

## The Organic Functional Group in Copper-containing Amine Oxidases

RESONANCE RAMAN SPECTRA ARE CONSISTENT WITH THE PRESENCE OF TOPA QUINONE (6-HYDROXYDOPA QUINONE) IN THE ACTIVE SITE\*

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Resonance Raman spectroscopy has been used to probe the structure of the organic cofactor in copper-containing amine oxidases from bovine plasma, porcine kidney, pea seedlings, and the bacterium *Arthrobacter* P1. The enzymes were first derivatized with phenylhydrazine or *p*-nitrophenylhydrazine; resonance Raman spectra were obtained on the intact derivatized enzymes and on a derivatized active-site peptide isolated from bovine plasma amine oxidase. Spectra of the intact amine oxidase phenylhydrazones are practically identical, consistent with the enzymes examined containing a similar cofactor. Only minor frequency shifts and some intensity variations are detected between the resonance Raman spectra of intact bovine plasma amine oxidase and the isolated peptide. These spectral perturbations are attributable to differences in the micro-environment between the intact, folded protein and the isolated small peptide in aqueous solution. This rules out the possibility that a new structure is formed during the isolation of the derivatized active-site peptide. Importantly, the resonance Raman spectra of the phenylhydrazine and *p*-nitrophenylhydrazine derivatives of the bovine plasma amine oxidase peptide are identical to the spectra of the corresponding derivatives of topa quinone (6-hydroxydopa quinone). Hence these data provide strong, independent support for the recent identification of topa as the organic functional group in bovine plasma amine oxidase (Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., and Klinman, J. P. (1990) *Science* 248, 981-987).

In 1984 Duine and co-workers (1) and Ameyama and co-workers (2) independently presented evidence that the covalently bound, organic cofactor in copper-containing amine

oxidases was pyrroloquinoline quinone (PQQ,<sup>1</sup> 1) (see Scheme 1). These reports rationalized a considerable body of existing data that was consistent with the presence of a carbonyl cofactor in amine oxidases (3-5) and greatly stimulated further research. Prior to 1984, a covalently bound pyridoxal derivative was widely considered to be the most plausible candidate for the organic cofactor in amine oxidases, although not all the available data were consistent with the structure or chemistry of pyridoxal derivatives (3). Over the past few years a variety of evidence has accumulated that is consistent with a quinone cofactor in copper-containing amine oxidases. For example, the resonance Raman spectra of several amine oxidase phenylhydrazones or dinitrophenylhydrazones were found to be very different from the spectra of analogous derivatives of pyridoxal and other simple carbonyl compounds, but (for the dinitrophenylhydrazones) similar to the PQQ derivative (6-9). Reductive trapping experiments are readily understood in terms of quinone chemistry but are difficult to rationalize on the basis of known pyridoxal reactions (10, 11). In addition, cyanide or *t*-butylisocyanide can be used to trap an enzyme radical, which displays EPR parameters typical of semiquinones (9, 12). Duine and co-workers (13, 14) have refined and extended their original methodology and claimed to have detected stoichiometric quantities of PQQ in numerous eukaryotic enzymes, in addition to the amine oxidases. However, the evidence for PQQ or any quinone cofactor in copper-containing amine oxidases has not been universally accepted (15, 16).

Recently, topa quinone (6-hydroxydopa quinone, 2) has been identified as the cofactor in bovine plasma amine oxidase (17). This conclusion was based on detailed mass spectroscopic and NMR data obtained on an active-site peptide isolated in high yield. These data unequivocally rule out PQQ as the cofactor, which raises questions about how PQQ came to be misidentified as the cofactor. It should also be noted that this is the first example of a 6-hydroxydopa-derived functional group in any enzyme. Therefore, confirmation by independent methods is highly desirable. In this paper we present resonance Raman spectra that confirm the presence of topa quinone in bovine plasma amine oxidase. Resonance Raman spectroscopy also provides evidence in support of topa quinone as an active site component in other copper-containing amine oxidases, isolated from a variety of sources. Finally, and importantly, the data rule out the possibility that topa is produced as a consequence of the chemical or the chromatographic steps used in the isolation of the peptide.

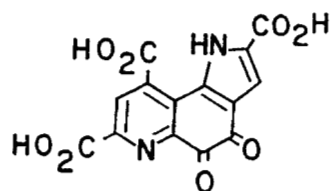
### EXPERIMENTAL PROCEDURES

Enzymes used in these experiments were purified by methods previously described in the literature (17-20). Phenylhydrazine- or *p*-nitrophenylhydrazine-labeled peptides were isolated from bovine plasma amine oxidase using the published procedure (17). Raman spectra were collected with a Spex Ramalog system, as described in greater detail elsewhere (6, 9); excitation wavelengths were provided by a Coherent Ar ion laser. Resonance Raman spectra of the enzymes and active-site peptides were recorded using 457.9 nm excitation. We also collected Raman spectra (not resonance-enhanced) of phenylhydrazine and nitrophenylhydrazine in saturated aqueous solution using 488.0 nm as the exciting line. Base-line subtraction, smoothing, and plotting were done using routines in SpectraCalc (v. 2.1, Galactic Industries, Inc.).

