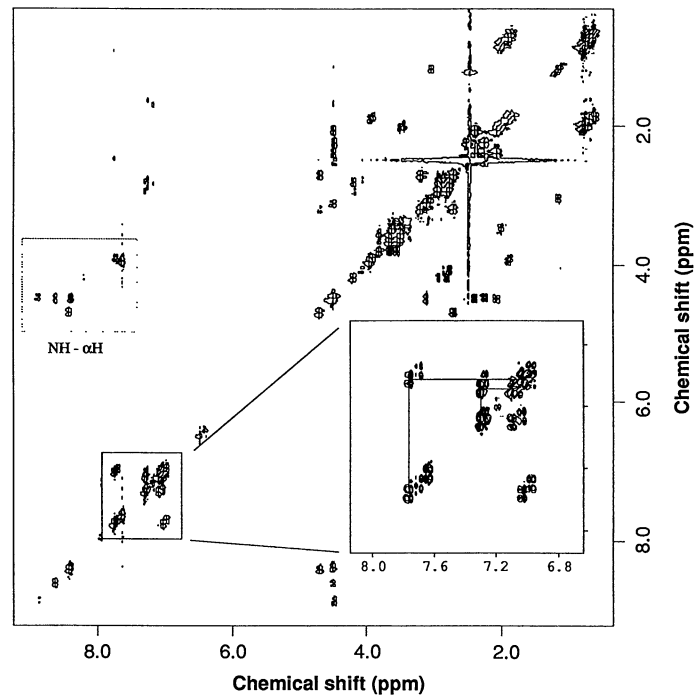


Fig. 4. The ^1H DQF COSY spectrum of the bis(tripeptidyl)-cofactor in DMSO-d_6. The dashed box indicates amide to alpha proton cross peaks from the peptide portion of the molecule that were used with data in Fig. 5 to obtain assignments. The expanded region shows aromatic-aromatic connectivities for the two conformers (dashed lines for one and solid lines for the other conformer).

frame provided an amino acid sequence identical to the sequence determined for the small MADH subunit by chemical sequencing (*22*) with one exception, residue 17 was Asp rather than Asn. The two residues designated XX_1 and XX_2 in Fig. 2 were Trp (*25*). This information suggests that the MADH cofactor might be a derivative of two cross-linked Trp residues. If so, it is very likely that these are also Trp residues in the enzymes from bacterium W3A1 and *T. versutus*. With this hypothesis in mind, we interpreted the NMR and MS data obtained for the MADH bispeptidyl-cofactor from bacterium W3A1 (Fig. 2).

NMR analysis. The bis(tripeptidyl)-cofactor (3.8 mg) (Fig. 2) was purified from 1.09 g of semicarbazide-derivatized and carboxymethylated MADH from bacterium W3A1 according to a published procedure (*24*). The NMR studies of this material were undertaken in D_2O and dimethyl-sulfoxide-d_6 (DMSO-d_6) solutions. In D_2O, two sets of resonances with relative intensities of ~2:1 were observed in a one-dimensional (1-D) spectrum taken at room temperature. When the sample was heated to 40°C, corresponding peaks for each conformational isomer broadened and started to merge. When the sample was then cooled to room temperature, the original NMR spectrum was recorded. These observations are the result of two forms of the bispeptidyl cofactor, whose interconversion is slow on a NMR time scale. The isomers possibly arise by rotation about the semicarbazone C=N bond or by rotation about the bond interconnecting the indole rings. (The latter possibility seems more likely because of the significant changes of proton chemical shifts for amino acid protons far from the site of the semicarbazone moiety).

Double quantum filtered correlated spectroscopy (^{1}H DQF COSY) (*30*) revealed a set of four coupled aromatic protons in addition to two downfield aromatic singlets that were evident in the 1-D spectrum. Sets of resonances for the attached amino acyl residues of the two cross-linked peptides were observed: two valyl, one carboxymethylcysteinyl, one seryl, and the α and β protons of the residues that constitute the cofactor. In DMSO-d_6 solution, again two sets of resonances were seen in the 1-D spectrum but with relative intensities of ~1:1 (Fig. 3). In addition to the resonances seen in D_2O solution, peaks from exchangeable N–H or O–H protons were seen. These included three far downfield-shifted peaks (arising from nonpeptide N–H groups), a cluster of amide doublets from the peptide bonds, and broad resonances from the NH_2-terminal amino groups of the two peptides and the semicarbazone nitrogens. By comparison to a NMR spectrum of the semicarbazide derivative of 2,7,9-tricarboxy-PQQ, the N–H peak furthest downfield is probably from the =N–NH–CO– proton of the semicarbazone.

