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The organic cofactor in plasma amine oxidase: evidence for pyrroloquinoline quinone and against pyridoxal phosphate

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Plasma amine oxidases (EC 1.4.3.6) are classified as containing the organic cofactor pyridoxal phosphate. Biochemical and bioassays on the pig plasma amine oxidase fail to reveal the presence of pyridoxal phosphate and ^{31}P n.m.r. evidence is also inconsistent with pyridoxal phosphate in the enzyme. Resonance Raman spectral studies on phenylhydrazone derivatives of the pig and bovine plasma enzymes have been carried out and comparisons made with the corresponding derivatives of pyridoxal phosphate and pyrroloquinoline quinone (PQQ). The resonance Raman evidence indicates that the cofactor in both plasma amine oxidases is PQQ or a closely related species and not pyridoxal phosphate. The results substantiate earlier reports concerning the identity of the organic cofactor.

INTRODUCTION

The identity or even existence of an organic cofactor in plasma amine oxidases (EC 1.4.3.6) has been a controversial area (see Knowles & Yadav, 1984, for review). Yamada & Yasunobu (1963) were the first to suggest that the cofactor in the beef plasma enzyme might be pyridoxal phosphate, although a positive identification could not be made at that time (Watanabe *et al.*, 1972). For the pig plasma enzyme, a wide body of evidence indicated pyridoxal phosphate to be the cofactor (Blaschko & Buffoni, 1965). Lobenstein-Verbeek *et al.* (1984) and Ameyama *et al.* (1984) have suggested, on the basis of chromatographic and spectroscopic evidence, that the cofactor in beef plasma amine oxidase is pyrroloquinoline quinone (PQQ; Fig. 1), which is covalently bound to the enzyme. This cofactor had been found previously in a number of prokaryotic oxidoreductases (Duine & Frank, 1981), but the papers by Lobenstein-Verbeek *et al.* (1984) and Ameyama (1984) were the first to report its presence in eukaryotes. The potential importance of this finding with respect to eukaryotic amine metabolism is considerable but caution urges that further evidence be obtained to identify PQQ as the cofactor rather than pyridoxal phosphate. Recently Moog *et al.* (1986) presented strong evidence from resonance Raman spectroscopy for PQQ as the organic cofactor in beef plasma amine oxidase. Their data were inconsistent with the presence of pyridoxal phosphate.

The present paper gives the results of experiments which indicate that PQQ rather than pyridoxal phosphate is the organic cofactor in pig plasma amine oxidase, and which establish that the cofactors in the beef plasma and pig plasma enzymes are closely similar.

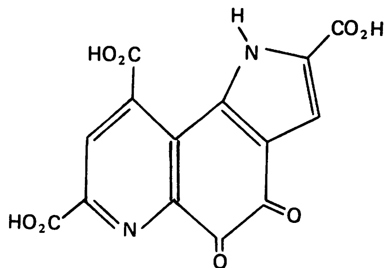

Fig. 1. Structure of pyrroloquinoline quinone (PQQ)

MATERIALS AND METHODS

Amine oxidase from pig plasma was isolated and characterized as described by Rius *et al.* (1984). Glycogen phosphorylase (EC 2.4.1.1) was purified by the method of Fischer & Krebs (1958); L-aspartate-2-oxoglutarate aminotransferase (EC 2.6.1.1), type 1, from pig heart, was purchased from Sigma, Poole, Dorset, U.K. Other reagent quality chemicals were purchased from Sigma or BDH Chemicals, Poole, Dorset, U.K.

Phenylhydrazine assay (Wada & Snell, 1961)

Pig plasma amine oxidase was dialysed (three changes each of 2000 volume excess) against potassium phosphate buffer (50 mM, pH 7.0); the final protein concentration was 9.4 mg/ml. A sample of glycogen phosphorylase (18 mg/ml) was prepared similarly. Portions (0.3 ml) of each enzyme were mixed with 0.6 M-perchloric acid (0.3 ml), allowed to stand on ice for 30 min and then centrifuged (15000 rev./min for 2 min on an Eppendorf microcentrifuge). Portions (0.5 ml) of the supernatant were mixed with 0.3 M-perchloric acid (1.5 ml), followed by a 2% solution of phenylhydrazine hydrochloride in 10 M-H_2SO_4 (0.1 ml). After a reaction time of 10 min at room temperature, the absorbance at 410 nm was read. A calibration curve was obtained similarly with standard pyridoxal phosphate solutions.

