Studies on the active site of pig plasma amine oxidase

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Amine oxidase from pig plasma (PPAO) has two bound Cu^{2+} ions and at least one pyrroloquinoline quinone (PQQ) moiety as cofactors. It is shown that recovery of activity by copper-depleted PPAO is linear with respect to added Cu^{2+} ions. Recovery of e.s.r. and optical spectral characteristics of active-site copper parallel the recovery of catalytic activity. These results are consistent with both Cu^{2+} ions contributing to catalysis. Further e.s.r. studies indicate that the two copper sites in PPAO, unlike those in amine oxidases from other sources, are chemically distinct. These comparative studies establish that non-identity of the Cu^{2+} ions in PPAO is not a requirement for amine oxidase activity. It is shown through the use of a new assay procedure that there are two molecules of PQQ bound per molecule of protein in PPAO; only the more reactive of these PQQ moieties is required for activity.

INTRODUCTION

Amine oxidase from pig plasma (PPAO) has been shown to contain bound Cu^{2+} ions (Buffoni *et al.*, 1968; Lindström & Pettersson, 1978) and pyrroloquinoline quinone (PQQ) (Knowles *et al.*, 1987) as cofactors. The holoenzyme, which is composed of two identical or closely similar subunits, has two Cu^{2+} ions per molecule of protein in chemically distinct non-interacting environments (Barker *et al.*, 1979). It is clearly important to establish whether one or both Cu^{2+} ions are required for catalytic activity; the reports by Lindström & Pettersson (1973, 1978) that the enzyme has a single active-site carbonyl grouping (now known to be PQQ) suggest that a single active-site Cu^{2+} ion might be adequate. For amine oxidase from bovine plasma, however, there is evidence that forms of the enzyme having a single bound Cu^{2+} ion are inactive (Suzuki *et al.*, 1986; Morpurgo *et al.*, 1987). The present paper addresses such questions of cofactor stoichiometry in PPAO with respect to enzyme activity. Enzyme activity as well as spectral properties characteristic of the holoenzyme are found to be reconstituted linearly through addition of Cu^{2+} ions to the apoenzyme. It is also shown through the use of a new assay procedure that there are two molecules of PQQ bound per molecule of enzyme; only the more reactive of these PQQ moieties is required for activity.

MATERIALS AND METHODS

Materials

PQQ was purchased from Fluka A.G., Buchs, Switzerland, and used as a stock solution (1 mM) in double-distilled water. 2-Hydrazinopyridine (2-HP), obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.,

was converted into the hydrochloride salt and recrystallized from methanol; a stock solution of the salt (10 mM) in double-distilled water was prepared. Other chemicals (reagent grade) were purchased from BDH Chemicals, Poole, Dorset, U.K. Benzylamine was converted into the hydrochloride salt and recrystallized three times from aqueous ethanol. All reagents used in studies of the copper-depleted forms of the enzyme, including those for enzyme activity assays, were prepared in double-distilled water and passed through chelating resin (Sigma Chemical Co., Poole, Dorset, U.K.) to remove extraneous metal ions.

PPAO was purified by the method of Rius *et al.* (1984) and assessed to be pure by analytical ultracentrifugation and SDS/polyacrylamide-gel electrophoresis as described by Barker *et al.* (1979). Loosely bound copper was removed by dialysis against 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA; after such treatment the copper content of the holoenzyme was found to be 2.1 ± 0.1 g-ions/mol of protein. The M_r of the enzyme was taken to be 186 000 (Barker *et al.*, 1979). The copper contents of holo, apo and reconstituted forms of the enzyme were determined by atomic absorption spectrometry on either a Pye–Unicam SP.2900 or a Perkin–Elmer 2380 instrument and checked by chemical assay by using the procedure of Van de Bogart & Beinert (1967). Methods for the determination of protein concentration and enzyme activity are described in Barker *et al.* (1979).