A DQF COSY spectrum run in DMSO-d_6 (Fig. 4) showed the same aromatic coupling pattern (with small differences in chemical shift relative to the D_2O spectrum) and allowed association of most of the amide resonances with the α-hydrogens of the amino acids. Both 1-D NOE (nuclear Overhauser effect) and 2-D NOESY (nuclear Overhauser and exchange spectroscopy) spectra (Fig. 5) in DMSO-d_6, were used to further identify resonances. In the NOESY spectra, there were clear exchange cross peaks between the resonances arising from the two conformations of the molecule. These cross peaks are quite strong, and this exchange process also leads to observation of exchange transferred NOE peaks. In addition, chemical exchange between a few of the NH resonances and water dissolved in the DMSO-d_6 was observed. In spite of these complications, it was possible to observe clear sequential connectivities (*31*) for the amino acids, which allowed their resonances to be absolutely assigned (Fig. 6). In addition to the sequential connectivities, several other NOE peaks, particularly involving the aromatic and downfield NH protons, were observed. These proved useful later in elucidation of the structure.

The preliminary FAB-MS data indicated that the MH^+ ion of the semicarbazide-derivatized bispeptidyl cofactor had a mass of 940 (*24*). To obtain a structural formula, peak matching was used to obtain the exact mass of 940.3262 mass units for this ion. Twenty-nine possible formulas have good agreement with the observed mass [within 5 millimass units (mmu)]. Many of these were eliminated because of conflict with the known peptidyl fragments of the molecule and the semicarbazone modification. However, there were a number remaining which were reasonable: $C_3H_{58}N_{13}O_{15}S_2$ with 0.0-mmu deviation, $C_3H_{58}N_{11}O_{13}S_2$ with −4.0-mmu deviation, and $C_{41}H_{53}N_{11}O_{13}S_1$ with 0.4-mmu deviation.

To provide more information, ^{13}C NMR spectra were run on the material in DMSO-d_6. A DEPT (distortionless enhancement polarization transfer) sequence (*30*) was used to identify hydrogen-bearing carbons. This procedure gave rise to the number of peaks expected based on the information in the proton spectrum, including six protonated unsaturated carbons. More important was a normal proton-decoupled carbon spectrum which included the unprotonated carbons. Although the spectrum was complex because of the doubling of resonances, a reasonable tally of the total number

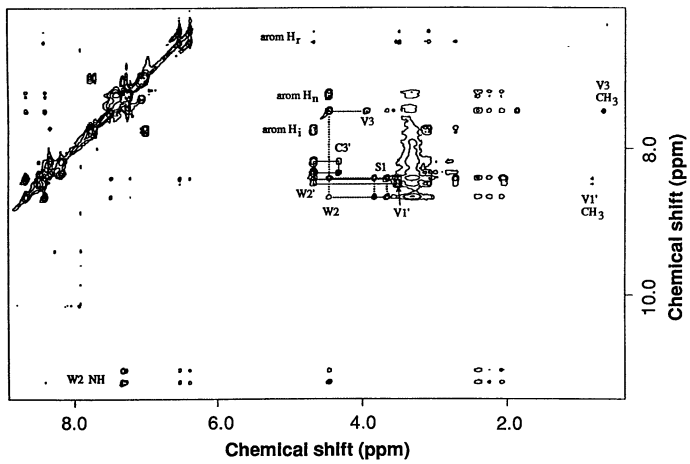


Fig. 5. Part of the ^{1}H NOESY spectrum of the bis(tripeptidyl)-cofactor in DMSO-d_6. The sequential connectivities for the two tripeptides are traced with solid and dashed lines. Cross peaks between the cofactor aromatic protons and peptide protons are also seen. These are identified schematically in Fig. 6.

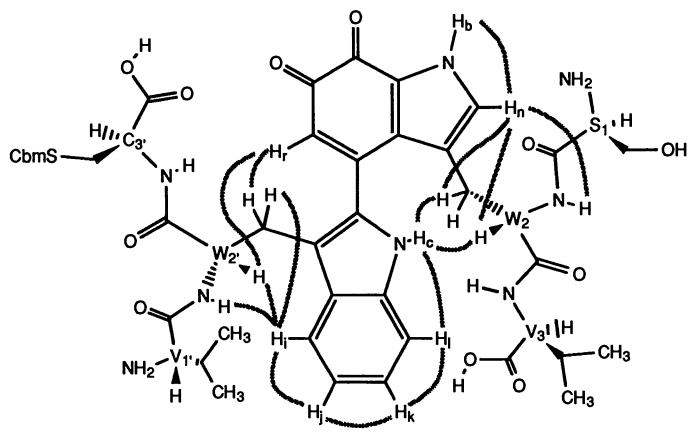
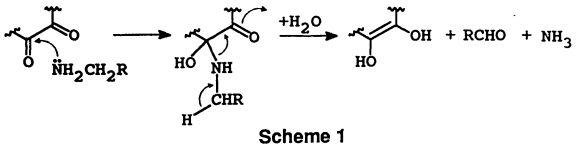


Fig. 6. Structure of the bis(tripeptidyl)-cofactor with observed NOE contacts indicated with stippled lines. The letters identifying particular protons are used in labeling Figs. 3, 4, and 5; CbM, carboxymethyl. For the structural analysis, one of the two carbonyl oxygens was substituted with a semicarbozone moiety.