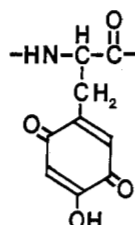
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<sup>1</sup> The abbreviation used is: PQQ, pyrroloquinoline quinone.



1



2

SCHEME 1

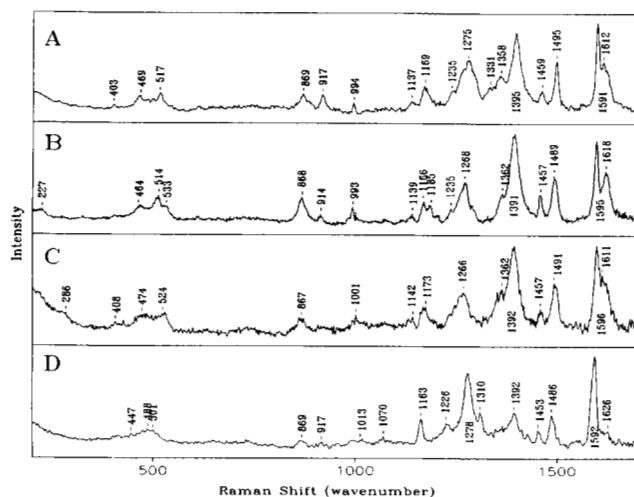


FIG. 1. Resonance Raman spectra of amine oxidase phenylhydrazones. A, bovine plasma amine oxidase; B, *Arthrobacter* P1 methylamine oxidase; C, porcine kidney diamine oxidase; D, pea seedling diamine oxidase. Excitation wavelength was 457.9 nm and the laser power was 25–40 milliwatts.

## RESULTS AND DISCUSSION

It has been recognized for some time that phenylhydrazine reacts rapidly with copper-containing amine oxidases to yield intensely absorbing ( $\epsilon \geq 30,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) chromophores in a manner that is competitive with substrate (3, 4, 17). Similar covalent adducts can be prepared with several substituted phenylhydrazines as well. These derivatives are ideally suited for resonance Raman spectroscopy.<sup>2</sup> Fig. 1 displays the spectra obtained on the phenylhydrazones of amine oxidases from bovine plasma, porcine kidney, pea seedlings, and *Arthrobacter* P1. With the possible exception of the pea seedling enzyme, the spectra are strikingly similar, with regard to both the band frequencies and relative intensities. This is also true of the spectra of the *p*-nitrophenylhydrazones (data not shown). Control experiments with phenylhydrazine and *p*-

<sup>2</sup> Despite numerous attempts we have not yet been able to obtain resonance Raman spectra of underivatized amine oxidases, in part owing to fluorescence. Efforts along these lines will continue.

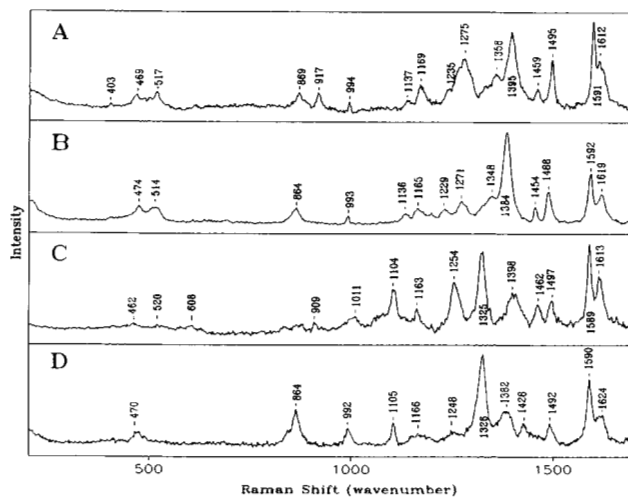


FIG. 2. Resonance Raman spectra of labeled bovine plasma amine oxidase and the isolated active-site peptide. A, the enzyme phenylhydrazine; B, the phenylhydrazine-labeled peptide; C, the enzyme *p*-nitrophenylhydrazine; D, the *p*-nitrophenylhydrazine-labeled peptide. Excitation conditions were as stated in Fig. 1.

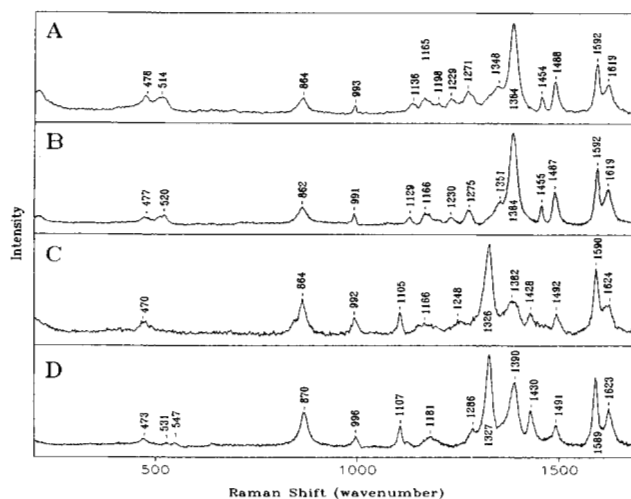


FIG. 3. Comparison of the resonance Raman spectra of the labeled peptides from bovine plasma amine oxidase to the corresponding synthetic topa-hydantoin derivatives. A, the peptide phenylhydrazine; B, the phenylhydrazine of topa-hydantoin; C, the peptide *p*-nitrophenylhydrazine; D, the *p*-nitrophenylhydrazine of topa-hydantoin.