Abbreviations used: DMSO, dimethyl sulphoxide; DNP, dinitrophenol; PQQ, pyrroloquinoline quinone.
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Bioassay (Barton-Wright, 1961)

Pig plasma amine oxidase was dialysed against a 2000 volume excess of potassium phosphate buffer (5 mM, pH 7.4). Portions (100 μl) of the dialysed enzyme (2.3 mg/ml) were mixed with 12 M-hydrochloric acid (AristaR; 100 μl) and heated in sealed ampoules at 100 °C for 21 h. The acid was evaporated away under reduced pressure and the residue dissolved in distilled water (100 μl). Portions (5 μl) of the hydrolysate were sterilized and 5 ml of sterilized vitamin B6 assay medium added (Barton-Wright, 1961). The medium was inoculated with 1 drop of a cell suspension of the yeast *Kloekera apiculata* and incubated at 25 °C with vigorous shaking in the dark for 22 h. The absorbance values at 540 nm were then measured and compared against calibration curves based on pyridoxal phosphate, pyridoxine and pyridoxamine, which had also been hydrolysed in 6 M-hydrochloric acid before the bioassay.

^{31}P n.m.r. spectroscopy

The ^{31}P n.m.r. spectra were recorded on a Bruker WH400 n.m.r. spectrometer at 162 MHz in a 10 mm tube with a broad band (43–170 MHz) probe. A 45° pulse was applied every 0.819 s and the spectra accumulated overnight (approx. 15 h). The chemical shifts are referenced to external H_3PO_4 and the sign convention of high frequency being positive is used. The n.m.r. samples were prepared as follows: 100 mg of pig plasma amine oxidase was dialysed against 5 × 2000 volume excess changes of Tris/HCl buffer (5 mM, pH 7.2) over a 48 h period in order to remove inorganic phosphate. Cu^{2+} was removed from the enzyme by dialysis against potassium cyanide under dithionite-reduced conditions, as described by Suzuki *et al.* (1983), and the apoenzyme separated from low molecular weight species by chromatography on a G-25 column equilibrated with 5 mM-Tris/HCl buffer, pH 7.2. The apoenzyme sample was concentrated to approximately 0.2 ml by vacuum dialysis (Sartorius ultrafilter; Göttingen, Germany) and 1 ml of 99% enriched deuterium oxide (D_2O) added. The sample was again concentrated to a small volume. This D_2O exchange procedure was repeated three times and the sample finally diluted to 1.2 ml with D_2O. The ^{31}P n.m.r. spectrum was run immediately at 5 °C; 0.2 mg of Na_2HPO_4 was then mixed with the sample and the spectrum re-run under identical conditions. As a control, the ^{31}P n.m.r. spectrum of a known pyridoxal phosphate-containing enzyme was run. Aspartate–2-oxoglutarate aminotransferase (30 mg) was dialysed against three changes (each of 2000 volume excess) of Tris/HCl buffer (5 mM, pH 7.2), concentrated to low volume and exchanged into D_2O as described above for the plasma amine oxidase. To the exchanged sample (volume 1.2 ml) were added 0.025 mg of pyridoxal phosphate from stock solution (5 mg of pyridoxal phosphate in 1 ml of D_2O) in order to replace loosely bound cofactor lost during dialysis. The ^{31}P n.m.r. spectrum was run under conditions identical with those described for the amine oxidase, and then 0.04 mg of Na_2HPO_4 was added and the spectrum re-run.