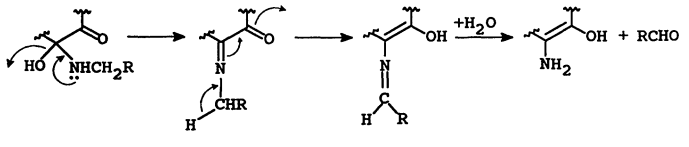
of carbons could be made. The tally agreed with the formula $C_{41}H_{53}N_{11}O_{13}S_1$ for the semicarbazide-derivatized bispeptidyl cofactor. Although there was some uncertainty in the carbon counting because of overlap of lines in the spectrum, this was the only formula that agreed with the ^{13}C NMR and MS data.

By subtracting out the known portions of the peptide chains and the semicarbazone moiety, a formula for the cofactor of $C_{16}H_8N_2O_2$ was derived. The ^1H NMR data showed that the cofactor contained a benzenoid ring with four neighboring protonated positions, two isolated unsaturated C–H groups, and two heterocyclic ring N–H groups. From the ^{13}C NMR data, the cofactor should have two carbonyl groups and eight other unsaturated carbons. When this information is coupled with the amino acid sequence data that showed that the prosthetic group is composed of two Trp residues, structure **6** in Fig. 1 is readily apparent. This structure agrees completely with the exact mass, all of the ^{13}C and ^1H NMR data (including the NOE data), and with the quinone nature of the prosthetic group. Several structurally related molecules could be proposed having the quinone carbonyl groups and the cross-link at different positions on the indole and the indole-dione ring systems. However, only structure **6** is consistent with all of the NOE data. Furthermore, NOESY connectivities within the amino acyl groups indicate that of the two Trp residues that constitute the cofactor, the one containing the indole-6,7-dione moiety (the indole labeled A of structure **6** in Fig. 1) is first in the amino acid sequence of the small MADH subunit (that is, Trp^{55} of the *M. extorquens* AM1 enzyme and Trp^{57} of the *T. versutus* enzyme) (Fig. 2). The deduced NOE connectivities are shown on the structural diagram in Fig. 6.

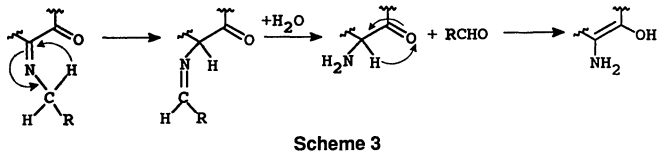
Fit of the deduced cofactor structure to electron density maps of MADH (*32*). After structure **6** in Fig. 1 was deduced, Mathews *et al.* were notified of our findings. The structure of **6** was fit to their 2.6 Å x-ray crystallographic map of MADH from *P. denitrificans*. At the same time Hol *et al.* were also made aware of our cofactor structural analysis. Again they fit cofactor structure **6** to their 2.25 Å x-ray structural map of MADH from *T. versutus* (*23*). This structure fit the electron density quite well for both the *P. denitrificans* and *T. versutus* enzymes. The fit to the electron density for both enzymes also indicates that of the two Trp residues of the cofactor, the one consisting of the indole-6,7-dione occurs first in the amino acid sequence of the small subunit, in agreement with the conclusion from the NOESY experiment. The results from the fit of the electron densities offer further evidence that our deduced structure of the quinonoid prosthetic group is correct for all known forms of MADH and that the cofactor is not an isomer of structure **6**. We considered the possibility that the indole rings are not cross-linked in the native enzyme, but that the covalent linkage occurred during isolation of the bispeptidyl-cofactor. However, the x-ray analysis data for the *P. denitrificans* and *T. versutus* enzymes show that this is not the case.



Scheme 1



Scheme 2



Scheme 3

Perspective. The data we present show that the redox prosthetic group of MADH from bacterium W3A1, *M. extorquens* AM1, *P. denitrificans*, and *T. versutus* is definitely not a PQQ or pro-PQQ derivative, but is instead derived from the cross-linking of two Trp residues. We propose to call this new cofactor tryptophan tryptophylquinone, or TTQ. A more systematic name would be 2,4′-bitryptophan-6′,7′-dione for the oxidized form and 6′,7′-dihydroxy-2,4′-bitryptophan for the reduced form (*33*).

