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CHANGES IN THE COPPER CENTRES OF BENZYLAMINE OXIDASE FROM PIG PLASMA DURING THE CATALYTIC CYCLE

J. Grant[a], I. Kelly[a], P. Knowles[a], J. Olsson[b] and G. Pettersson[b]

[a]Astbury Department of Biophysics, University of Leeds, Leeds, U.K.

[b]Chemical Center, University of Lund, Lund, Sweden

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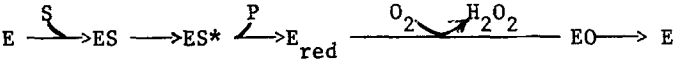
SUMMARY
The Cu^{2+} sites in benzylamine oxidase for various points in the catalytic cycle have been studied by 35GHz EPR spectroscopy in conjunction with the rapid freeze technique. No evidence has been obtained for reduction or oxidation of Cu^{2+} in any intermediate. The only change detected is in the E reduced intermediate for which the ligand environment of one of the two Cu^{2+} sites is modified. This observation provides direct evidence for the participation of Cu^{2+} in the catalytic mechanism and is consistent with other reports that the enzyme has only one active site.

Benzylamine oxidase (amine:oxygen oxidoreductase (deaminating) EC 1.4.3.6) from pig plasma has been shown to contain two tightly bound Cu^{2+} ions and one catalytically active carbonyl grouping (probably pyridoxal phosphate) (1,2). Kinetic studies of the enzyme have led to the detection of several intermediates in the catalytic cycle(3,4). The inter-relationship between these intermediates is illustrated in Scheme 1.



Scheme 1. Intermediates appearing during the catalytic cycle of benzylamine oxidase from pig plasma.

The first intermediate (ES) is probably a Schiff's base formed by rapid pre-equilibration between enzyme (E) and the amine substrate (S). The ES complex is converted into a spectrally modified form (ES*) at an early time in the reaction sequence; ES* appears to represent a tautomeric form of the primary Schiff's base. Subsequent release of the aldehyde product

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(P) leads to a reduced enzyme species (E_{red}), which lacks the 470 nm absorption band present in the native enzyme and the two enzyme-substrate complexes. E_{red} is the species which accumulates when enzyme is reacted with amine substrates under anaerobic conditions; it has been tentatively identified as a pyridoxamine complex. In the presence of oxygen, E_{red} is reoxidised to the native (oxidised) form by a comparatively slow process involving intermediate formation of a species (EO) which exhibits a 290 nm difference absorption band compared to native enzyme. Hydrogen peroxide and ammonia have been shown to be produced during the re-oxidation process, (5) hydrogen peroxide being released concomitantly with formation of EO.

From the results of the kinetic studies, the times at which maximal concentrations of these intermediates accumulate can be calculated. The aim of the present investigation was to use the rapid freeze technique to prepare samples containing maximal concentrations of each of the intermediates and to study them by electron paramagnetic resonance (EPR) spectroscopy. This is expected to allow further characterisation of the intermediates in terms of changes at the Cu^{2+} sites.

MATERIALS AND METHODS

Benzylamine oxidase was purified to homogeneity from pig plasma according to the procedure of Taylor et al. (6). Criteria of enzyme purity have been given previously (2). The enzyme was stored as a crystalline suspension in ammonium sulphate solution at 4°C. The crystalline enzyme had a specific activity of 0.097 units per mg protein; one unit is defined as the amount of enzyme required to catalyse the production of 1 µmole of benzaldehyde per min at 25°C under the standard assay conditions of Tabor et al. (7).

p-Methoxybenzylamine and benzylamine were purchased from BDH Chemicals (Poole, U.K.), converted to the hydrochloride salt and re-crystallised twice from aqueous ethanol before use. Other chemicals were of reagent grade quality.

Preparation of samples for the EPR studies

Enzyme was dialysed to remove ammonium sulphate, then passed down a column of Chelex 100 resin to eliminate extraneous Cu^{2+}; the column had been pre-equilibrated with 0.1 M potassium phosphate buffer (pH 9.0). The enzyme was concentrated to the required concentration by vacuum dialysis (Sartorius ultrafilter).

