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Evidence for methoxatin (pyrroloquinolinequinone) as the cofactor in bovine plasma amine oxidase from resonance Raman spectroscopy

(copper-containing/quinoprotein/2,4-dinitrophenylhydrazone)

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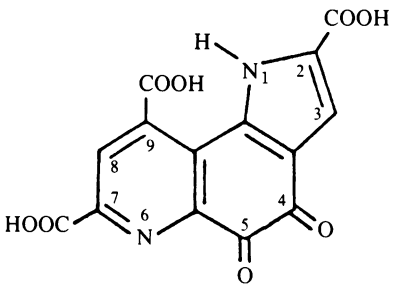
ABSTRACT **Resonance Raman spectra of the 2,4-dinitrophenylhydrazine derivatives of bovine plasma amine oxidase [amine:oxygen oxidoreductase (deaminating) (copper-containing), EC 1.4.3.6] have been measured. Detailed comparisons to the spectra of the corresponding derivatives of methoxatin (pyrroloquinolinequinone), pyridoxal, and other aldehydes and diones provide further evidence that covalently bound methoxatin or a closely similar derivative is the organic cofactor in copper-containing amine oxidases.**

Copper-containing amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper-containing), EC 1.4.3.6] are a widespread and diverse class of enzymes. They can be isolated from microorganisms, plants, and mammals (1, 2). A variety of primary amines, including mono-, di-, and polyamines, can serve as substrates, depending on the enzyme source. In addition to copper, these amine oxidases also contain an organic cofactor that is tightly bound, probably covalently, to the enzyme; recent experiments have established a subunit:copper:cofactor stoichiometry of 2:2:1 for most amine oxidases (1–8). Extensive spectroscopic and ligand binding studies have defined the basic structure of the copper sites (1, 2, 9–15). The presence of an organic cofactor was first inferred from the sensitivity of amine oxidases to carbonyl reagents—e.g., phenylhydrazine, semicarbazide, and hydroxylamine (16). Despite considerable effort, the organic cofactor has not been conclusively identified. Many different techniques have been brought to bear on this problem, but the results have often been ambiguous or even conflicting. For many years the most likely candidate for the cofactor was pyridoxal or a closely related derivative (1, 2, 17–24), but strong evidence against pyridoxal had also been reported (1, 2, 6, 25–28). Knowles and Yadav (2) have summarized concisely and analyzed the available data in a recent review. In 1984 two groups independently presented spectroscopic (absorbance and fluorescence) and chromatographic evidence that methoxatin (pyrroloquinolinequinone, PQQ), structure **1**, was the organic factor in copper-containing amine oxidases (29, 30). Previously methoxatin had been identified as a coenzyme only in prokaryotic organisms (31). Methoxatin contains electrophilic carbonyl groups and displays one- and two-electron redox chemistry (31). In this context it should be noted that free radicals have been detected in some amine oxidase reactions (32) and that sequential one-electron steps may be involved in the reoxidation of substrate-reduced amine oxidases by O_2 (33–37). Several properties of methoxatin that are relevant to its potential functions in amine metabolism have already been delineated. For example, data pertaining to redox potentials, carbonyl chemistry, mechanisms of reactions with amines, and metal ion complexation are available for methoxatin and various analogues (38–41).



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The identification of the organic cofactor as methoxatin is potentially very important to understanding the mechanisms and functions of copper-containing amine oxidases. Substantiating this proposal by independent means is therefore highly desirable. The original reports were based on the strategy of attempting to isolate the cofactor itself (30) or a stable derivative (29). In the latter case, the yield was less than 6%; no yield was reported in the former study, but at least two species that resembled methoxatin were obtained following ion-exchange chromatography of amine oxidase digests. Further, methoxatin and its 2,4-dinitrophenylhydrazone (DNPH) are unstable under the conditions used by both groups in order to remove the cofactor from the enzyme (29, 30). Another complication is that some amino acids (produced by enzymatic or acid hydrolysis) react with methoxatin (29). As a result, empirical comparisons between authentic PQQ and the isolated cofactor are problematic. Therefore, resonance Raman spectroscopy was chosen to probe the structure of the organic cofactor for two reasons: (*i*) the technique may be applied to intact enzymes under physiological conditions; and (*ii*) molecular vibrational spectra are a very powerful approach to structure determination. Resonance Raman spectroscopy has been used extensively to investigate the structure of natural protein-bound chromophores and protein–probe molecule complexes (42–44). The principal advantage of resonance Raman spectroscopy is that the vibrational spectrum of a chromophore in a complex macromolecule can be selectively obtained by resonance enhancement—that is, by using an excitation frequency that is within the linewidth of an electronic transition associated with the chromophore. Vibrational frequencies of normal