Amine oxidases from pea seedlings and bovine plasma were prepared as described by Kluetz *et al.* (1980) and Summers *et al.* (1979) respectively. *Arthrobacter* P1 methylamine oxidase was purified by a new procedure (McIntire, 1989). Removal of extraneous copper from these amine oxidase samples followed by concentration for e.s.r. study used similar procedures to those described for PPAO.

Abbreviations used: PPAO, pig plasma amine oxidase; PQQ, pyrroloquinoline quinone; 2-HP, 2-hydrazinopyridine.

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Preparation of apoenzyme

The procedure is based on that of Suzuki *et al.* (1983) for removal of copper from the bovine plasma enzyme. Enzyme (typically 1 ml at a concentration of approx. 50 mg/ml) in 0.1 M-potassium phosphate buffer, pH 7.0, was reduced overnight at 4 °C under anaerobic conditions through addition of a 25-fold molar excess of solid $\text{Na}_2\text{S}_2\text{O}_4$; e.s.r. study on a control sample indicated that the copper was at least 90% reduced by this treatment. The reduced enzyme was dialysed anaerobically for 12–20 h at 4 °C against 1 litre of 1.0 M-potassium phosphate buffer, pH 7.0, containing 25 mM-KCN and 2.5 mM- $\text{Na}_2\text{S}_2\text{O}_4$. Cu^{2+} ions and cyanide were removed and the buffer was exchanged by passage through a Sephadex G-25 column equilibrated with 0.05 M-Pipes/KOH buffer, pH 7.0.

Reconstitution of the apoenzyme

The apoenzyme (10 mg/ml) in 0.05 M-Pipes/KOH buffer, pH 7.0, was reconstructed by slow addition with stirring of a stock solution of $^{63}\text{Cu}(\text{NO}_3)_2$ (8 mM in water, pH adjusted to 7.0) followed by incubation at room temperature (approx. 22 °C); the $^{63}\text{Cu}(\text{NO}_3)_2$ was a gift from Dr. N. J. Blackburn. The reconstituted enzyme was concentrated on either a Sartorius (Göttingen, Germany) type SM 16305 ultrafilter or an Amicon (Lexington, MA, U.S.A.) model M-3 ultrafilter. The protein concentration was 0.5–1.0 mM for the e.s.r. and 0.04–0.3 mM for the optical spectroscopic studies. Alternative methods of reconstitution, including addition of Cu^{2+} ions to concentrated samples of the apoenzyme, were explored in attempts to avoid the ultrafiltration step, but it was found that these samples gave non-reproducible results.

E.s.r. measurements were obtained with a Varian E112 spectrometer operating at Q-band (35 GHz) frequencies. The sample temperature during measurement was controlled at 150 K. Simulations of the e.s.r. spectra were performed on the Amdahl 470/V7-CDC 7600 computer at the University of Manchester as described in Collison & Mabbs (1982) and Gahan & Mabbs (1983). All simulations have been performed on the assumption of coincidence between the principal axes of g and A tensors. These two parameters are treated as variables in the simulation procedures together with principal linewidth (ΔB_i) components capable of having a fractional ratio of Gaussian to Lorentzian lineshape functions. Adequate simulations were achieved by using the criterion of best visual fit.

Optical absorbance measurements were made on a Pye-Unicam SP.8-100 or a Cary 219 (interfaced to an IBM/AT model 339 personal computer) spectrophotometer with matched 10 mm-pathlength quartz cells.

Assay conditions for free PQQ

Portions containing not more than 0.5 μmol of PQQ were taken from the stock solution, and 1.0 ml of the stock 2-HP solution was added; the pH of the reaction mixture was approx. 7. The reaction mixture was incubated at 55–60 °C for 2 h and then cooled to room temperature, 2.0 ml of 0.1 M-sodium phosphate buffer, pH 7.0, was added and the volume was adjusted to 5.0 ml with distilled water. The absorbance was measured at 415 nm against distilled water. These conditions are the standard ones used for the assays.