It is appropriate to speculate why near stoichiometric amounts of derivatized 2,7,9-tricarboxy-PQQ were obtained from the *T. versutus* MADH and from other enzymes believed to contain covalently bound PQQ (*8*). It is possible this compound was misidentified, since these analyses relied mainly on the comparison of the physical and chemical properties of the cofactor, which can never substitute for direct structural analysis. Even ^1H NMR analysis of the isolated cofactors (*5, 7*) is unconvincing, since ^1H NMR for 2,7,9-tricarboxy-PQQ is not very revealing because of the presence of only two aromatic hydrogens.

Perhaps some unusual chemistry occurring during the cofactor isolation procedures produces a PQQ-like compound, such as a reaction between released Tyr and Glu, the precursors in the biosynthesis of 2,7,9-tricarboxy-PQQ (*34*). An interesting reaction was proposed by Janes *et al.* for plasma amine oxidase (*10*). For this proposal, the free amino acyl 6-hydroxy-dopaquinone derived from the oxidase cyclizes to produce 2-carboxy-4,6,7-trihydroxyindole, which would oxidize to its quinonoid form. This product could react with Glu in "two sequential Michael additions" followed by oxidation to yield an isomer of 2,7,9-tricarboxy-PQQ having the N-1 and C-3 switched in the pyrrole ring when compared to normal PQQ. This compound could behave like "normal" PQQ in the various analytical methods used. Whatever the nature of these speculative reactions, it is unlikely that near stoichiometric amounts of PQQ (*8*) would be detected.

Another possible explanation is the tenacious binding of 2,7,9-tricarboxy-PQQ to various proteins. In these cases, it has no function and its presence is coincidental. Many methylotrophs, including bacterium W3A1, synthesize and excrete large amounts of unadulterated 2,7,9-tricarboxy-PQQ (*35*), and this compound behaves like an essential nutrient in mice (*36*). Thus, this cofactor could be available to mammals from food or from enteric bacteria that might produce it. Once absorbed, this quinone could interact nonspecifically with various blood and tissue proteins, such as bovine serum albumin and human immunoglobulin G (*37*). However, it is unlikely that binding would explain the near 1:1 PQQ-subunit stoichiometry reported for various enzymes (*8*). The reason for these results remains unknown.

Clearly, 2,7,9-tricarboxy-PQQ is not a cofactor for MADH, the copper-containing amine oxidases (*10*), dopamine β-hydroxylase (*14*), galactose oxidase (*11*), or soy bean lipoxygenase-1 (*15, 16*), and these facts bring into question all of the reports of enzymes suggested to contain covalently bound 2,7,9-tricarboxy-PQQ. For these and "new" quinoproteins, cofactor identification methods that rely on the physical and chemical properties of a compound must be viewed as screening procedures. After a likely quinoprotein candidate is identified, the putative quinone must be converted to a stable derivative to eliminate undesired reactions, removed from the enzyme, purified, and then subjected to a rigorous structural analysis.

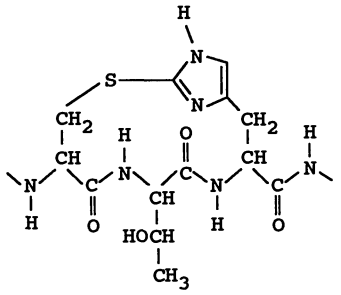


Fig. 7. The cross-linked Cys^{94}-His^{96} in tyrosinase. Intervening residue 95 is a threonine (*44*).

Our data, coupled with findings that the copper-containing amine oxidases have topa quinone as their redox cofactor (*10*), indicate that no amine oxidizing enzyme is now known to contain PQQ as its redox prosthetic group. This is quite surprising, since this quinone seems well designed for the oxidation of amines (*4*).

Since TTQ and topa quinone, like PQQ, are ortho-quinones, we expect much of what has been learned about the chemistry of PQQ (*4*) would be applicable to these as well. The two simplest mechanisms for amine oxidation by ortho-quinones are presented in Schemes 1 and 2 (R, alkyl oraryl group). Benzylamine oxidation by PQQ in solution can occur by either pathway, with the mechanism shown in Scheme 2 (the aminotransferase mechanism) predominating at pH <10 (*4*). The modified aminotransferase mechanism in Scheme 3 seems to be operating for the topa quinone–copper-containing bovine plasma amine oxidase (*3, 38, 39*). The modification was required in order to explain all of the available data for the oxidation of 2-phenylethylamine by this oxidase (*39*).

