A New Redox Cofactor in Eukaryotic Enzymes: 6-Hydroxydopa at the Active Site of Bovine Serum Amine Oxidase

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An active site, cofactor-containing peptide has been obtained in high yield from bovine serum amine oxidase. Sequencing of this pentapeptide indicates: Leu-Asn-X-Asp-Tyr. Analysis of the peptide by mass spectrometry, ultraviolet-visible spectroscopy, and proton nuclear magnetic resonance leads to the identification of X as 6-hydroxydopa. This result indicates that, contrary to previous proposals, pyrroloquinoline quinone is not the active site cofactor in mammalian copper amine oxidases. Although 6-hydroxydopa has been implicated in neurotoxicity, the data presented suggest that this compound has a functional role at an enzyme active site.

THE NATURE OF THE COVALENTLY BOUND COFACTOR IN mammalian copper amine oxidases has been intensively investigated for several decades. Copper amine oxidases appear to be widespread and to catalyze physiologically important reactions such as the oxidative removal of biogenic amines from blood plasma (*1*), the cross-linking of collagen and elastin in connective tissue biogenesis (*2*), and the regulation of intracellular spermine and spermidine (*3*). However, our understanding of these proteins has been limited by the absence of a structural proof of the active site cofactor.

In early studies of the mechanism of action, the copper amine oxidases were shown to contain a reactive carbonyl group capable of forming a chromophoric complex with phenylhydrazine (*4*). Although this property initially suggested pyridoxal phosphate as the cofactor (Fig. 1, **1**), confirmation of such a structure was not forthcoming. An apparent structural resolution occurred in 1984, with two independent reports (*5, 6*) that the copper amine oxidase from bovine serum (BSAO) contained covalently bound pyrroloquinoline quinone (PQQ) (Fig. 1, **2**). While the evidence for PQQ was indirect in both cases, these reports were well received since they offered a reasonable explanation of existing data. Although PQQ has been demonstrated to be a dissociable cofactor in numerous bacterial enzymes (*7*), the studies of Ameyama *et al.* (*5*) and Lobenstein-Verbeek *et al.* (*6*) provided the first intimation of a possible PQQ requirement in higher organisms.

In subsequent studies, a battery of physical and chemical probes were used to examine the problem. For example, Dooley and co-workers performed resonance Raman studies on phenylhydrazine derivatives of several copper amine oxidases for comparison with the phenylhydrazones of pyridoxal phosphate and PQQ (*8–10*). These studies conclusively eliminated pyridoxal phosphate as a possible cofactor, strongly implicating PQQ. In our own laboratory, a reductive trap method was developed for the assay of a cofactor that contained an α-dicarbonyl, providing chemical evidence for quinone-like structures at the active sites of the copper amine oxidases from bovine serum (*11*) and *Arthrobacter* P1 (*12*). Pursuing their initial study of BSAO (*6*), Duine and co-workers derivatized a large number of enzymes with either phenylhydrazine or hexanol; in each case protein digestion was reported to yield a low molecular weight adduct which co-eluted on HPLC (high-performance liquid chromatography) with an authentic derivative of PQQ. Using this approach, investigators have reported that PQQ is present in proteins as disparate as dopamine β-monooxygenase (*13*), lipoxygenase (*14*), dopa decarboxylase (*15*), and galactose oxidase (*16*).

A major drawback throughout the above-described studies has been the lack of direct evidence for the presence of PQQ at the active site of a single mammalian protein. Although an active site cofactor-containing peptide has been isolated from pig kidney diamine oxidase (*17*), the reported yield was very low (0.1 percent). Since characterization of this peptide was limited to sequencing, it must be assumed that insufficient material was available for cofactor identification. The absence of an unambiguous structural proof of PQQ becomes particularly compelling in light of the recent x-ray studies of Hol and co-workers on methylamine dehydrogenase from *Thiobacillus versutus* (*18*): despite the prior evidence for covalently bound PQQ in this protein (*19*), x-ray diffraction patterns indicate an active site dicarbonyl which lacks the pyridine ring present in authentic PQQ (*18*).

We have now isolated an active site, cofactor-containing peptide from bovine serum amine oxidase. The high yield (40 percent) and small size (five residues) of this peptide have allowed a complete structural characterization. Our data demonstrate that the cofactor is

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not PQQ, and they provide evidence that 6-hydroxydopa is a redox cofactor in mammalian proteins.

Peptide isolation and sequencing. Previous efforts to obtain underivatized, cofactor-containing peptides from the copper amine oxidases have been unsuccessful. In part, this is due to the inherent reactivity of the active site cofactor, which can be attributed to a rapid reaction of the cofactor with amino acid side chains during the course of proteolytic digestion. Like earlier investigators, we have focused on the more stable, phenylhydrazone derivatives of BSAO.

In our screening for a useful proteolytic protocol, BSAO was labeled with [U-^{14}C]phenylhydrazine to facilitate the identification of cofactor-containing proteolytic fragments. In the initial experiments with reduced and carboxymethylated BSAO, trypsin, thrombin, V8, and chymotrypsin failed to yield detectable levels of a ^{14}C-labeled peptide (or peptides), and elastase generated multiple, ^{14}C-containing peaks under analytical HPLC conditions. Although a potentially promising result was obtained with thermolysin, which indicated a dominant ^{14}C-labeled peptide, repeated efforts to increase the peptide yield above 4 to 8 percent were unsuccessful. Instituting proteolytic digestion on native BSAO led to increased yields (to 16 percent). The addition of 2M urea further increased the yield to 40 percent.