The "rapid-freeze" technique was used to obtain samples of enzyme

which had reacted at 22°C with p-methoxybenzylamine in air saturated potassium phosphate buffer (0.1 M, pH 9.0) for 16 and 76 ms, respectively. The final concentrations of enzyme were 1.4 and 0.8 mM, whilst the final concentrations of p-methoxybenzylamine were 10 and 15 mM, respectively. Computer simulations based on the results of stopped flow studies (3,4) indicated that these samples should contain 75% ES and 50% ES*, respectively. The rapid freeze apparatus was based on that described by Bray (8) but utilised a ram driven by an electric motor instead of a hydraulic system. The ram speed was measured using a linear transducer and recorded on a Tektronix 535A oscilloscope, which showed that the ram speed was uniform and allowed an accurate estimate of its value. The full procedure for calculating the times for reaction has been described by Bray (8). The reaction mixture was quenched into isopentane at -140°C, contained in a funnel connected by a tygon sleeve to a 35 GHz EPR tube. The frozen crystals were packed into the EPR tube using a stainless steel rod. Excess isopentane was removed from the tubes, which were then stored in liquid nitrogen prior to EPR study at 35 GHz.

A sample calculated to be at least 90% EO was prepared as follows. Benzylamine oxidase (1.5 mM) was oxygenated by three cycles of successive evacuation and exposure to a pure oxygen atmosphere, then transferred into a 35 GHz tube fitted with a dual taphead using a Hamilton 705 gas tight syringe. A pure oxygen atmosphere was maintained during the subsequent addition of oxygenated p-methoxybenzylamine (18 mM) in potassium phosphate buffer (0.1 M, pH 9.0) from a Pasteur pipette. The mixture was frozen after 5 s reaction time by immersion in liquid nitrogen and the EPR tube sealed under oxygen.

The preparation of a sample calculated to be 100% E_{red} followed a similar procedure to that described above for EO, except that a deoxygenated argon atmosphere (6) was used and a reaction time of 10 s allowed before freezing. Benzylamine (40 mM final concentration) was used as substrate in this sample, the final enzyme concentration being 1.1 mM.

Other samples were prepared for EPR study at 9 GHz. The procedures described above were used to obtain samples calculated to be 100% E_{red} and at least 90% EO. In addition, samples were prepared containing enzyme (about 0.1 mM) together with the substrate analogues amphetamine (10 mM) or phenylhydrazine (0.15 mM) in 0.1 M phosphate buffer (pH 9.0).

EPR spectroscopy

9 GHz EPR spectroscopy was performed at 77 K on a Varian E-3 spectrometer. 35 GHz spectra were run on a Varian 4500 series spectrometer in conjunction with a Varian 23 cm magnet and a low temperature accessory. The magnetic field was calibrated using a Lithium resonance probe (Newport Instruments, Newport Pagnell, U.K.) and the microwave frequency using a standard of α,α-diphenyl-β-picrylhydrazyl.

The concentration of EPR detectable Cu^{2+} in the samples was determined by comparison with a standard of Cu^{2+} (2 mM) in disodium EDTA (25 mM). Integrations of signal intensities were performed according to Wyard (9). Computer simulation of the 35 GHz EPR spectra was carried out as described by Barker *et al.* (2).

RESULTS

The 35 GHz EPR spectra obtained from the various samples are shown in Fig. 1. It should be noted that three separate samples for each of the reaction conditions summarised in the legend gave the same spectrum