The identification of TTQ as the MADH cofactor raises a series of questions about its biosynthesis. Obviously, a distinct type of co- or posttranslational modification of amino acyl side groups must occur. In order to produce active MADH the ortho-quinone must be formed and the indole groups must be cross-linked. Although many examples of posttranslational modifications of proteins are known (*40*), this is an unusual example of two polypeptide chain residues being linked and modified to form a cofactor that acts in the absence of other cofactors or metal ions.

Galactose oxidase from *Dactylium dendroides* offers another example of a prosthetic group formed by cross-linking two amino acyl side groups. In this case, a thioether cross-link exists between Cys^{228} to Tyr^{272} (Fig. 1, **3**). The phenolic hydroxyl group of Try^{272} is the axial ligand of the Cu(II) in the active site. This arrangement, along with noncovalent interactions with other amino acyl side groups (*11*), is responsible for stabilizing the Tyr radical that is essential for activity of this enzyme (*41*).

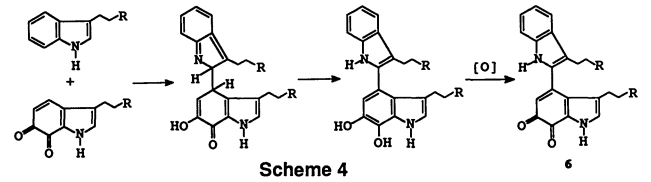
The disulfide linkage is also an example of cross-linked amino acyl residues directly involved in the catalytic function of some enzymes. *Escherichia coli* ribonucleotide reductase (*42*), and the flavoprotein disulfide oxidoreductases (FDOR) glutathione reductase, thioredoxin reductase, lipoamide dehydrogenase, and trypanothione reductase, belong to this group (*43*). Unlike TTQ in MADH, all of these enzymes require other factors besides the disulfide for catalysis [for example, the external reductants NAD(P)H (either NADH, reduced nicotinamide adenine dinucleotide, or NADPH, reduced nicotinamide adenine dinucleotide phosphate) for FDOR and reduced thioredoxin for ribonucleotide reductase, enzyme-bound flavin for FDOR, and enzyme-bound Tyr radicals for ribonucleotide reductase]. Also unlike TTQ, the disulfide bond is broken during catalysis.

Tyrosinase reportedly contains the Cys^{94}-His^{96} thioether cross-link shown in Fig. 7. Although the posttranslational formation of this link may be required to activate protyrosinase, it does not participate in the catalytic mechanism (*44*).

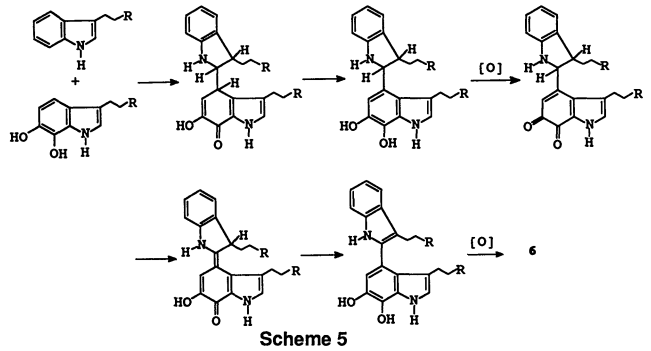
Other examples of posttranslational modifications involve conversion of polypeptide residues to an active site moiety. One of these is the pyruvoyl-dependent enzymes, such as histidine decarboxylase, in which an internal Ser residue is directly transformed into a pyruvoyl moiety in the active site. Formation of this catalytic group in the active site requires no special external factors or enzymes, that is, each molecule of prohistidine decarboxylase catalyzes the formation of its own pyruvoyl group. In the process of forming this catalytic group, the NH_2-terminal peptide bond of the Ser residue is cleaved, so that this enzyme is converted from a hexameric π_6 to an $\alpha_6\beta_6$ enzyme (*45*). A second example is the cofactor of the copper-containing amine oxidases, topa quinone (Fig. 1, **2**). It has been proposed that this quinone cofactor is derived from an amino acyl side group (most likely a Tyr phenolic moiety) (*10*). Other examples of amino acyl groups acting as cofactors, (such as Tyr and Trp radicals) in proteins and enzymes (such as ribonucleotide reductase, cytochrome c peroxidase, prostaglandin H synthase, and photosystem II) are described in recent articles (*42, 46*). For all of the examples mentioned in this paragraph, in addition to the modified amino acyl groups, other organic cofactors or metal ions are required for catalysis.

Since MADH is a periplasmic enzyme, obvious questions arise concerning when, where, and how TTQ is biosynthesized. There are two distinct processes taking place to produce the cofactor, the formation of the ortho-quinone and the cross-linking. Whether these processes are coupled or occur sequentially is unknown. It is reasonable to speculate that formation of the (dihydro)quinone precedes cross-linking. Tryptophylquinone would then be activated for a nucleophilic attack by the unmodified indolyl group (Scheme 4). Alternatively, the dihydroindoloquinone could act as a nucleophile for attack at the other indolyl group (Scheme 5). Another possible route is through an enzyme-mediated radical mechanism (Scheme 6). Other mechanisms can be proposed, and further work is necessary to determine the biosynthetic route.