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Abbreviations: PQQ, pyrroloquinolinequinone; DNPH, 2,4-dinitrophenylhydrazone.

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modes that produce distortions in the molecule, similar to the distortions produced by the transition to the excited electronic state, will display the greatest resonance enhancement (45, 46). This rule is especially applicable for resonant scattering coupled to electronically allowed transitions (42–46), such as those of the enzyme derivatives studied here. Thus, the observed resonance Raman spectra should be structurally diagnostic for the chromophore. It has proved to be very difficult to obtain high-quality resonance Raman spectra from the native enzymes; consequently, we decided to investigate the 2,4-dinitrophenylhydrazine derivatives.

MATERIALS AND METHODS

PQQ was obtained from Fluka and as a gift of J. A. Duine. 1,10-Phenanthroline-5,6-dione was used as purchased (ALFA, Danvers, MA), and 1,7-phenanthroline-5,6-dione was synthesized by published procedures (38). The enzyme was isolated from bovine plasma (bovine plasma amine oxidase) by using a new procedure to be published elsewhere. Pyridoxal, pyridoxal phosphate, and 2-pyridinecarboxaldehyde (Aldrich) were all used without further purification. All solvents were reagent grade.

PQQ-dinitrophenylhydrazone (DNPH) was prepared by dissolving 8 mg of 2,4-dinitrophenylhydrazine in 0.5 ml of 4 M HCl. The solution was warmed to aid in solubilization and then filtered. A solution of 1 mg of PQQ in 0.5 ml of 4 M HCl was added to the filtrate. The red precipitate was collected by centrifugation and washed with water. All other model derivatives were prepared by mixing a solution containing 10 mg of the appropriate ketone or aldehyde in 1 ml of 4 M HCl with a solution of 40 mg of dinitrophenylhydrazine hydrochloride in 5 ml of 4 M HCl. The precipitates were recrystallized from methanol.

The enzyme derivative was prepared by adding a 10% molar excess of dinitrophenylhydrazine (dissolved in absolute ethanol) to the enzyme in 0.1 M potassium phosphate buffer (pH = 7.2) and incubating at room temperature for 16 hr. The protein concentrations ranged from 0.07 to 0.11 mM.

The Raman scattering experiments were performed with a Spex Ramalog system and a Coherent INNOVA-18 argon ion laser. Samples of about 10 μl were placed in a glass melting-point capillary, and the scattered signal was collected at 90° from the transverse excitation. The collected light was focused through a polarization scrambler onto the slits of a 0.85-m double monochrometer (Spex 1403) with the bandwidth set at 4 cm^{-1}. The detection system consisted of a Hamamatsu R928 photomultiplier in a thermoelectrically cooled housing (PFR). Typical powers at the sample were 20–60 mW. Scans were collected by using 1-cm^{-1} increments and averaging for 1 sec at each point. A photodiode monitored a fraction of the excitation beam, and its output was used for normalization of the observed scattering signal. Generally, several scans were collected to check for consistency, summed to enhance the signal-to-noise ratio, and then transferred to an IBM personal computer for further analysis and storage. The Raman spectra themselves were found to provide the most sensitive indication of photochemical or thermal degradation of these complexes. If necessary, the data were smoothed by using the Savitsky–Golay procedure (47), and a straight-line background level was substracted from the data. Sodium sulfate was used as an internal standard for excitation profiles.