Alternatively, a methanolic solution of 0.55 mM-PQQ

and 11 mM-2-HP was heated for 1 h at 60 °C. The resulting orange precipitate was dissolved in double-distilled water and the pH was adjusted to 8.0 with NaOH.

Assay conditions for PQQ in PPAO samples

Enzyme samples containing approx. 0.25 μmol of protein were allowed to react with 1.0 ml of the stock 2-HP solution for 4 h at room temperature (approx. 22 °C). Then 2.0 ml of 0.1 M-sodium phosphate buffer, pH 7.0, was added and the volume was adjusted to 5.0 ml. The absorbance of the solution, which remained transparent throughout, was measured at 415 nm. This procedure gives the standard assay conditions.

Alternatively, enzyme samples in 0.1 M-potassium phosphate buffer, pH 7.2, were allowed to react with a 20-fold excess of 2-HP, added so that the dilution of the enzyme sample was no more than 10%.

RESULTS

Properties of the apoenzyme

The apoenzyme had 0.12 and 0.09 g-ion of copper/mol of protein according to atomic absorption spectrometry and chemical assay respectively. The small difference between these assay results is probably due to interference by protein in the atomic absorption measurement or to random errors. The apoenzyme typically had a specific activity of 50 units/mg immediately after preparation compared with the specific activity of the holoenzyme of 1800 units/mg.

Studies on the reconstituted enzyme

Enzyme activity. It was found that recovery of activity is slow (Fig. 1), requiring at least 6 days at room temperature to attain equilibrium. The linear dependence of activity on copper content is shown in Fig. 2; it should be noted that these activities are the equilibrium values.

Electron absorption spectra. The 480 nm absorption band characteristic of the holoenzyme (Lindström *et al.* 1976) is lost during removal of copper (Fig. 2 inset), undoubtedly owing to the coincident reduction of the PQQ moiety by $\text{Na}_2\text{S}_2\text{O}_4$. Addition of Cu^{2+} ions, resulting

Fig. 1. Effect of incubation time at 22 °C on the specific activity of samples of copper-reconstituted PPAO

Apoenzyme was incubated with a 2-fold excess of Cu^{2+} ions. Enzyme activity is expressed in spectrophotometric units (Tabor *et al.*, 1954).

Fig. 2. Relationship between the Cu/enzyme molar ratio and the enzyme activity (○) and A_{480}/A_{430} ratio (◆) after 8 days incubation of PPAO at 22 °C

Enzyme activity is expressed in spectrophotometric units (Tabor *et al.*, 1954). The inset shows the visible-region absorption spectra of (1) apoenzyme, (2) single-copper form and (3) holoenzyme. The protein concentration was 70 μM .

in the reoxidation of the reduced PQQ, restores this absorption band. This reoxidation is irreversible under the conditions of the experiment. As copper content increases, the 480 nm band not only increases but becomes better defined. Therefore the peak-to-trough ratio (480 nm/430 nm) was used to determine the relationship between the PQQ chromophore and copper (Fig. 2).

35 GHz e.s.r. spectra. The 35 GHz e.s.r. spectra of various samples of PPAO are shown in Fig. 3. Visual inspection of all of the spectra indicated the presence of two spectral components, and it has proved possible to simulate the spectra on this basis (see Fig. 3 and Table 1). The strongest indicator of two components is the clear doubling on the fourth hyperfine feature in the 'z' region of the spectrum (arrowed in Figs. 3a and 4a). This feature of the PPAO spectrum is further emphasized by comparison with the 'z' region of the spectra of amine oxidases from a number of different sources (see Fig. 4), where the width of the hyperfine lines in the 'z' region remains constant in all cases except for PPAO. Clearly there is a difference in the e.s.r. behaviour of PPAO and the other amine oxidases, a difference that we attribute to 'distinct' copper sites in PPAO as opposed to 'identical' copper sites in amine oxidases from other sources. We rule out 'g' strain (Guigliarelli & Cannistrano, 1985; Cannistrano & Guigliarelli, 1986) as being the major contribution to the form of the e.s.r. spectrum of native PPAO, since this would lead to a progressive sequential line broadening across the 'z' region of the spectrum and not to the doubling effect on the fourth hyperfine line that we observe.