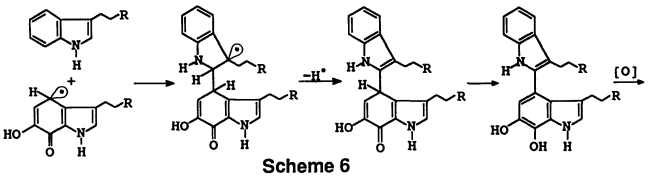
How a Trp residue in MADH is hydroxylated in the first place is of interest. Of the hydroxylated indoles found in nature, the best



Scheme 4



Scheme 5



Scheme 6

known is 5-hydroxytryptophan, which is formed in mammals by hydroxylation of Trp by tryptophan hydroxylase. Decarboxylation produces the neurotransmitter, 5-hydroxytryptamine (5-HT or serotonin). From 5-HT, the neurotoxins 4,5-, 5,6-, and 5,7-dihydroxytryptamine (DHT) are produced by unknown side reactions. Cross-linking of hydroxylated indoles results in the formation of melanins in the central nervous system. Radical-mediated cross-linking of 5-HT and of 4,5-, 5,6-, and 5,7-DHT has been studied in order to provide better understanding of the neurotoxicity (*33, 47*). Whether these hydroxylation or coupling reactions have relevance to the formation of the MADH cofactor is unknown.

Little is known about the chemistry of 6,7-dihydroxyindole or its oxidized form. To our knowledge, the MADH cofactor represents the only occurrence of a useful 6,7-dihydroxyindole derivative in nature.

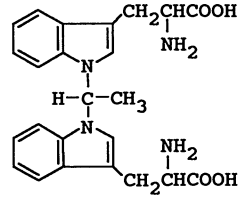
Unraveling when and where the hydroxylation and cross-linking processes take place will be a complicated process. The enzymes involved could be cytoplasmic, inner or outer membrane, or periplasmic proteins. Is the formation of the cofactor coupled to transport across the membrane, as has been proposed for mitochondrial cytochrome c (*48*)? It seems unlikely that the cofactor-containing subunit would be fully formed before transport across the cell membrane, but does some presecretion modification occur? Is transfer to the periplasmic space dependent on a specific transporter? Does the membrane potential play a role?

Not only is the small MADH subunit cross-linked through two Trp residues, but it also contains numerous disulfide linkages. For various species, the small MADH subunit has from 9 to 13 Cys residues (*21*). The x-ray structure of the *T. versutus* MADH shows that all 12 of its cysteines are tied up in disulfide linkages (*23*); thus, for this ~13-kD subunit, about one out of every nine residues is involved in a cross-link. As a result, the formation of native cofactor-containing subunits becomes an interesting and complex folding problem.

Although the questions of when, where, and how TTQ is formed can be addressed by future experimentation, why this cofactor exists is a matter for speculation. Because the formation of cofactors like TTQ, topa quinone, the galactose oxidase cofactor, and the pyruvoyl group require fairly simple modifications of amino acyl residues in proteins, it may be that these cofactors came into existence early in evolution. TTQ, topa quinone, and the galactose oxidase cofactor originally could have been formed by some combination of oxidative, photo, or radical reactions, possibly mediated by metal ions, either free or protein-bound. The predecessor of TTQ may have been the uncross-linked tryptophyl-6,7-dione derivative, which should have amine-oxidizing capabilities, and eventually given rise to the cross-linked form to produce a more efficient catalyst. Free in solution, TTQ or topa quinone are unstable; however, tightly bound in proteins, their reactivities would be diminished.