RESULTS AND DISCUSSION

The electronic absorption spectra of the DNPH adducts of bovine plasma amine oxidase, PQQ, and pyridoxal are shown in Fig. 1. Other relevant DNPH compounds displayed wavelengths of maximum absorbance as follows: 1,7-phenanthroline-5,6-dione-DNPH, 465 nm (MeOH); 1,10-phenanthroline-5,6-dione-DNPH, 475 nm (MeOH); and 2-pyridinecarboxaldehyde-DNPH, 415 nm (H_2O, pH <5). Both dione derivatives displayed a pronounced shoulder on the high-energy side of the peak. Note that the DNPH derivatives of PQQ and the two diones display absorption spectra similar to that of the modified enzyme, whereas pyridoxal-DNPH and 2-pyridinecarboxaldehyde-DNPH exhibit markedly blue-shifted absorption bands. Based on the absorption spectra, pyridoxal and the oxidized, ring-opened flavin derivative proposed by Hamilton (36) are not likely to be the organic cofactor when compared with PQQ. The similarity between the absorption spectra of PQQ-DNPH and bovine plasma amine oxidase-DNPH has been noted previously by Duine and coworkers (29); they also found that the adduct isolated after denaturation and proteolysis of bovine plasma amine oxidase-DNPH exhibits an absorption spectrum essentially identical to that of PQQ-DNPH. These results establish that the electronic structure of the cofactor-DNPH adduct is altered upon displacement from the enzyme into solution, reflecting one or more of the following differences in the two environments: (*i*) the cofactor is covalently bound to the enzyme in an unknown manner (1, 2, 17, 18, 23–25); (*ii*) the microscopic environment of the enzyme-bound cofactor-DNPH adduct is undoubtably different from that of the free adduct in aqueous solution; and (*iii*) there may be significant interactions between the copper ion(s) and the cofactor. Thus, the resonance Raman spectra of the derivatized enzyme and the isolated adduct would be expected to be similar but not identical.

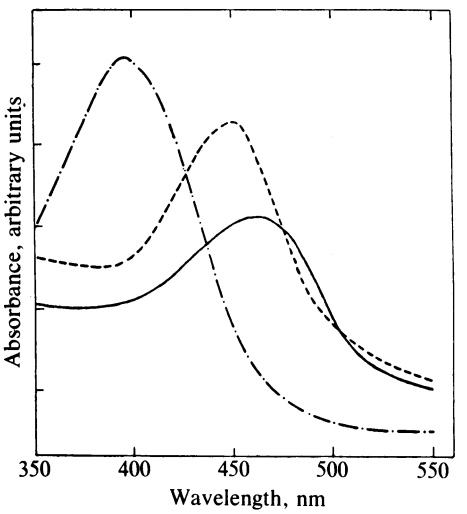

FIG. 1. Electronic absorption spectra of key derivatives. ——, bovine plasma amine oxidase-DNPH; ------, PQQ-DNPH; -·-·-, pyridoxal-DNPH.

Resonance Raman spectra of bovine plasma amine oxidase-DNPH and PQQ-DNPH in aqueous solution are shown in Fig. 2. Although the relative intensities of the various bands are not uniformly comparable between the two, the correspondence in peak positions is excellent. The energies of the peaks in Fig. 2 are collected in Table 1. With only a few exceptions, each peak in the bovine plasma amine oxidase-DNPH spectrum can be correlated to a similar peak in the PQQ-DNPH spectrum within 20 cm^{-1}, with a majority of those peaks within 10 cm^{-1} of each other. Such frequency shifts between the free and protein-bound forms of a chromophore are not unusual (42, 43). Of the four features (603, 877, 1078, 1454 cm^{-1}) in the bovine plasma amine oxidase-DNPH spectrum that are not this close in energy to observed features of the PQQ-DNPH spectrum, reasonable correlations can be made for two of them by obtaining excitation profiles. All of the bands in the 700–1000 cm^{-1}