Considering the simulation parameters (Table 1), the values of the 'z' parameters are fairly well defined, and it is the relative signal amplitude in this region of the

spectrum that is the best indicator of the fractional composition in these two-component spectra. A word of caution is, however, necessary with regard to the simulation parameters reported for the 'x' and 'y' components of the spectra. There is a strong correlation between the linewidth, lineshape function and hyperfine splitting constants for these largely unresolved band envelopes, although the difficulty found in obtaining exact overlay simulations in this region does indicate that particular values of these simulation variables are characteristic of the species present.

For the native PPAO, simulation of the spectrum was achieved by a 50/50 mixture of an almost axial and a rhombic component (Fig. 3a). This result and the parameters used on the simulation are in good agreement with previous work reporting that the two Cu^{2+} ions in PPAO are chemically distinct (Barker *et al.*, 1979). Addition of excess benzylamine hydrochloride to the native PPAO under anaerobic conditions (Fig. 3b) leads to minor changes in the axial and rhombic components of the spectrum, the main difference from the native PPAO being that the 'g_⊥' region is better resolved. In this case also it is essential to invoke two distinct copper sites in order to simulate the whole spectrum (see Table 1). It should be emphasized that the copper remains bivalent after the addition of benzylamine, as has been shown in an earlier report (Buffoni *et al.*, 1968).

Apo-PPAO reconstituted with one Cu^{2+} ion/molecule of protein (Fig. 3c) gives a spectrum that is effectively the same as for the native PPAO except for a poorer signal-to-noise ratio and the presence of a trace of Mn^{2+} impurity. This result indicates that reconstitution of the 'one-copper' form, under the reported conditions, leads to 50/50 mixture of the same two copper sites found in the native enzyme. Addition of benzylamine hydrochloride under anaerobic conditions to the one-copper

Fig. 3. 35 GHz e.s.r. spectra of (a) holoenzyme, (b) holoenzyme reduced under anaerobic conditions with a 10-fold molar excess of benzylamine hydrochloride and (c) single-copper form of PPAO

The structure to high field in (c) is due to trace amounts of Mn^{2+} contaminant. Simulations of the various spectra according to the parameters in Table 1 are also included. The arrow indicates the region of the spectra that gives the clearest evidence for two underlying components. Note that the amplitude scales for (a), (b) and (c) are not the same.

form of PPAO led to an e.s.r. spectrum that differed from that of the similarly reduced form of native PPAO only in requiring a slightly larger value of ΔBx of component 1 for adequate spectral simulation (results not shown).

Fig. 4. 35 GHz e.s.r. spectra ($g_{\parallel}$ region only) of amine oxidases from different sources

(a) Pig plasma amine oxidase; (b) pea seedling amine oxidase; (c) *Arthrobacter* P1 amine oxidase; (d) *Arthrobacter* P1 amine oxidase on $10 \times$ expanded scale; (e) bovine plasma amine oxidase; (f) bovine plasma amine oxidase reduced anaerobically with a 10-fold excess of benzylamine hydrochloride. See Fig. 3 legend for explanation of the significance of the arrow.

Table 1. Parameters required to simulate the 35 GHz e.s.r. spectra of PPAO

Enzyme form	g_z	g_x	g_y	A_z (mT)	A_x (mT)	A_y (mT)	${}^D B_z$ (mT) (fractional Lorentzian)	${}^D B_x$ (mT) (fractional Lorentzian)	${}^D B_y$ (mT) (fractional Lorentzian)	Fractional composition
Holoenzyme										
Component 1	2.2760	2.059	2.030	16.2	1.5	1.5	7.0 (0.0)	10.0 (0.0)	7.5 (0.0)	0.5
Component 2	2.2862	2.079	2.068	14.8	2.0	2.0	7.0 (0.0)	16.5 (0.3)	16.5 (0.3)	0.5
Holoenzyme + benzylamine										
Component 1	2.2760	2.059	2.057	16.2	1.5	1.5	7.0 (0.3)	7.2 (0.0)	7.5 (0.0)	0.5
Component 2	2.2860	2.072	2.072	14.8	1.5	1.5	7.0 (0.3)	19.5 (0.0)	19.5 (0.0)	0.5