REFERENCES AND NOTES

1. S. A. Salisbury, H. S. Forrest, W. B. T. Cruse, O. Kennard, *Nature* **280**, 843 (1979).
2. J. A. Duine, *Biofactors* **2**, 87 (1989).
3. D. M. Dooley, W. S. McIntire, M. A. McGuirl, C. E. Cote, J. L. Bates, *J. Am. Chem. Soc.* **112**, 2782 (1990).
4. E. J. Rodriguez and T. C. Bruice, *J. Am. Chem. Soc.* **111**, 7947 (1989).
5. C. L. Lobenstein-Verbeek, J. A. Jongejan, J. Frank, J. A. Duine, *FEBS Lett.* **170**, 305 (1984).
6. R. A. van der Meer, J. A. Jongejan, J. Frank, J. A. Duine, *ibid.* **206**, 111 (1986); Z. Glatz, J. Kovár, L. Macholán, P. Pec, *Biochem. J.* **242**, 603 (1987).
7. R. A. van der Meer, J. A. Jongejan, J. A. Duine, *FEBS Lett.* **221**, 299 (1987).
8. R. A. van der Meer, A. C. Mulder, J. A. Jongejan, J. A. Duine, *ibid.* **152**, 99 (1989).
9. M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa, O. Adachi, *Agric. Biol. Chem.* **48**, 561 (1984).
10. S. M. Janes *et al.*, *Science* **248**, 981 (1990).
11. N. Itoh *et al.*, *Nature* **350**, 87 (1991).
12. E. Karhunen *et al.*, *FEBS Lett.* **267**, 6 (1990).
13. R. A. van der Meer, B. W. Groen, J. A. Duine, *ibid.* **246**, 109 (1989); E. J. M. Pennings, B. W. Groen, H. A. Duine, R. Verpoorte, *ibid.* **255**, 97 (1989); B. W. Groen, R. A. van der Meer, H. A. Duine, *ibid.* **237**, 98 (1989).
14. J. G. Robertson, A. Kumar, J. A. Mancewicz, J. J. Villafranca, *J. Biol. Chem.* **264**, 19916 (1989).
15. G. A. Veldink *et al.*, *FEBS Lett.* **270**, 135 (1990).
16. I. Michaud-Soret *et al.*, *Biochem. Biophys. Res. Commun.* **172**, 1122 (1990).
17. O. Jenkins and D. Jones, *J. Gen. Microbiol.* **133**, 453 (1987).
18. W. C. Kenney and W. S. McIntire, *Biochemistry* **22**, 3858 (1983).
19. _____, in *Microbial Growth on C_1 Compounds*, R. L. Crawford and R. S. Hanson, Eds. (American Society for Microbiology, Washington, DC, 1984), pp. 165–169.
20. V. L. Davidson and J. W. Neher, *FEMS Microbiol. Lett.* **44**, 121 (1987); M. Husain, V. L. Davidson, K. A. Gray, D. B. Knaff, *Biochemistry* **26**, 4139 (1987); G. W. Haywood, N. S. Janschke, P. J. Large, J. M. Wallis, *FEMS Microbiol. Lett.* **15**, 79 (1982); T. Matsumoto, *Biochim. Biophys. Acta* **522**, 291 (1978); _____, B. Y. Hiraoka, J. Tobari, *ibid.*, p. 303; T. Matsumoto, and J. Tobari, *J. Biochem. (Tokyo)* **83**, 1591 (1978); F. M. D. Villieux, *et al.*, *Eur. J. Biochem.* **154**, 383 (1986); G. Backes, V. L. Davidson, J. A. Duine, J. Sanders-Loehr, unpublished results.
21. M. Husain and V. L. Davidson, *J. Bacteriol.* **169**, 1712 (1987).
22. Y. Ishii, T. Hase, Y. Fukumori, H. Matsubara, J. Tobari, *J. Biochem. (Tokyo)* **93**, 107 (1983).
23. F. M. D. Villieux *et al.*, *EMBO J.* **8**, 2171 (1989); F. M. D. Villieux and W. G. J. Hol, *FEBS Lett.* **255**, 460 (1989).
24. W. S. McIntire and J. T. Stults, *Biochem. Biophys. Res. Commun.* **141**, 562 (1986).
25. A. Y. Chistoserdov, Y. D. Tsygankov, M. E. Lidstrom, *ibid.* **172**, 211 (1990).
26. A. A. Kasprzak, and D. J. Steenkamp, *J. Bacteriol.* **156**, 348 (1983); M. Husain and V. L. Davidson, *J. Biol. Chem.* **260**, 14626 (1985); C. Anthony, in *PQQ and Quinoproteins*, J. A. Jongejan and J. A. Duine, Eds. (Kluwer Academic, Dordrecht, 1989), pp. 1–13; P. J. Large and C. W. Bamforth, *Methylotrophy and Biotechnology* (Wiley, New York, 1988), pp. 18–76; R. J. M. van Spanning, C. W. Wansell, W. N. M. Reijinders, L. F. Oltmann, A. H. Stouthamer, *FEBS Lett.* **275**, 217 (1990).
27. R. De Beer, J. A. Duine, J. Frank, P. J. Large, *Biochim. Biophys. Acta* **622**, 370 (1980).
28. E. Lee, K. Laufer, M. E. Lidstrom, unpublished results.
29. W. S. McIntire, J. L. Bates, D. E. Brown, D. M. Dooley, *Biochemistry* **30**, 125 (1991).
30. G. E. Martin and A. S. Zekter, *Two Dimensional NMR Methods for Establishing Molecular Connectivity* (VCH Publishers, New York, 1988).
31. K. Wüthrich, *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
32. F. S. Mathews and W. G. J. Hol, personal communication.
33. G. Dryhurst, *Chem. Rev.* **90**, 795 (1990).
34. D. R. Houck, J. L. Hanners, C. J. Unkefer, *J. Am. Chem. Soc.* **110**, 6920 (1988); _____, M. A. G. van Kleef, J. A. Duine, in *PQQ and Quinoproteins*, J. A. Jongejan and J. A. Duine, Eds. (Kluwer Academic, Dordrecht, 1989), pp. 177–185; M. A. G. van Kleef and J. A. Duine, *FEBS Lett.* **237**, 91 (1988).
35. See W. S. McIntire and W. Weyler, *Appl. Environ. Microbiol.* **53**, 2183 (1987), and references therein.
36. J. Kilgore *et al.*, *Science* **245**, 850 (1989); R. Rucker *et al.*, in *PQQ and Quinoproteins*, J. A. Jongejan and J. A. Duine, Eds. (Kluwer Academic, Dordrecht, 1989), pp. 159–161.
37. O. Adachi *et al.*, in *PQQ and Quinoproteins*, J. A. Jongejan and J. A. Duine, Eds. (Kluwer Academic, Dordrecht, 1989), pp. 145–147; P. M. Gallop, M. A. Paz, R. Flückiger, H. M. Kagan, *Trends Biochem. Sci.* **14**, 343 (1989).
38. C. Hartmann and J. P. Klinman, *J. Biol. Chem.* **262**, 962 (1987); *FEBS Lett.* **261**, 441 (1990).
39. _____, *Biofactors* **1**, 41 (1988).
40. R. B. Rucker and F. Wold, *FASEB J.* **2**, 2252 (1988).
41. M. M. Whittaker, V. L. DeVito, S. A. Asher, J. W. Whittaker, *J. Biol. Chem.* **264**, 7104 (1989); K. Clark, J. E. Penner-Hahn, M. M. Whittaker, J. W. Whittaker, *J. Am. Chem. Soc.* **112**, 6433 (1990).
42. J. Stubbe, *J. Biol. Chem.* **265**, 5329 (1990); *Adv. Enzymol.* **63**, 349 (1990); R. C. Prince, *Trends Biochem. Sci.* **13**, 286 (1988).
43. S. Ghisla and V. Massey, *Eur. J. Biochem.* **181**, 1 (1989).
44. K. Lerch, *J. Biol. Chem.* **257**, 6414 (1982).
45. P. D. van Poelji and E. E. Snell, *Annu. Rev. Biochem.* **59**, 29 (1990).
46. R. C. Prince and G. N. George, *Trends Biochem. Sci.* **15**, 170 (1990).
47. A Japanese company has used a genetically engineered bacterium to mass produce L-tryptophan, which was subsequently marketed as a sleeping aid. A few lots of this L-tryptophan contained a contaminant that caused eosinophilia-myalgia syndrome in over 5000 individuals and resulted in 27 deaths. The contaminant is formed by the crosslinking of two Trp molecules to form the ditryptophan $N(\alpha)$-aminal of acetaldehyde, that is, 1,1′-ethylidenebis-[tryptophan]:



Currently, it is not known if this species is formed by the bacterium or during the purification process. See P. Raphals, *Science* **249**, 619 (1990); A. N. Mayano *et al.*, *ibid.* **250**, 1707 (1990); Center for Disease Control, *Morb. Mortal. Wkly. Rep.* **39**, 589 (1990); *ibid.*, p. 789.
48. D. W. Nicholson, C. Hergersberg, W. Neupert, *J. Biol. Chem.* **263**, 19034 (1988).
49. We thank J. Leary for measuring the exact $[M+H^+]$ mass of the bis(tripeptidyl) cofactor, P. F. Knowles and J. Sanders-Loehr for prepublication copies of their manuscripts, and L. Chen, V. L. Davidson, H. Duine, W. Hol, F. S. Mathews, E. G. Huizinga, and F. M. D. Villieux for their input and cooperation in the preparation of this manuscript. We also thank J. P. Klinman, C. Hartmann, and D. M. Dooley for support, discussions, and suggestions throughout the research. Funded by the Department of Veterans Affairs, NIH Program Project grant no. HL 16251, NSF grant DMB 87-18741 (W.S.M.), the Office of Energy Research, Office of Health Environment Research, Health Effects Research Division of the USDOE, contract DE AC03-76SF00098, instrumentation funding from USDOE grant DE FG05-86ER75281, NSF grants DMB 86-09035 and BBS 87-20134 (D.E.W.), and NIH grant GM 36296 (M.E.L.).

25 January 1991; accepted 19 March 1991