Resonance Raman spectroscopy

The 2,4-dinitrophenylhydrazone (DNP) and phenylhydrazone derivatives of pig plasma amine oxidase were prepared by adding a 10% molar excess of the appropriate hydrazine (dissolved in absolute ethanol) to the enzyme in 0.05 M-Pipes buffer (pH 7.0). Final protein concentrations were 0.08 mM. Whereas the phenylhydrazone adduct was used immediately, the DNP derivative was incubated at room temperature for 3 h before the Raman experiments. The reactions were monitored by absorption spectroscopy: $\lambda_{max.}$(DNP) = 462 nm, $\lambda_{max.}$(phenylhydrazone) = 437 nm. Sodium sulphate (final concentration ≈ 0.08 M) was added to each solution to provide an internal frequency and intensity standard. Resonance Raman spectra were collected as previously described (Moog *et al.*, 1986). The 457.9 nm Ar^+ laser line provided 50 mW of excitation power, and the spectral band width of the monochromator was set to 4 cm^{-1}. Only a single scan at 1 s/cm^{-1} was necessary because of the excellent signal-to-noise ratio obtained. The comparison resonance Raman spectra (given below in Figs. 4, 5 and 6) have been published by Moog *et al.* (1986).

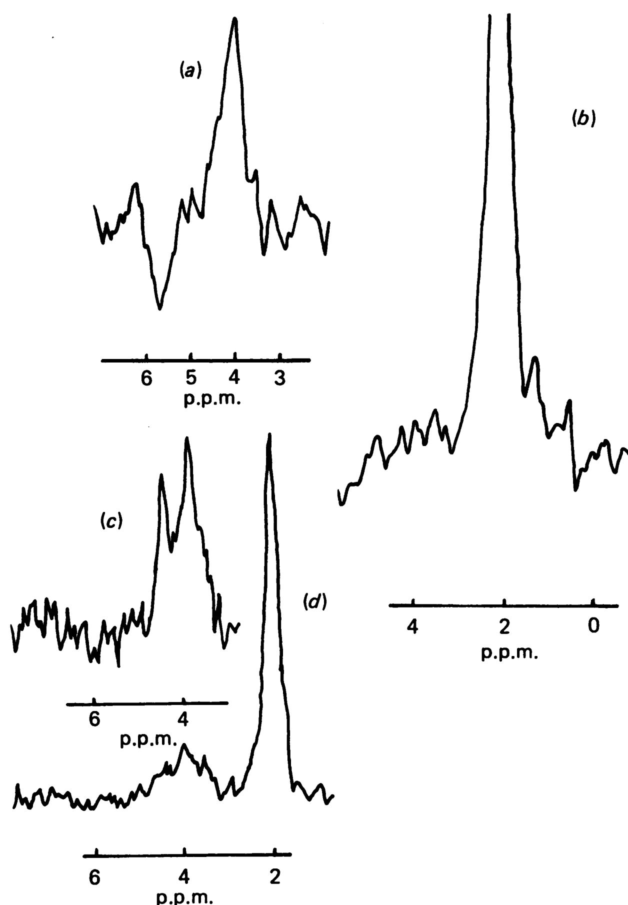

Fig. 2. ^{31}P n.m.r. spectra

(*a*) Pig plasma amine oxidase (100 mg in 1.2 ml of 5 mM-Tris/HCl buffer, pH 7.2). (*b*) As in (*a*) but with further addition of 0.2 mg of Na_2HPO_4. (*c*) Aspartate–2-oxoglutarate aminotransferase (30 mg in 1.2 ml of 5 mM-Tris/HCl buffer, pH 7.2). (*d*) As in (*c*) but with further addition of 0.04 mg of Na_2HPO_4.

RESULTS

Phenylhydrazine assay

Glycogen phosphorylase released 0.93 mol of pyridoxal phosphate per subunit, which compares favourably