Fig. 5. Spectra of 2-HP derivatives of free and PPAO-bound PQQ

—, PPAO (415 nm form); ·····, PPAO (525 nm form);
 -----, PQQ (415 nm form); ———, PQQ (500 nm form). For
 further details on reaction conditions see the text.

Reaction between PQQ and 2-HP

The production of an orange chromophore with $\lambda_{\max}$ 415 nm through reaction between PQQ and 2-HP at pH 7.0 was found to be slow at room temperature but could be accelerated by warming at 55–60 °C. Maximum colour development under these conditions required 2 h. The absorbance at 415 nm was constant in the pH range 4.5–9.0. Below pH 3.0 the chromophore exhibited a shoulder at 390 nm, whereas above pH 11.0 the chromophore had absorbance bands at 460 nm and 500 nm. Addition of Cu^{2+} ions to the reaction mixture at pH 7.0 gave a red chromophore with $\lambda_{\max}$ 500 nm (Fig. 5). Maximal colour development required a 20-fold excess of 2-HP over PQQ. Beer's Law was found to be

Fig. 6. Time-dependence of the reaction of PPAO-bound PQQ with 2-HP

The reaction was carried out with a 40-fold excess of 2-HP over PPAO at 22 °C.

Table 2. PQQ content of PPAO and its derivatives

The assays were carried out under the standard conditions and the values given are the average of three determinations that agreed to within $\pm 5\%$. The protein concentration range used was 0.045–0.3 mM.

Enzyme form	PQQ content (mol/mol of enzyme)
1. Holoenzyme	1.9
2. Holoenzyme + 1000-fold SCN^-	2.3
3. Holoenzyme + 1000-fold CN^-	1.6
4. Holoenzyme + 600-fold N_3^-	2.2
5. 1 Cu-enzyme	1.7
6. 1 Cu-enzyme + 1000-fold SCN^-	1.7
7. 1 Cu-enzyme + 1000-fold CN^-	1.1
8. 1 Cu-enzyme + 600-fold N_3^-	1.9
9. Apoenzyme*	1.9
10. Reduced holoenzyme†	0.08

* Reaction of the apoenzyme with 2-HP under the standard assay conditions was slow. Full chromophore development required reaction either for 16 h at room temperature (approx. 22 °C) or 2 h at 60 °C.

† Holoenzyme was reduced by anaerobic incubation for 16 h at 4 °C in the presence of a 25-fold molar excess of $\text{Na}_2\text{S}_2\text{O}_4$; dithionite was removed by gel filtration under anaerobic conditions.

obeyed up to 0.5 μmol of PQQ under the standard assay conditions (see the Materials and methods section). The molar absorption coefficient at 415 nm was $1.54 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Job's (1928) method of continuous variation was used to establish that PQQ and 2-HP reacted with 1:1 stoichiometry. It was found that no colour developed after reduction of PQQ by $\text{Na}_2\text{S}_2\text{O}_4$ at

Fig. 7. Effect of 2-HP on the activity of PPAO

Enzyme activity is expressed in spectrophotometric units (Tabor *et al.*, 1954).

pH 7.0; also it was observed that addition of $\text{Na}_2\text{S}_2\text{O}_4$ after the standard assay bleached the chromophore.