Monitoring a thermolytic digest of the phenylhydrazone of BSAO (Fig. 2) at 214 nm (Fig. 2A) revealed a large number of peptide peaks, consistent with the low specificity of thermolysin. In contrast, monitoring of the phenylhydrazone adduct of the active site cofactor (at 350 nm) revealed a single dominant peak which

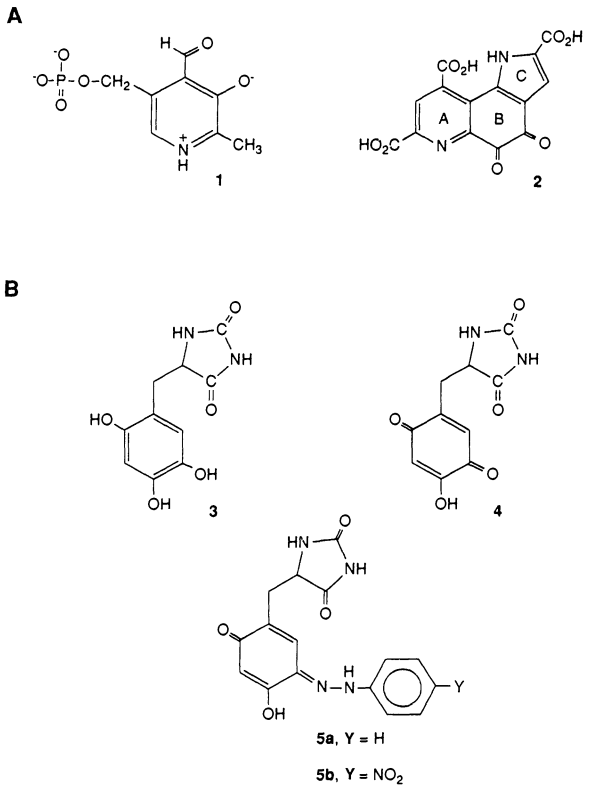


Fig. 1. (**A**) Structures of previously proposed cofactors in the copper amine oxidases: **1**, pyridoxal phosphate; **2**, pyrroloquinoline quinone. (**B**) Structure of hydantoin derivatives of 6-hydroxydopa (topa): **3**, the reduced form of topa; **4**, oxidized topa, shown as a *p*-quinone (this compound will exist as a mixture of *o*- and *p*-quinones in solution); **5a** and **5b**, the phenylhydrazone and nitrophenylhydrazone derivatives of oxidized topa. Since equilibration between *o*- and *p*-quino structures is expected, the carbonyl at position 5 of the benzene ring is expected to be the most reactive toward phenylhydrazine as well as other nucleophiles.

Table 1. Characterization of phenylhydrazones of BSAO and topa hydantoin by ultraviolet-visible spectroscopy.

Derivative	Wavelength λ_{max} (nm)		
	Protein*	Peptide†	Topa hydantoin‡
Phenylhydrazone	448	434	433
Nitrophenylhydrazone	468	457	456

*BSAO was reacted with either phenylhydrazine or nitrophenylhydrazine using methods described in the legend to Fig. 2. †Phenylhydrazine or nitrophenylhydrazine containing pentapeptides were derived from modified BSAO by proteolytic digestion and HPLC purification (Fig. 2, legend). Nitrophenylhydrazone containing peptide was eluted from HPLC within 0.5 min of the retention time of phenylhydrazone peptide. ‡Structures **5** in Fig. 1. Phenylhydrazones prepared as described in the text and purified by HPLC with gradient elution (solvent A, 0.11 percent trifluoroacetic acid, 5 percent acetonitrile; solvent B, 0.10 percent trifluoroacetic acid, 80 percent acetonitrile, increased to 70 percent in 30 minutes). The retention time for **5a** was 27.5 minutes and the retention time for **5b** was 28 minutes.

coeluted with a minor peptide band at a retention time of 37 to 38 minutes (Fig. 2B). Subsequent determination of radioactivity in peptide fractions showed coincidence of ^{14}C with the major 350-nm absorbing peak (Fig. 2C), confirming that the 350-nm absorbance was due to phenylhydrazone adduct formation.

Prior to further characterization of the 37-minute fraction, some samples of this material were subjected to an additional purification step by HPLC (the conditions of Fig. 2, but with a trifluoroacetic acid gradient consisting of solvent A, 0.11 percent trifluoroacetic acid and 5 percent acetonitrile; solvent B, 0.10 percent trifluoroacetic acid and 80 percent acetonitrile). Although this step was often unnecessary and reduced the yield by about 30 percent, it ensured the removal of minor contaminating peptides that could have complicated sequencing and mass spectral analyses. The sequence of the 37-minute peptide fraction is shown below (*20*).

—LEU—ASN—X—ASP—TYR—

Because only one amino acid was detected at each round of peptide sequencing, we conclude that the cofactor has a single (stable) point of attachment to protein. The sequence shown above has been verified in all subsequent peptide preparations used for the spectrometric characterizations described below.