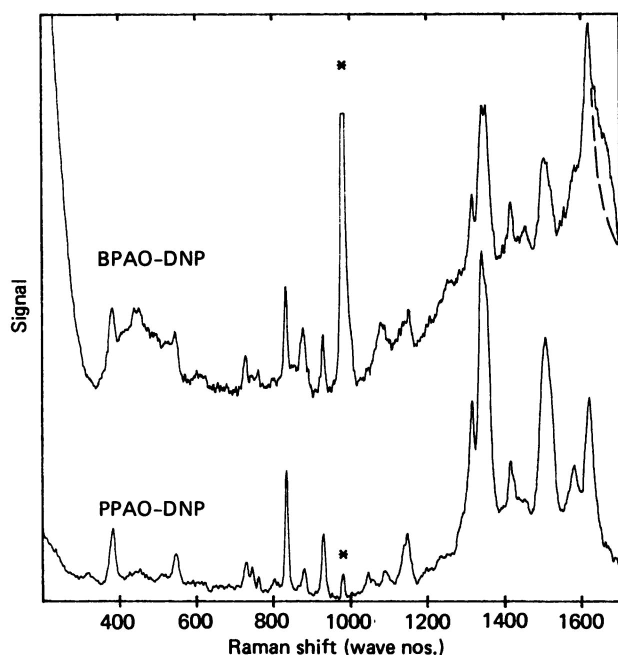

Fig. 3. Resonance Raman spectra of plasma amine oxidase 2,4-dinitrophenylhydrazones

Top: beef plasma amine oxidase (BPAO-DNP); bottom: pig plasma amine oxidase (PPAO-DNP). $*SO_4^{2-}$ peaks. The true band shape of the $1620\,cm^{-1}$ peak in the beef plasma enzyme spectrum is indicated by --- (top right); significant intensity $> 1620\,cm^{-1}$ was not observed in other spectra that are otherwise identical with that shown here (e.g. Fig. 5). Spectra were obtained at ambient temperature using 457.9 nm excitation and have been subjected to a 9-point smoothing routine (Savitzky & Golay, 1964).

with literature values (Cohen *et al.*, 1971). Pig plasma amine oxidase under the same conditions released < 0.012 mol of pyridoxal phosphate per subunit, which is taken to be an insignificant amount.

Bioassay

Pyridoxal phosphate, pyridoxine and pyridoxamine can all be assayed by the procedure described (calibration curves not shown). If the pig plasma amine oxidase contained 1 mol of pyridoxal phosphate/mol of protein, the portions taken should have released 0.062 nmol of this cofactor, which is in the bioassay calibration range. However, triplicate assays on two separate hydrolysed samples gave no detectable growth in the bioassay.

^{31}P n.m.r. studies

The ^{31}P n.m.r. spectrum of copper-depleted pig plasma amine oxidase is shown in Fig. 2(*a*) and reveals a small peak with a chemical shift of ~ 4 p.p.m. Addition of inorganic phosphate to the sample causes this peak to be replaced by one with a chemical shift of ~ 2 p.p.m. (Fig. 2(*b*). The small peak with a chemical shift of 4 p.p.m. could be explained reasonably by phosphate in a strongly hydrogen-bonded environment; the shift to a single peak with chemical shift at 2 p.p.m. on addition of inorganic phosphate is consistent with fast exchange ($> 10^4$ s) between free and bound inorganic phosphate environments. Although the removal of Cu^{2+} from the enzyme before the n.m.r. study eliminates the possibility that an organic phosphate peak has been broadened beyond detection owing to its proximity to a paramagnetic centre, the possibility remains that slow tumbling of an organic phosphate cofactor firmly bound to the protein obscures its detection. The control studies on aspartate–2-oxoglutarate aminotransferase, a pyridoxal phosphate enzyme of high molecular weight (Kirsch *et al.*, 1984), are shown in Figs. 2(*c*) and 2(*d*) and render the latter possibility unlikely. It can be seen (Fig. 2*c*) that the aminotransferase sample gives a clear ^{31}P

Table 1. Vibrational frequencies (cm^{-1}) observed in the resonance Raman spectra of the 2,4-dinitrophenylhydrazone derivatives of plasma amine oxidases and of possible carbonyl cofactors

Excitation profiles suggest that frequencies connected by the broken lines (----) are associated with the same mode, despite the relatively large shift between PQQ and the enzymes.

Pig plasma amine oxidase-DNP	Beef plasma amine oxidase-DNP	PQQ-DNP	Pyridoxal-DNP
320		343	
383	382	362	
	439	442	
452	452	471	472
510			
546	547	535	
~ 620	603	576	583
			702
729	729	724	719
745	743	750	743
762	762	764	
803	802	800	
835†	834†	841†	838†
	856	860	856
880 ----	---- 877 ----		
930	928†	928†	928†
		---- 954	
1046		1031	‡
1091	1078		1104
	1135	1122	
1148†	1152†	1142†	1145†
		1162	
1198	1207	1208	
1234	1251	1255	1230
1282			1281
1317	1317	1322	1310
1341†	1342†	1340†	1337†
1354	1351	1354	1384
1416	1415	1410	
1455	1454	1429	1473
1506	1503	1489	1509
			1521
			1549
			1579
1581	1580	1596	1603
1620	1620	1616	1620

* Pyridoxal-DNP data were compiled from spectra obtained in CH_3OH and DMSO.