PQQ in PPAO and its derivatives

PQQ in the native enzyme reacted rapidly with 2-HP at room temperature to give the chromophore with λ_{max} 415 nm; the wavelength maximum is identical with that observed with free PQQ (see Fig. 5). Prolonged warming of the enzyme/2-HP reaction mixture at 60 °C produced a purple chromophore (λ_{max} 525 nm), possibly the result of further reaction involving the copper cofactor (Fig. 5). E.s.r. studies showed that this reaction does not involve reduction of the copper, though the e.s.r. spectrum is perturbed (results not shown). The time-dependence in development of the 415 nm chromophore is shown in Fig. 6. It can be seen that there are two phases: a rapid phase completed within 60 s corresponding to reaction with one molecule of PQQ, then a slow phase reaching a plateau value after 200 min that corresponds to reaction with two molecules of PQQ. For these calculations a molar absorption coefficient at 415 nm of $1.54 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for PQQ (see above) was used. The presence of NH_4Cl (50 mM) in the reaction mixture did not alter the kinetics of the two phases (results not shown).

Copper had a catalytic effect on the rate of reaction of enzyme-bound PQQ with 2-HP. The apoenzyme reacts slowly with 2-HP at room temperature (see Table 2), possibly owing to partial reduction of PQQ during removal of copper. Addition of Cu^{2+} ions to the apoenzyme restored its facility to react with 2-HP at room temperature, which is consistent with reoxidation of reduced PQQ being catalysed by Cu^{2+} ions.

The amounts of PQQ determined in various samples of PPAO under the standard assay conditions are also given in Table 2. Azide is known to inhibit the enzyme activity through reaction with the copper cofactor (Barker *et al.*, 1979). Thiocyanate inhibits the enzyme activity and, by analogy with azide, this probably occurs through binding to copper (Dooley & Cote, 1985). The data show that neither azide nor thiocyanate has any effect on reaction of the PQQ cofactor with 2-HP. By contrast, the presence of cyanide, which also binds to the copper cofactors (Barker *et al.*, 1979), interferes with the 2-HP assay; this may be due to reaction of PQQ with cyanide. Reduction of holoenzyme with $\text{Na}_2\text{S}_2\text{O}_4$ eliminates reaction with 2-HP under the standard assay conditions; this is consistent with reduction of the PQQ cofactor. Dithionite interferes with the assay and prior removal is therefore essential.

Effects of 2-HP on PPAO activity

Fig. 7 shows the effects on enzyme activity of incubating PPAO with various amounts of 2-HP at room temperature for 10 min. It can be seen that activity is completely inhibited by one molecule of 2-HP per molecule of enzyme.

DISCUSSION

The data in Fig. 2 demonstrate that restoration of the 480 nm band characteristic of native enzyme (Lindström *et al.*, 1976) through addition of Cu^{2+} ions to apoenzyme parallels recovery of enzyme activity. These changes are relatively slow, requiring several days to reach equilibrium (Fig. 1); the reason for the slow reconstitution

may relate to the organic cofactor being present as PQQH₂ from the reducing conditions used to produce the apoenzyme (Suzuki *et al.*, 1983). Addition of one Cu^{2+} ion/molecule of apoenzyme restores 50% of the activity and 50% of the absorbance at 480 nm. These observations correlate reasonably well with conclusions drawn from 35 GHz e.s.r. spectra in apoenzyme reconstituted with one Cu^{2+} ion/molecule of enzyme, where 1:1 mixing of rhombic and 'close-to-axial' components gave good simulation of the experimental spectra. Taken together, the results indicate that there are two binding sites for Cu^{2+} ions in the apoenzyme and that the affinity for Cu^{2+} ions at these sites is similar. It is not possible at present to draw conclusions on whether one or both Cu^{2+} ions are required for enzyme activity. Addition of benzylamine to the holoenzyme leads to 35 GHz e.s.r. spectral changes that have been simulated (Table 1) on the basis of both the axial and rhombic sites being modified. The changes, however, are small, and alternative simulation parameters involving only one of the copper sites cannot be ruled out. Since benzylamine is known to bind to the PQQ cofactor in amine oxidases (Hartmann & Klinman, 1988), the changes in e.s.r. lineshape are consistent with the possibility of interactions between the PQQ and copper sites in PPAO. Our observations that activity and copper content correlate directly between no and two Cu^{2+} ions per enzyme molecule for PPAO are inconsistent with the observations by Suzuki *et al.* (1986) and Morpurgo *et al.* (1987) that the single-copper form of bovine plasma amine oxidase is inactive. This may be due to differences in methods used to prepare the single-copper forms for the two enzymes rather than to differences in active-site structure/function. Finally in this section it is noteworthy that the presence of non-identical copper sites in PPAO is in contrast with other amine oxidases in which the two copper sites appear by 35 GHz e.s.r. criteria to be in identical environments. Clearly, non-identity of the copper sites is not a prerequisite for amine oxidase function.