Peptide characterization by mass spectrometry. In an initial study, liquid secondary ion mass spectrometry (LSIMS) showed a molecular ion (MH^+) of 807.5 daltons for the phenylhydrazone pentapeptide isolated from BSAO (shown above). Peptide isolated from protein derivatized with [1-^{15}N]phenylhydrazine underwent a mass increase of 1 dalton (MH^+ 808.3), establishing that the phenylhydrazone moiety had remained intact during isolation. To identify the number of free carboxylate groups and improve the mass spectral characteristics (*21*), the peptide (MH^+ 807.5) was derivatized with acidic hexanol, producing a new MH^+ at 975.5 daltons. Since derivatization of each carboxylate adds 84.1 daltons to the molecular mass and the total increase observed is 168.2, we conclude that there are two carboxylate groups in the pentapeptide. These can be accounted for in the peptide itself (at Asp and the carboxyl terminus), indicating that there are no additional carboxylate groups in the cofactor. This result rules out PQQ (Fig. 2, **2**) as the cofactor, which in its underivatized form contains three carboxylate groups. Although one of the carboxylate groups of PQQ could be involved in an ester or amide linkage to the peptide, at least two carboxylate groups would be expected to remain available for derivatization with hexanol.

Subsequent studies of the dihexyl derivative of the pentapeptide led to an exact molecular mass measurement of 974.5123, which, after subtraction for two hexyl groups, gave a mass of 806.3245 for the peptide. After subtraction of accurate masses for Leu, Asn, Asp, and

Tyr, a value for X of 283.0967 was obtained. Again, this is not consistent with a PQQ type of structure, which would have a minimum mass of 489 for attachment of PQQ via an ester linkage to serine. Computer permutation of possible elemental compositions for X led to five empirical formulae that were within ±5 ppm of its value. Of these, only one empirical formula, $C_{15}H_{13}N_3O_3$, was compatible with both the ultraviolet-visible (UV/Vis) absorbance properties of active site cofactor and the presence of phenylhydrazone in X. Two structures are consistent with the formula $C_{15}H_{13}N_3O_3$ (Fig. 3). Structure A is a serine residue, attached to the phenylhydrazone of a catechol ring through an ether linkage; and structure B is the phenylhydrazone of a trihydroxyphenylalanine derivative, referred to as 6-hydroxydopa (topa).

The exact structure of the cofactor was investigated further by means of high-sensitivity tandem mass spectrometry. In this technique the ^{12}C isotope in the molecular ion cluster MH^+ is selectively introduced into a collision chamber containing an inert gas, which imparts sufficient energy to result in collision-induced dissociation (CID). The high-energy CID mass spectrum for the molecular ion (MH^+ *m/z* 807.5) of the BSAO-derived pentapeptide is shown in Fig. 4. Analysis of a second CID spectrum, with peptide that had been derivatized with [^{15}N]phenylhydrazine, revealed an increase by one atomic mass unit for the molecular ion and all X-containing peptide fragments. As indicated in the upper corner of Fig. 4, peak assignments provide excellent agreement with the sequence deduced from protein sequencing (Leu-Asn-X-Asp-Tyr). The intense peak at *m/z* 256 in Fig. 4 corresponds to the immonium ion derived from X; the high relative abundance of this peak, together with the fact that it was possible to sequence past X by the micro-Edman technique, establishes the presence of a peptide backbone structure in X. The ions at *m/z* 239 and 227 are consistent with loss of NH_3 and NH_2CH, respectively, from the immonium ion at *m/z* 256. In an effort to distinguish structures A and B (Fig. 3), CID spectra were examined for side chain fragmentation from the molecular ion. In no instance was loss of a mass 213 detected for cleavage between the β-carbon and oxygen of structure A (Fig. 3). In contrast, an intense peak at *m/z* 580 arose in spectra of both [^{14}N]- and [^{15}N]phenylhydrazone-containing peptides, which is attributed to loss of a mass 227 (or 228) for cleavage between the α- and β-carbons of structure B (Fig. 3) from the molecular ion. Although *m/z* 580 was isobaric with the y_3 ion in the ^{14}N-spectrum, the spectrum for the ^{15}N-labeled peptide indicated ions at both *m/z* 580 and 581, corresponding to side chain loss and the y_3 ion, respectively. These observations permitted a tentative assignment of the active site cofactor in BSAO to structure B (Fig. 3).

Synthesis of a peptide analog of 6-hydroxydopa. At this juncture, we considered it essential to have an authentic sample of a peptide analog of 6-hydroxydopa for use in confirmatory spectral studies. Synthesis of such a material followed a modification of existing procedures (*22*) where 2,4,5-tribenzyloxybenzaldehyde was reacted with hydantoin to give 5-(2,4,5-tribenzyloxybenzylidine)hydantoin in 48 percent yield. Selective reduction of the benzylidine hydantoin side chain double bond with 3 percent Na(Hg) gave 5-(2,4,5-tribenzyloxybenzyl)hydantoin in 77 percent yield. The protecting benzyl groups were then removed by hydrogenation in dimethylformamide over a 5 percent Pd-C catalyst, generating 5-(2,4,5-trihydroxybenzyl)hydantoin (Fig. 1, **3**). Compound **3** was too unstable to isolate, undergoing rapid air oxidation to a quinone (Fig. 1, **4**). Since all the peptides obtained from BSAO have been in the form of phenylhydrazones, the crude quinone was further reacted with either a tenfold molar excess of phenylhydrazone in 80 mM potassium phosphate, pH 7.2, yielding the phenylhydrazone (Fig. 1, **5a**), or with a 1.1-fold molar excess of *p*-nitrophenylhydrazone in methanol, yielding the nitrophenylhydrazone (Fig. 1, **5b**). After purification by HPLC, each was obtained in approximately 5 percent overall yield.