† Feature characteristic of all DNP derivatives (Moog *et al.*, 1986).

‡ 100–1060 cm^{-1} region is obscured by solvents

n.m.r. peak with a chemical shift of ~4 p.p.m. Addition of inorganic phosphate (Fig. 2*d*) leads to a second peak with a chemical shift of ~2 p.p.m. The n.m.r. behaviour of the two enzymes is clearly distinct and the results indicate that, had pyridoxal phosphate been present in the plasma amine oxidase, it would have been detected by its ^{32}P n.m.r. signal.

Resonance Raman results

Resonance Raman spectra of the 2,4-dinitrophenylhydrazones of pig plasma and beef plasma amine oxidase are displayed in Fig. 3. The energies of the bands in these two spectra are very similar. However, there are some significant differences in the relative intensities of the resonance-enhanced modes, especially in the 1300–1700 cm^{-1} region. Table 1 lists the energies of the peaks in the spectra shown in Fig. 3. Minor differences in peak positions between the enzyme spectra are mainly attributable to the better signal-to-noise ratio in the resonance Raman spectrum of pig plasma amine oxidase-DNP. Also presented in Table 1 are the energies of the peaks observed in the resonance Raman spectra of PQQ-DNP and pyridoxal-DNP. With few exceptions, each peak in both enzyme spectra can be correlated to a peak in the PQQ-DNP spectrum within 20 cm^{-1}, with most correlations within 10 cm^{-1}. Vibrational frequency shifts of this magnitude between the free and protein-bound forms of a chromophore are well documented (Tu, 1982; Carey, 1982; Spiro & Stein, 1977) and have been predicted theoretically (Hirakawa & Tsuboi, 1975). It is not possible to correlate satisfactorily the enzyme-DNP and pyridoxal-DNP vibrational energies, particularly in the three spectral regions previously identified as being diagnostic for the structure of the parent carbonyl compound: 300–600 cm^{-1}, 660–800 cm^{-1} and 1300–1700 cm^{-1} (Moog *et al.*, 1986). Fig. 4 shows a comparison in the crucial 1300–1700 cm^{-1} region between the spectra of the various DNP derivatives. The PQQ-DNP spectrum provides a clearly superior match to the enzyme spectra, compared with pyridoxal-DNP. Resonance Raman spectra of the beef and pig plasma amine oxidase phenylhydrazone derivatives are presented in Fig. 5. In contrast with the results for the DNP derivatives, both the peak positions and their relative intensities are essentially identical in the phenylhydrazone spectra.

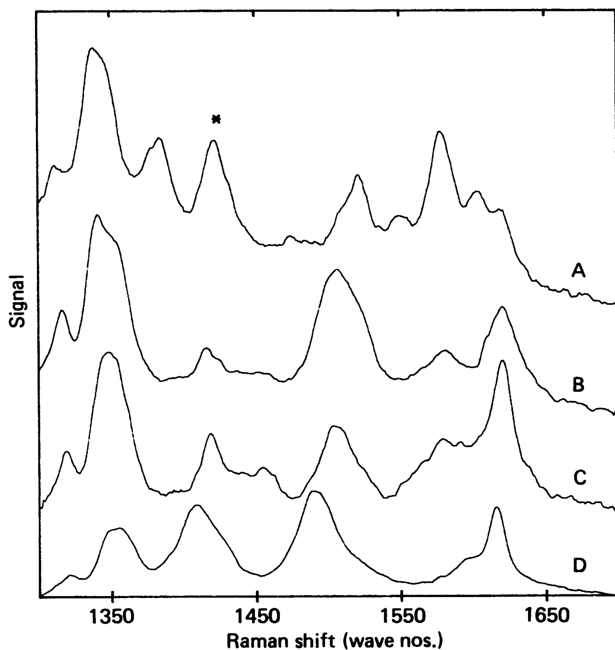

Fig. 4. Resonance Raman spectra of 2,4-dinitrophenylhydrazone (DNP) derivatives in the 1300–1700 cm^{-1} region

A, pyridoxal-DNP; B, pig plasma amine oxidase-DNP; C, beef plasma amine oxidase-DNP; D, PQQ-DNP. *Solvent peak. Spectra obtained in other solvents show no comparable feature in this region. See Fig. 3 for experimental details.