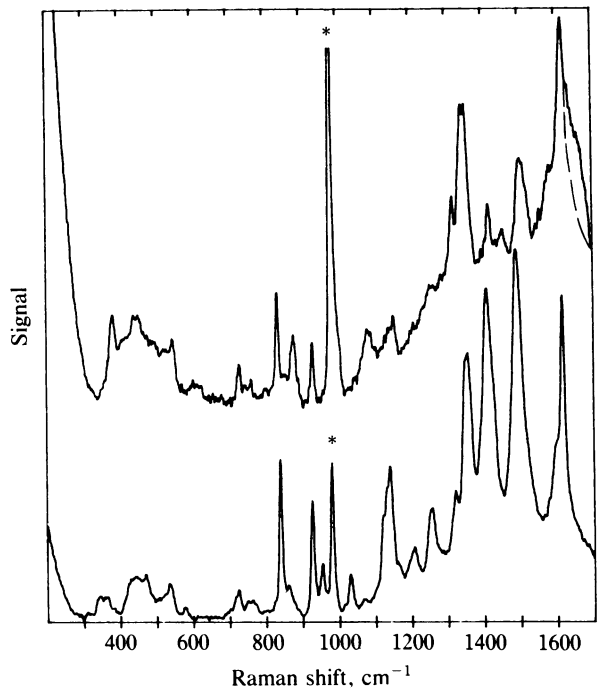

FIG. 2. Resonance Raman spectra of the bovine plasma amine oxidase-DNPH (upper spectrum) and PQQ-DNPH (lower spectrum) adducts obtained with 457.9-nm excitation. The true bandshape of the 1620-cm^{-1} peak in the enzyme spectrum is indicated by a dashed line; significant intensity at >1620 cm^{-1} was not observed in other spectra that were otherwise essentially identical to that shown here (see Fig. 4). Asterisks denote a SO_4^{2-} peak.

region undergo strong enhancement along the respective absorption profiles with the exception of the 877 cm^{-1} feature in bovine plasma amine oxidase-DNPH and the 954 cm^{-1} band of PQQ-DNPH. Thus, even though they are 77 cm^{-1} apart, these two features likely correspond to a similar vibrational mode in the two species. Excitation at lower energies also resolves the slight shoulder at 1429 cm^{-1} in the PQQ-DNPH spectrum into a definite separate peak. This relatively weakly enhanced band is only 25 cm^{-1} from the similar weak peak at 1454 cm^{-1} in the bovine plasma amine oxidase-DNPH spectrum. Thus, PQQ-DNPH in aqueous solution appears to be an excellent model for the DNPH-modified enzyme cofactor, based on the vibrational frequencies observed in the resonance Raman spectra.

It is well known that the relative intensities in resonance Raman spectra can be quite sensitive to the microscopic environment of the chromophore (42, 43). Such variations in the relative intensities are readily rationalized by theory (44–46, 48, 49). For example, the intensity of a mode in a resonance Raman spectrum can be related to the slope of the excited state surface (in the Franck–Condon region) along the ground-state normal-mode coordinate (48, 49). Hence, any variation in these slopes between the protein and aqueous environment will primarily appear as intensity differences in the Raman spectra.

Line representations of the spectra obtained from the DNPH derivatives of the five model compounds and bovine plasma amine oxidase are shown in Fig. 3. Each line represents the position of a peak or shoulder in the 457.9-nm resonance Raman spectra. Approximate relative intensities are represented by the heights of the lines. However, we reiterate that it is the peak positions and not their magnitudes that are the most significant factors for comparison. Careful examination of Fig. 3 indicates which spectral regions are most sensitive to the structure of the original carbonyl compound. First, there are several peaks that are common to

Table 1. Observed energies of peaks in resonance Raman spectra of bovine plasma amine oxidase-DNPH and PQQ-DNPH

Energy in cm^{-1}, relative intensity*	
bovine plasma amine oxidase-DNPH	PQQ-DNPH
	343,m
382,m	362,m
439,m	442,m
452,m	471,m
547,m	535,m
603,m	576,w
729,m	724,m
743,w	750,w
762,w	764,w
802,w	800,?
834,s	841,s
856,?	860,w
877,m	
928,m	928,s
	954,m
1078,m	1031,m
1135,w,sh	1122,m,sh
1152,m	1142,s
	1162,w
1207,w	1208,m
1251,w	1255,m
1317,m	1322,m
1342,s	1340,s,sh
1351,s	1354,s
1415,m	1410,s
1454,w	1429,sh
1503,s	1489,s
1580,w,sh	1596,w,sh
1620,s	1616,s