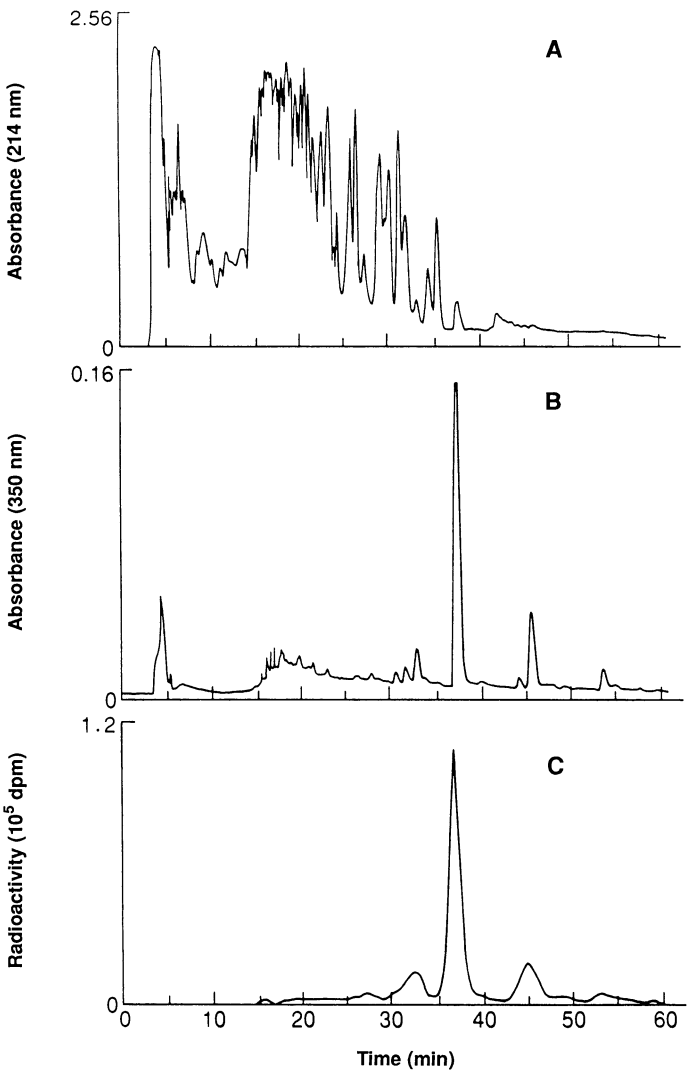


Fig. 2. HPLC purification of a labeled active site peptide from bovine plasma amine oxidase. BSAO was prepared from 20 liters of cow blood by a modification of existing procedures (*42*). The initial anion exchange step was replaced by a Q-Sepharose fast flow chromatographic step, and Ultrogel AcA34 was used for gel filtration. In addition, back to back pH 8 and pH 6 Q-Sepharose columns were used for final purification. The enzyme showed a single band on SDS gel electrophoresis and showed a specific activity of 0.41 to 0.45 U/mg according to the assay conditions of Neumann *et al.* (*43*). Enzyme (13.4 mg) was dissolved in 2.4 ml of 10 mM potassium hydrogen phosphate (pH 8) and inactivated by the addition of 156 nmol of [U-^{14}C]phenylhydrazine in five portions over a 30-minute period. Excess radioactivity was removed by desalting on a Bio-Rad 10DG column that had been equilibrated with 100 mM NH_4HCO_3. Derivatized enzyme was added to 100 mM NH_4HCO_3 containing 2M urea and placed on a shaker bath at 37°C. Proteolytic digestion was initiated by the addition of 2 percent (w/w) thermolysin. A second portion of protease was added after 12 hours, and the digestion was quenched by the addition of EDTA (final concentration 10 mM) after 48 hours. Digested enzyme was injected onto a Dynamax-C8 column (5 μm, 300 Å material), equilibrated with 0.3 percent triethylamine acetate, pH 6.8 (solvent A) on a Shimadzu HPLC system. Peptides were eluted with 0.3 percent triethylamine acetate, pH 6.8, containing 60 percent acetonitrile (solvent B). Gradient elution involved the linear addition of solvent B to 20 percent at 10 minutes, 45 percent at 70 minutes, and 100 percent at 80 minutes. Elution of peptides was monitored at 214 nm (**A**), at 350 nm (**B**), or by radioactivity in collected fractions (**C**). The yield, based on radioactivity in the injected digest compared to radioactivity in the 37-minute peak, was 34 percent. The yield varied somewhat with the preparation ranging from a low 30 to 57 percent.

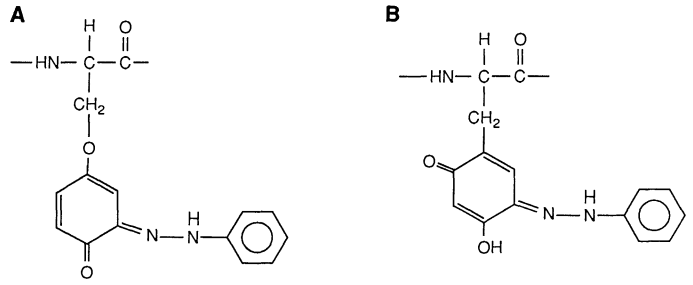


Fig. 3. Structures compatible with an empirical formula of $C_{15}H_{13}N_3O_3$ for residue X in the pentapeptide. (**A**) A serine residue, attached to the phenylhydrazone of a catechol via an ether linkage. (**B**) The phenylhydrazone of 6-hydroxydopa.