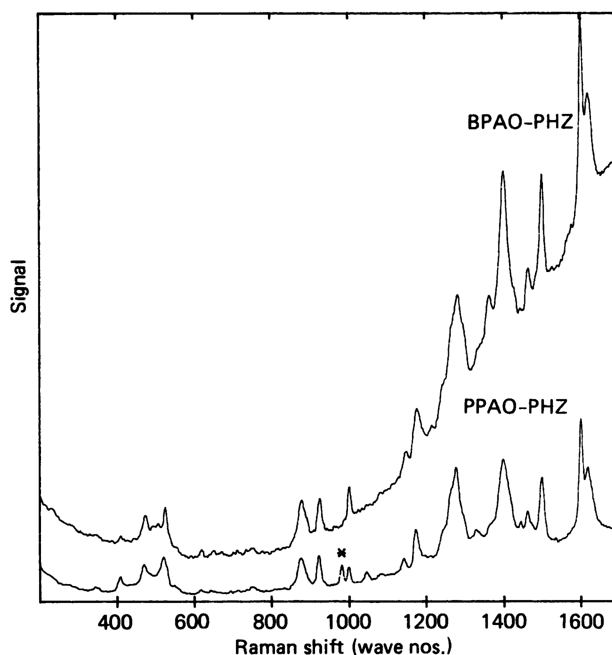

Fig. 5. Resonance Raman spectra of plasma amine oxidase phenylhydrazones

Top: beef plasma amine oxidase (BPAO-PHZ); bottom: pig plasma amine oxidase (PPAO-PHZ). See Fig. 3 for experimental details.

DISCUSSION

The phenylhydrazine assay rules out loosely bound pyridoxal phosphate in pig plasma amine oxidase but leaves open the possibility that the pyridoxal phosphate has remained attached to protein during precipitation by perchloric acid. The assay also indicates that PQQ is not liberated from the enzyme by treatment with 0.6 M-perchloric acid, since PQQ would also react with phenylhydrazine to give absorbance at 410 nm (Moog *et al.*, 1986). The bioassay for pyridoxal derivatives was negative, even though the protein had been completely hydrolysed. Similarly, the ^{32}P n.m.r. study failed to reveal any organic phosphate in the enzyme. It might, of course, be argued that removal of copper from the sample prepared for n.m.r. has simultaneously removed the organic cofactor, but this is refuted by the observation (Suzuki *et al.*, 1983; P. F. Knowles, F. X. Rius & I. Singh, unpublished work) that activity is restored to the apoenzyme by simple addition of cupric ions.

Thus we have failed to obtain any evidence in favour of pyridoxal phosphate as organic cofactor. Suva & Abeles (1978) reported that bioassays for pyridoxal phosphate in hydrolysates of bovine plasma amine oxidase gave negative results. From this and other evidence (Berg & Abeles, 1980) it was concluded that the beef plasma enzyme operated by an imine mechanism which does not require the participation of any organic cofactor. However, the imine mechanism predicts liberation of product ammonia under anaerobic conditions; in the case of pig plasma amine oxidase, strong evidence against this mechanism has come from rapid quench kinetic studies of ammonia release (Rius *et al.*, 1984). An amino transferase mechanism requires a carbonyl grouping at the active site. The resonance Raman evidence now presented shows that the carbonyl grouping is provided by an organic cofactor similar to PQQ in both pig and bovine plasma amine oxidases.