*s, strong; m, moderate; w, weak; sh, shoulder; ?, very weak but reproducible.

all of the DNPH derivatives (around 840, 930, 1145, and 1340 cm^{-1}). Second, there are some features common only to the three diones (around 1200 and 1350 cm^{-1}), and others are

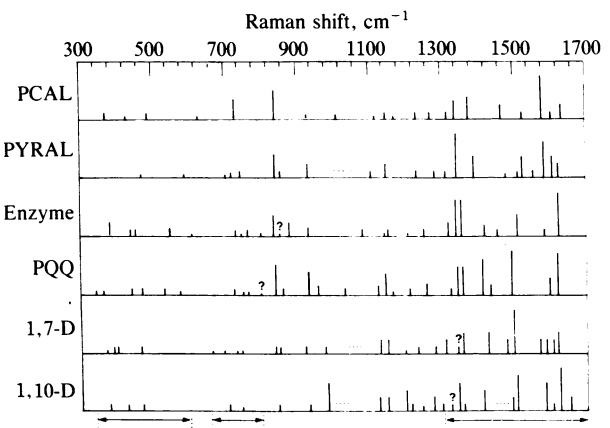

FIG. 3. Line-representations for the resonance Raman spectra of several DNPH derivatives. PCAL, 2-pyridinecarboxaldehyde; PYRAL, pyridoxal; enzyme, bovine plasma amine oxidase; 1,7-D, 1,7-phenanthroline-5,6-dione; 1,10-D, 1,10-phenanthroline-5,6-dione. Approximate relative intensities are represented by the heights of the lines. The arrows at the bottom of the figure indicate the spectral regions that are especially sensitive to the structure of the carbonyl compound. Dashed lines appear in regions dominated by Raman scattering from the solvent. Question marks identify a very weak peak or shoulder that is reproducible. All spectra were obtained with 457.9-nm excitation.

found only in the pyridinecarboxaldehyde derivatives (around 1380 and 1520 cm^{-1}). In general, the position and number of peaks between 750 and 1300 cm^{-1} are relatively constant throughout. Three distinct segments can be identified that are significantly variable among these spectra: 300–600 cm^{-1}, 660–800 cm^{-1}, and 1300–1700 cm^{-1}. These "fingerprint" regions contain only one peak (1340 cm^{-1}) that is common to all of the spectra and only a few peaks that are characteristic of either diones or pyridinecarboxaldehydes. Thus, these regions are particularly sensitive to the structure of the carbonyl compound. The spectrum of PQQ-DNPH unquestionably provides an excellent match to that of bovine plasma amine oxidase-DNPH in each of these three regions. Therefore, the similarity in the spectra may be attributed to a close similarity in the structure of the two nonderivatized species.

Data presented in Figs. 3 and 4 effectively rule out pyridoxal as the cofactor. In the lowest energy region, 300–600 cm^{-1}, the enzyme derivative shows a series of four peaks (382, 438, 453, and 547 cm^{-1}) over a broad background. The PQQ-DNPH Raman spectrum corresponds most closely to that of the enzyme in this region. Comparison with pyridoxal-DNPH is complicated in this region by the presence of solvent peaks. Because both ethanol and dimethyl sulfoxide exhibit strong features in this spectral region, neither solvent alone permits a complete 200- to 600-cm^{-1} spectrum of the pyridoxal-DNPH adduct to be obtained. However, a careful consideration of both spectra indicates that there are probably no features between 300 and 450 cm^{-1}. Two weak sharp bands at 472 cm^{-1} and 583 cm^{-1} are observable, as shown in Fig. 3. Thus, the correlation of pyridoxal-DNPH and bovine plasma amine oxidase-DNPH spectra in this region is poor.