UV/Vis spectroscopy. One of the earliest observations on BSAO was the generation of a new chromophore at 440 to 450 nm on derivatization with phenylhydrazines (*4*). More recently, investigators have invoked the similarity of λ_{max} (maximum absorbancy wavelength) for the phenylhydrazone of PQQ (445 nm) as evidence in favor of PQQ as the active site cofactor (*23*). Like others before us, we routinely see a λ_{max} at 448 nm for phenylhydrazine derivatized BSAO. Isolated pentapeptide shows a blue shift of 14 nm to 434 nm (Table 1). This is important in view of the absorbance of the phenylhydrazone of topa hydantoin (Fig. 1, **5a**) at 433 nm. Analogous trends have been seen with nitrophenylhydrazone derivatives. The nitrophenylhydrazone of BSAO absorbs at 468 nm, undergoing a blue shift to 457 nm for the isolated pentapeptide (Table 1). Once again, the nitrophenylhydrazone of topa hydantoin shows almost exact correspondence with derivatized pentapeptide, absorbing at 456 nm. It appears that protein side chains lead to a red-shifted λ_{max} for phenylhydrazones of the active site cofactor, producing a fortuitous agreement with absorbance data for PQQ. Removal of protein effects on electronic transitions leads to almost exact correspondence between λ_{max} values for phenylhydrazone-containing peptides and the phenylhydrazones of topa quinone (Table 1).

Characterization by proton NMR. Although the above documented mass spectral analyses and absorption spectroscopy implicate topa as the active site cofactor in BSAO, we undertook proton nuclear magnetic resonance (NMR) studies as a final proof of structure. Initial studies in deuterated water (D_2O) were performed on the phenylhydrazone pentapeptide, producing a spectrum containing resonances due to the peptide backbone and aliphatic side chains below 6 ppm and resonances arising from the tyrosine side chain and derivatized cofactor above 6 ppm. For the region of the spectrum above 6 ppm (Fig. 5A), the two doublets centered at 6.8 and 7.1 ppm integrate to four hydrogens and are easily assigned to the tyrosine side chain at position five of the peptide (Leu-Asn-X-Asp-Tyr). Three additional peaks were identified at 7.5, 7.2, and 6.9 ppm. Since these integrated in the ratio of 4 to 5:1:1, they were initially assigned to the five hydrogens in the phenylhydrazine ring (7.5 ppm) and the two hydrogens in the cofactor ring (7.2 and 6.9 ppm) (Fig. 3, structure B). However, in a decoupling experiment, irradiation of the 7.2-ppm peak led to a noticeable sharpening solely in the 7.5-ppm resonance, suggesting that the 7.2-ppm peak was due to one of the five hydrogens on the phenylhydrazine ring.

In an effort to resolve the origin of the 7.2-ppm peak in Fig. 5A, we examined the pentapeptide prepared from BSAO derivatized with nitrophenylhydrazine. Comparison of the NMR spectrum of such a nitrophenylhydrazone peptide (Fig. 5C) with that in Fig. 5A indicated two new doublets at 7.5 and 8.3 ppm. Integration of these peaks revealed four hydrogens, which were assigned to the nitrophenylhydrazone ring. This assignment, together with the absence of a peak at 7.2 ppm in Fig. 5C, indicated that the 7.2-ppm resonance in Fig. 5A was due to the phenylhydrazine ring, not to cofactor. A corollary of these results is that NMR experiments in D_2O permit detection of only one of the two ring hydrogens of cofactor (*24*).

This property of cofactor-containing peptide can be rationalized from the structure of the phenylhydrazone of 6-hydroxydopa (Fig. 3B). As shown, the C–H bond at position 5 of the cofactor ring lies between an enol and a ketone functional group. An acid-base–catalyzed enol tautomerization is to be expected, which in the

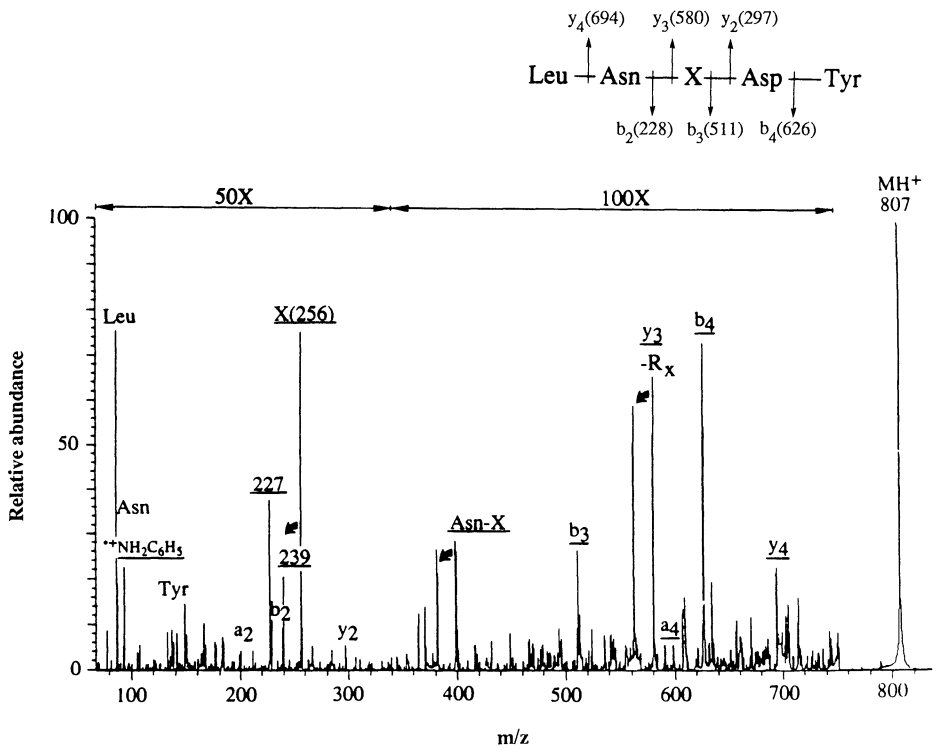
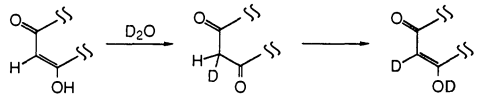