Resonance Raman spectra of the phenylhydrazone and 2,4-dinitrophenylhydrazone derivation of bovine plasma amine oxidase have been measured previously. Detailed comparisons with the spectra of the corresponding derivatives of PQQ, pyridoxal and other aldehydes and diones confirm that covalently bound PQQ is the organic cofactor in the bovine amine oxidase (Moog *et al.*, 1986). These comparisons have now been extended to include derivatives of pig plasma amine oxidase. The relatively small differences in vibrational energies between the 2,4-dinitrophenylhydrazone derivatives of PQQ and both pig and bovine plasma amine oxidase (see Table 1) are well within the range generally observed for the differences in energy between the vibrational modes of free versus protein-bound chromophores (Spiro & Stein, 1977; Tu, 1982; Carey, 1982). This evidence suggests strongly that PQQ, or a closely similar species, is the cofactor in both pig and bovine plasma amine oxidase.

The intensity of a mode in a resonance Raman spectrum depends on the differences between the structures of the ground state and the relevant electronic excited state, i.e. the excited state (or states) produced by laser excitation (Heller, 1981; Heller *et al.*, 1982). Consequently, the relative band intensities in resonance Raman spectra are determined by the excited state displacements of the nuclei from the ground state equilibrium geometry, projected on the ground state normal modes (Heller, 1981; Heller *et al.*, 1982). Thus it is significant that the relative intensities in the enzyme–phenylhydrazone spectra (Fig. 6) are practically identical, but that this is not the case in the enzyme–DNP spectra (Fig. 4). A plausible explanation is that the nitro groups interact differently (e.g. via steric or electrostatic forces) with local protein structural elements in the two enzymes, such that particular nuclear displacements may be more (or less) favoured in one protein environment compared with the other. Once vibrational assignments are available, detailed resonance Raman studies of derivatized amine oxidases should be an informative approach to investigating the active site structures. Some variation in the active site structure is expected since the enzymes have distinct substrate specificities (Knowles & Yadav, 1984).

The evidence herein presented substantiates the reports by Lobenstein-Verbeek *et al.* (1984) and by Ameyama *et al.* (1984) that the organic cofactor in plasma amine oxidases is PQQ. Inhibition of amine

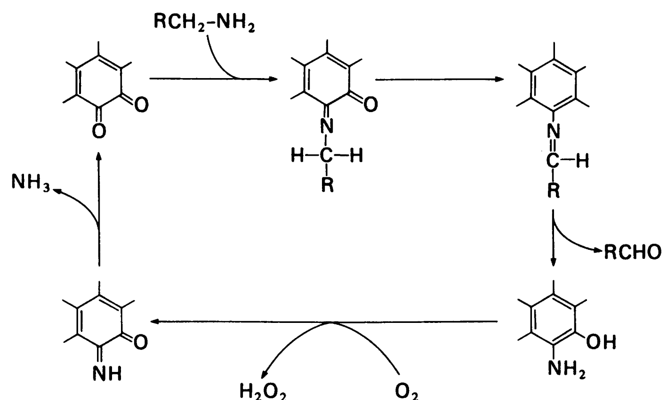

Fig. 6. Possible catalytic mechanism for plasma amine oxidase

Amine substrate reacts with enzyme-bound pyrroloquinoline quinone and is converted into aldehyde product. The copper cofactor is involved in the reactions following oxygen binding.

oxidases by phenylhydrazine and other carbonyl reagents, which has in the past been regarded as evidence for pyridoxal phosphate, is equally consistent with PQQ as the cofactor, and the report by Suzuki *et al.* (1983) that copper-depleted beef plasma amine oxidase has a visible chromophore which can be bleached by dithionite is consistent with PQQ but not with pyridoxal phosphate as cofactor. Collectively, the spectroscopic, chemical and chromatographic evidence for PQQ in plasma amine oxidases is convincing. Given the numerous similarities among amine oxidases isolated from various sources (Knowles & Yadav, 1984), it is probable that PQQ is the cofactor in all these enzymes.

For purposes of discussion, the following catalytic mechanism for plasma amine oxidase can be proposed, based on the order of product release (Rius *et al.*, 1984) and the presence of PQQ as a cofactor (Fig. 6). The role of copper in this mechanism is not fully defined, although it is known to be involved in re-oxidation of the reduced enzyme by oxygen (Barker *et al.*, 1979).

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