*p*-nitrophenylhydrazine established that the Raman spectra of these compounds are very different from the spectra of the enzyme or peptide derivatives shown in Figs. 1–4. Work on other enzymes (21) and numerous carbonyl and quinone compounds has shown that resonance Raman spectra will reflect the structure of the cofactor.<sup>3</sup> Therefore it is reasonable to conclude, on the basis of the spectra in Fig. 1, that the amine oxidases represented there contain an active-site component that is similar to topa.

Fig. 2 compares the resonance Raman spectra of the phenylhydrazine and *p*-nitrophenylhydrazine of intact bovine plasma amine oxidase to the spectra of the labeled pentapeptides, isolated and characterized as described (17). The peptide sequence is -Leu-Asn-X-Asp-Tyr- where X is the derivatized

<sup>3</sup> Significant differences exist among derivatives of phenylhydrazine (and among derivatives of other hydrazines) with simple carbonyl compounds and quinones (D. E. Brown, J. L. Bates, and D. M. Dooley, unpublished observations).

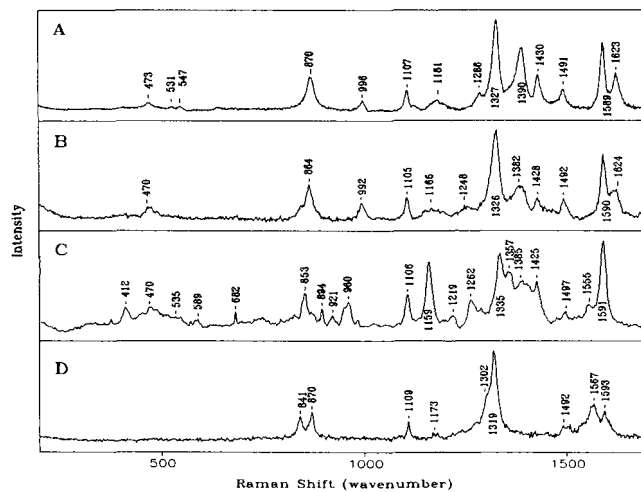


FIG. 4. Comparison of the resonance Raman spectra of the *p*-nitrophenylhydrazones of topa-hydantoin (A), the active-site peptide of bovine plasma amine oxidase (B), PQQ (C), pyridoxal phosphate (D).

topa moiety. Overall, the spectra of the two phenylhydrazones are closely similar; with few exceptions, the relative intensities of the peaks appear identical, and frequency shifts are generally less than  $10\text{ cm}^{-1}$ . The most notable differences are among the frequencies and relative intensities in the 1200–1300  $\text{cm}^{-1}$  region, and the absence of the  $917\text{ cm}^{-1}$  peak in the peptide spectrum. Such differences between free and protein-bound states of a chromophore are well precedented in, for example, resonance Raman studies of hapten binding to antibodies (22). Relatively greater differences are apparent when the *p*-nitrophenylhydrazones are compared; the decrease in intensity of the  $1254\text{-cm}^{-1}$  band in the peptide spectrum is particularly striking. Nevertheless, the overall similarity of the spectra of the *p*-nitrophenylhydrazones is still apparent, and the differences can reasonably be attributed to environmental effects and are consistent with theoretical considerations (23). We suggest that the presence of the *p*-nitro group may lead to different conformations of the chromophore in the protein and peptide. The critical result established by these comparisons is that *the same chromophore is present in the isolated peptides and in the intact enzyme*. In other words, the topa structure is not produced during the labeling and isolation of the peptides.

In Fig. 3 the resonance Raman spectra of the labeled peptides are compared to the spectra of identically labeled, synthetic topa-hydantoin. The identity of the phenylhydrazone spectra, and the near-identity of the *p*-nitrophenylhydrazone spectra, provide strong and unambiguous support for the deduction of Klinman and co-workers (17) that the organic functional group in resting bovine plasma amine oxidase

is topa quinone. As is evident from the spectra in Fig. 4, the resonance Raman spectra of the *p*-nitrophenylhydrazones of PQQ and pyridoxal phosphate are sufficiently different from the spectrum of the peptide that they may be unequivocally ruled out as the cofactor. Furthermore, as implied above, the similarity of the amine oxidase-phenylhydrazone resonance Raman spectra to each other, and to the spectrum of the topa-hydantoin phenylhydrazone, supports the view that topa is present in the enzymes represented here. Given the well documented similarities among copper-containing amine oxidases from various sources (3, 4), it is likely that all these enzymes contain topa, as pointed out previously (17).

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