Fig. 4. High-energy CID mass spectrum for the molecular ion (MH^+ *m/z* 807.5) of the BSAO-derived pentapeptide. Mass spectra were obtained on a Kratos Concept IIHH (Manchester, U.K.) four sector instrument of EBEB geometry. Ions that increased by 1 dalton in the spectrum for [1-^{15}N]-labeled phenylhydrazine are underlined. Fragments containing Asp or Tyr (or both) were further confirmed by mass shifts in the spectrum for the hexyl ester derivative of the peptide. Significant ions for the structure are labeled and shown in the sequence above. The solid arrows indicate a loss of NH_3 from the indicated ions. Ion types are as follows: a_2, $[NH_2CHR_1CONHCHR_2]^+$; b_2, $[H_2NCHR_1COHNCHR_2\text{-}CO]^+$; y_2, $[NH_3CHR_4CONHCHR_5COOH]^+$. For convenience, we have not differentiated between fragments where the number of protons can vary (for example, y_n refers to y_n and y_{m-2}).

presence of the D_2O used as solvent for NMR experiments would produce deuterated cofactor:



(1)

Further investigation of this exchange reaction was facilitated by the availability of the nitrophenylhydrazone derivative of topa hydantoin (**5b**, Fig. 1), which immediately after dissolution in D_2O gave the spectrum shown in Fig. 5B. After correction for the tyrosyl side chain of peptide, the region of this spectrum above 6 ppm indicated an almost exact correspondence with the BSAO-derived nitrophenylhydrazone peptide spectrum (Fig. 5C). A new peak appeared at 5.6 ppm which integrated to one hydrogen at early times. Since this peak disappeared into the base line after several hours' incubation, it was assigned to the exchangeable proton at position 5 of the cofactor ring.

Using NMR, we made a final effort to detect the missing ring hydrogen in cofactor-containing peptide by examining the spectrum for the nitrophenylhydrazone derivatized peptide in H_2O (Fig. 5D). In these studies the solvent water signal was suppressed with a $1:\bar{3}:3:\bar{1}$ sequence, using a peak of excitation at about 7 ppm. Although this excitation distorted peak intensities, for example, leading to a loss of the nitrophenylhydrazone peak at 8.3 ppm, the 5.6 ppm signal seen with the authentic topa derivative (Fig. 5B) is clearly also present in the cofactor peptide (*25*). This ability to detect the second ring hydrogen in BSAO-derived cofactor provides the final piece of evidence required for an unambiguous identification of structure.