Turning now to the organic cofactor in PPAO, our studies on the reaction of 2-HP with the enzyme confirm the observations by Lindström & Pettersson (1973, 1978) and Falk (1983) that titration of a single bound PQQ moiety per molecule eliminates enzyme activity. The stability of 2-HP over phenylhydrazine [as used by Lindström & Pettersson (1973, 1978) and Falk (1983)] has allowed us to extend the time for reaction with enzyme, and this has revealed the presence of a second PQQ moiety. Two PQQ moieties are also detected in enzyme from which copper has been wholly or partially removed and for enzyme in the presence of anion inhibitors such as azide, which are known to co-ordinate to active-site copper (Barker *et al.*, 1979). It has been proposed (Finazzi Agrò *et al.*, 1977) that copper is co-ordinated to the organic cofactor in the active site of amine oxidases. Our data show that, if such co-ordination occurs, it does not prevent interaction between protein bound PQQ and 2-HP. A similar conclusion has been drawn by Williams & Falk (1986) on the basis of ^{19}F -n.m.r. relaxation measurements with fluorinated derivatives of phenylhydrazine. The difference in reaction rate with 2-HP of PQQ in its free and amine oxidase-bound forms might well be a further indication of copper-PQQ interactions in the active sites of amine oxidases (Dooley *et al.*, 1987).

The role of the less-reactive PQQ moiety in PPAO is unclear. An attractive possibility is that the enzyme exhibits half-of-the-sites reactivity (Fersht, 1977). The enzyme has previously been shown to exhibit non-linearity in Eadie-Hofstee plots, which indicates cooperativity or other complexities in the mechanism (Kelly *et al.*, 1981). In this context it may be noted that NH_4^+ ions, which activate the activity of PPAO (Kelly *et al.*, 1981; Yadav & Knowles, 1981), have no effect on the rate of reaction of the second PQQ moiety with 2-HP; thus at present there is no supporting evidence for a second, latent, active site in the enzyme. It should also be borne in mind that, although the enzyme appears to be homogeneous by SDS/polyacrylamide-gel electrophoresis and analytical ultracentrifugation (Barker *et al.*, 1979), the holo form as isolated may not exhibit full activity; our data could equally well be explained if the enzyme as isolated were composed of 50% active and 50% inactive forms. Although we regard this possibility as unlikely, further attempts to purify the enzyme must be explored.

Finally, the availability through the present investigations of a convenient and sensitive assay procedure effective with both free and bound forms of PQQ should be valuable in quantitative studies of this novel cofactor, which is being reported to occur in diverse biological systems (Hartmann & Klinman, 1988; Duine & Jongejan, 1989).

This work was supported by an S.E.R.C. grant to P. F. K., an N.I.H. grant (GM27659) to D. M. D., a N.A.T.O. grant to P. F. K. and D. M. D., a Commonwealth Academic Staff Fellowship to I. S. and a fellowship awarded to F. X. R. by C.I.R.I.T. of Catalonia, Spain; D. C. thanks The Royal Society for award of a University Research Fellowship. We thank Mrs. V. Blakeley for skilled assistance in preparation of the enzyme.

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Received 2 May 1989/26 June 1989; accepted 29 June 1989