A comparison of the resonance Raman spectra of bovine plasma amine oxidase-DNPH, PQQ-DNPH, and pyridoxal-DNPH in the other two "fingerprint" regions is presented in Fig. 4. In the 660- to 800-cm^{-1} region shown in Fig. 4*A*, the three-peak sequence exhibited by the bovine plasma amine oxidase-DNPH spectrum (728, 744, and 762 cm^{-1}) is matched extremely well by PQQ-DNPH (724, 750, and 764 cm^{-1}). Although the spectrum of pyridoxal-DNPH is similar to the enzyme spectrum, the correlation is clearly worse than that of PQQ-DNPH and bovine plasma amine oxidase-DNPH. In the high-energy region (Fig. 4*B*) of the spectrum, bovine plasma amine oxidase-DNPH displays a fairly simple spectrum. Above 1400 cm^{-1}, there are five features of alternating intensity (Table 1). PQQ-DNPH exhibits a similar sequence in both peak position and relative intensities. The pyridoxal-DNPH spectrum is considerably different in this region. The prominent peak at 1384 cm^{-1} is not observed in the bovine plasma amine oxidase-DNPH spectrum. Although the dimethyl sulfoxide peak at 1422 cm^{-1} obscures the 1400- to 1450-cm^{-1} region in the pyridoxal-DNPH spectrum presented, the spectrum taken in ethanol shows no peaks between 1400 and 1440 cm^{-1}. Also, there are more features in this spectrum than in either the bovine plasma amine oxidase-DNPH or PQQ-DNPH spectrum. Overall, these results suggest that the derivatized organic cofactor of bovine plasma amine oxidase-DNPH closely resembles PQQ-DNPH but is quite different from pyridoxal-DNPH. Since the resonance Raman spectra are quite sensitive to the structure of the reactive carbonyl-containing species, this strongly implies that the cofactor is PQQ or a closely similar derivative.

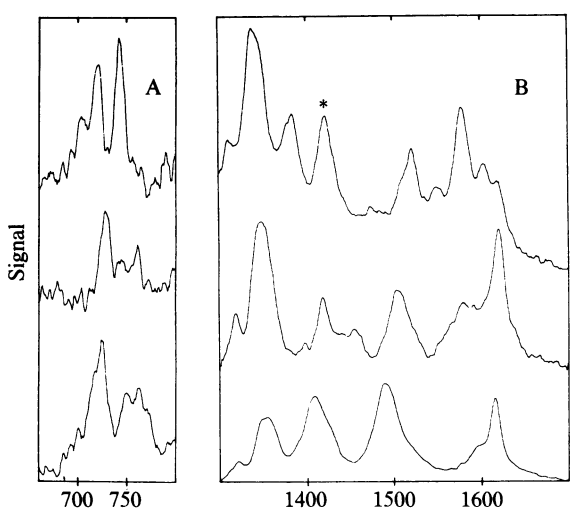

FIG. 4. Resonance Raman spectra of the DNPH adducts of pyridoxal (top spectrum), bovine plasma amine oxidase (middle spectrum). and PQQ (bottom spectrum) in the 660- to 800-cm^{-1} region (*A*) and the 1300- to 1700-cm^{-1} region (*B*). The asterisk indicates a solvent peak.

Collectively, the absorption and resonance Raman data presented here strongly support the proposal that PQQ (or a slightly modified derivative) is the organic cofactor in bovine plasma amine oxidase. We find the combined evidence from this and earlier work (29, 30) to be convincing. Furthermore, it is likely that the same cofactor is present in all copper-containing amine oxidases. We have directly confirmed this for the pig plasma amine oxidase by examining the resonance Raman spectra of the enzyme-phenylhydrazone and -DNPH derivatives. (R.S.M., D.M.D., and P. F. Knowles, unpublished observations). As pointed out previously (29, 30, 40, 41), the presence of PQQ in mammals raises the intriguing possibility that it is a heretofore unrecognized vitamin. Finally, the results described here suggest that resonance Raman spectroscopy may be an informative approach for structural studies of other methoxatin enzymes.

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