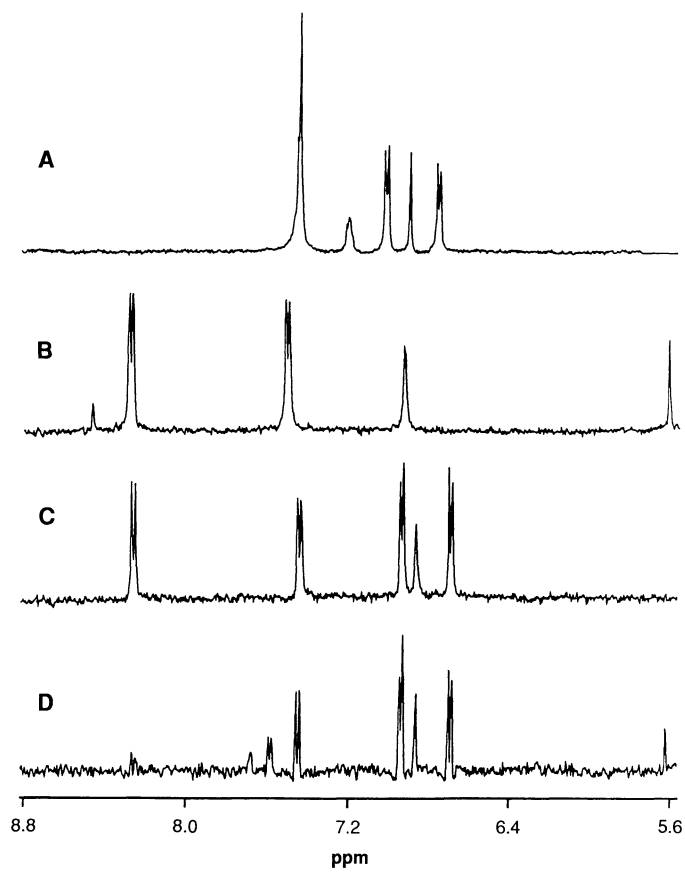


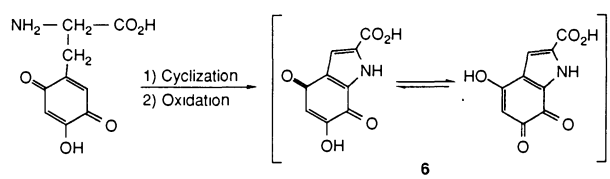
Fig. 5. Proton NMR spectra for peptides and model compounds. Data were collected on a GN 500 spectrometer. (**A**) Phenylhydrazone-containing pentapeptide. (**B**) Nitrophenylhydrazone-derivatized topa quinone hydantoin (Fig. 1, **5b**). (**C** and **D**). Nitrophenylhydrazone-containing peptide in D_2O and H_2O, respectively. For the spectrum in H_2O (containing 10 percent D_2O as a lock for the spectrometer), the solvent signal was suppressed with the use of a $1:\bar{3}:3:\bar{1}$ selective excitation sequence (*44*), with a delay of 750 μsec between pulses. The baseline was flattened by means of a cubic spline function. Cofactor-peptide samples were 30 to 50 nmol dissolved in 400 μl; spectra are the average of 128 to 1024 scans.

Relation of topa to studies of cofactor structure in copper amine oxidases. Our results show that the active site cofactor in BSAO is 6-hydroxydopa and not the recently proposed pyrroloquinoline quinone (*5, 6, 26*). Since it appears likely that all of the copper amine oxidases have a common cofactor, these results imply a role for topa in serum amine oxidases from other sources and in intracellular amine oxidases such as lysyl oxidase. We now consider why PQQ has been the accepted redox cofactor in these proteins.

There is little question that PQQ is produced by selected bacteria, undergoing excretion into the growth medium (*27*). The cofactor has been isolated and fully characterized by NMR (*7*) and x-ray crystallography (*28*). Significantly, PQQ has been shown to function as a dissociable cofactor in various bacterial enzyme reactions, largely through reconstitution studies that show a reappearance of enzyme activity after the addition of PQQ to apoenzyme. In the case of bacterial enzymes reputed to contain a covalently bound form of PQQ, the situation is less clear. The recent x-ray characterization of a methylamine dehydrogenase from *Thiobacillus versutus* indicates a structure for the active site cofactor that is distinct from PQQ and has been designated pro-PQQ (*18*).

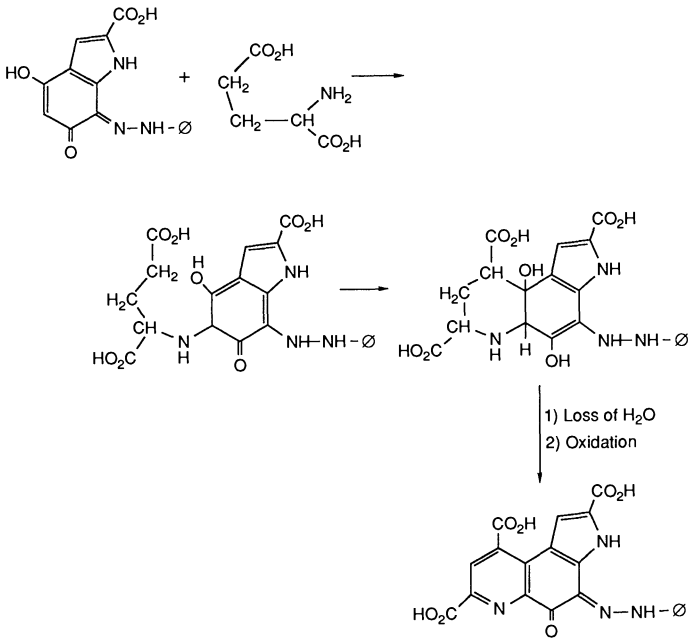
Many studies describing PQQ as a covalently bound cofactor have been focused on eukaryotic enzymes (*26*). Beginning with the initial reports of PQQ in BSAO (*5, 6*), proof of PQQ has relied on the isolation of compounds from phenylhydrazine inactivated enzymes that coelute on HPLC with authentic samples of PQQ phenylhydrazone. It has been noted that retention time on HPLC represents a fairly tenuous form of structural proof (*29*). In view of our results, we have considered the possibility that a derivative of topa phenylhydrazone, formed from BSAO during proteolysis, may fortuitously co-elute with the phenylhydrazone of PQQ.

Topa quinones undergo a rapid intramolecular cyclization reaction (*30*) that is expected to yield the tautomeric mixture designated **6** in Eq. 2.



6 (2)

Inspection of compound **6** indicates a close resemblance to rings B and C of PQQ (Fig. 1, **1**), as well as to the species Hol and coworkers refer to as pro-PQQ (*18*). We suggest that pronase treatment of phenylhydrazine derivatives of copper amine oxidases can release the phenylhydrazone of topa quinone, which then undergoes a rapid cyclization to the phenylhydrazone of **6**. While this compound itself may not be expected to show the same retention time on HPLC as PQQ (*31*), a derivative formed by reaction with glutamate would coelute with PQQ (Scheme 1). Both **6** and its phenylhydrazone are highly reactive and are expected to form Michael adducts with a range of nucleophilic compounds. Although amino acids with side chains more nucleophilic than glutamate can be expected to accumulate after pronase digestion, condensation of the phenylhydrazone of **6** with glutamate may be favored by a high concentration of this particular amino acid. Proteolysis of oxidatively damaged proteins leads to production of glutamate or its semialdehyde from a number of amino acids such as arginine, lysine, and proline (*32, 33*). In addition, the availability of



Scheme 1. Proposed mechanism for the generation of a PQQ-like product from the ring-cyclized form of topa quinone. The reaction is illustrated by the phenylhydrazone derivative of **6** (Eq. 2). Glutamate is proposed to undergo two sequential Michael additions to the phenylhydrazone of **6**. After elimination of H_2O and oxidation by molecular oxygen, the phenylhydrazone of a structural isomer of PQQ is formed. Although this product shows a reversal of carbon and nitrogen in the indole ring, relative to PQQ, it would be expected to have the same retention time on HPLC as the phenylhydrazone derivative of PQQ.

two nucleophilic positions in glutamate (at the α-amino group and the γ-carbon) may drive the condensation reaction (Scheme 1) via an entropically favored ring closure to form a six-membered ring. Analogous condensations can also be envisioned for the amino acids serine or cysteine (involving their α-amino and β-OH or SH functional groups); however, ring-closed products formed from these amino acids would be incapable of the final oxidation step to form stable aromatic species.

While the above arguments are rationalizations of experimental results from other laboratories, they offer a plausible explanation for many of the results on PQQ, as well as provide a framework for continuing study of the physical and chemical properties of topa quinone and its derivatives. Resonance Raman studies on phenylhydrazone derivatives of bovine and porcine serum amine oxidases and lysyl oxidase (*8–10*) have revealed spectral properties that are similar (although not identical) to those of the phenylhydrazone of PQQ. While these studies have provided clear evidence against a simple carbonyl cofactor such as pyridoxal phosphate, they have not yet addressed the extent of difference (or similarity) that may be expected for phenylhydrazone derivatives of other quinostructures.

As to a direct role for PQQ in mammalian cells, Gallop and co-workers have developed highly sensitive assays for the detection of PQQ in cell extracts (*34, 35*). Glycine (as reductant) and nitroblue tetrazolium (as oxidant) have been reported to detect PQQ in the nanomolar range; using this technique, investigators have reported PQQ to be present at high levels in adrenal tissue and brain (*36*). These tissues contain high concentrations of catechol hormones and neurotransmitters, as well as their precursors and oxidation products—dopa and topa. We therefore undertook the assay of topa hydantoin and found that the glycine-nitroblue tetrazolium redox cycling system of Gallop and co-workers works nearly as well with topa hydantoin as PQQ. These results suggest that the reported detections of PQQ in a range of mammalian tissues reflect, instead, the presence of catechols and their oxidation products. This view is compatible with the report (*37*) that, contrary to a previous finding (*13*), PQQ is not present in dopamine β-monooxygenase from bovine adrenal glands.

Despite the possibility that previous claims of PQQ in mammalian systems can be attributed to either topa or related compounds, it is important to bear in mind the recent nutritional studies by Rucker and co-workers (*38*). Using germ-free rats and controlled diets, these authors have demonstrated numerous abnormalities in animals deprived of PQQ. Of particular note is the occurrence of abnormalities in connective tissue formation, ascribed to an inability of nutritionally deprived animals to synthesize active, PQQ-containing lysyl oxidase. In our view, isolation and characterization of an active site peptide from lysyl oxidase is of the highest priority, since only through such studies will it be possible to resolve, in an unambiguous manner, the nature of its active site cofactor.

Whither topa as cofactor? For many years it has been recognized that the 6-hydroxy derivatives of dopa, dopamine, norepinephrine, and epinephrine are neurotoxic (*39*). Although the exact mechanism of cellular toxicity is not known, it is almost certainly related to the facile redox reactions carried out by these compounds. It is, therefore, astonishing that the redox properties of 6-hydroxydopa have been harnessed in a constructive manner through its use as an active site, enzymatic cofactor.

The presence of topa in BSAO raises the question (among others) of how topa arises. A reasonable hypothesis is that topa formation is a posttranslational process involving oxidation of an active site tyrosine. However, since dopa is a naturally occurring amino acid, the possibility of its direct incorporation into the growing protein chain, via a special transfer RNA, cannot be excluded. If tyrosine oxidation is the mechanism of topa formation, is this performed by a second enzyme (possibly with tyrosinase-like activity)? Or alternatively, do the active site metal atoms in the copper amine oxidases play a catalytic role in this posttranslational event?

It is important to ascertain the full scope of topa as a redox cofactor. We note that the x-ray studies of Hol and co-workers (*18*) on the bacterial methylamine dehydrogenase show a ring-cyclized isomer of topa, attached to protein via a glutamate side chain. This finding raises the possibility of a role for topa-like molecules either through their direct incorporation into the protein backbone (BSAO), or through their covalent attachment to active-site side chains (methylamine dehydrogenase).

There is also the relation of topa to PQQ. Although the misidentification of topa as PQQ in BSAO may be coincidental, these two structures may be interrelated in a more fundamental manner. Studies of PQQ in bacteria implicate a topa-like intermediate as a precursor of PQQ (*40, 41*). A similar scheme, illustrating a possible chemical conversion of ring cyclized topa quinone (**6**) to a PQQ-like molecule, is shown in Scheme 1. It is apparent that both PQQ and topa contain quino-structures, which are capable of covalent adduct formation with their substrates, before reduction to quinols. In addition, the relative instability of the reduced, quinol forms of PQQ and topa facilitates regeneration in each case. Therefore, these cofactors may reflect related strategies toward the biogenesis of quino-containing cofactors in pro- and eukaryotic proteins.

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