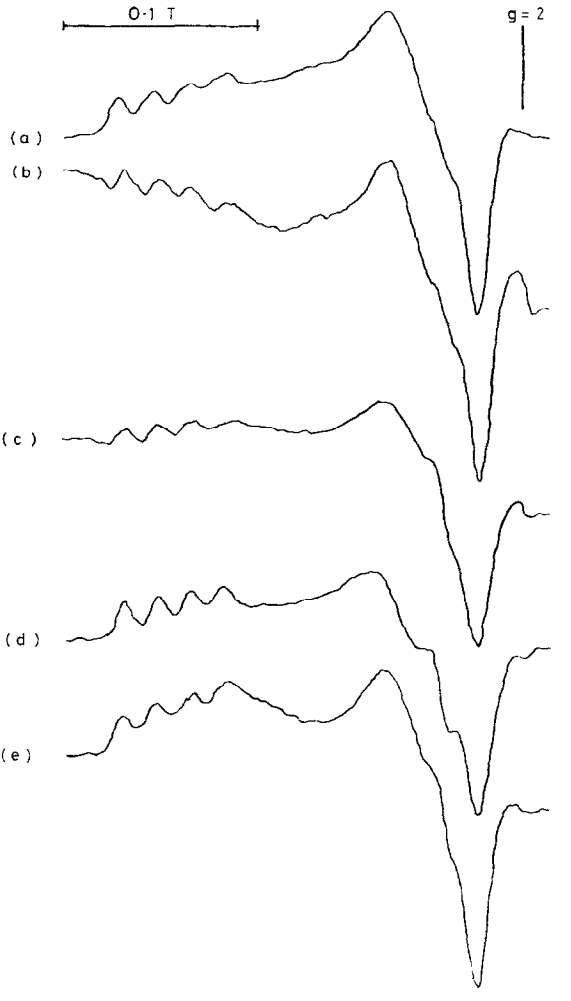


Fig. 1. 35 GHz EPR spectra of intermediates formed during the catalytic cycle of pig plasma benzylamine oxidase. (a) Native enzyme (1.07 m mol l^{-1}) (b) 16 mx "rapid freeze" sample (c) 75 ms "rapid freeze" sample (d) Anaerobically reduced enzyme (e) 5s "manual freeze" oxygenated sample. The EPR operating conditions were : temperature about 150 K, modulation 2.0 mT, microwave power 50 mW. For further details see text.

which gives us confidence in our conclusions. Spectra (a) - (c) and (e) are the same within experimental error and can be computer simulated on the basis of two components present in equal proportions, one showing axial symmetry, the other rhombic symmetry (see Fig. 2A). The EPR parameters used to achieve this simulation are given in Table 1. The conclusion drawn from these results is that the Cu^{2+} sites in the inter-

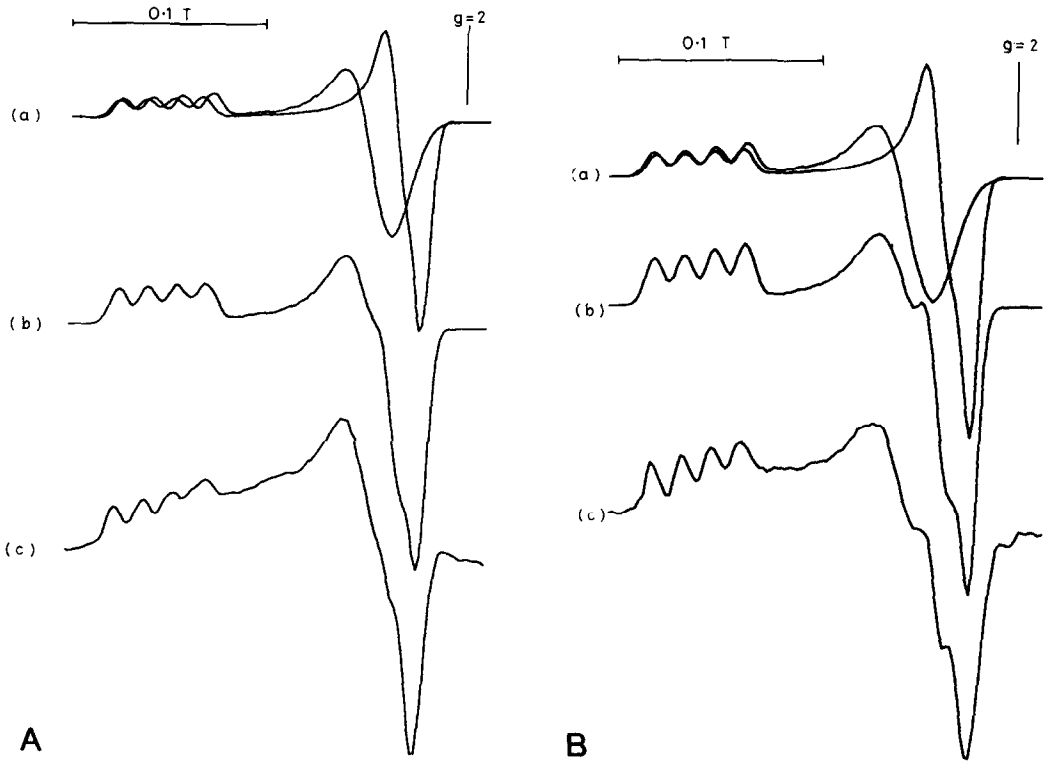


Fig. 2. Computer simulation of 35 GHz EPR spectra. A: Simulation of spectrum from native enzyme. The two component spectra shown in (a) were mixed in equal proportions to produce spectrum (b). The spectrum obtained experimentally is shown in (c). B: Simulation of spectrum from reduced enzyme. The two component spectra shown in (a) were mixed in equal proportions to produce spectrum (b). The spectrum obtained experimentally is shown in (c).

TABLE 1. EPR parameters used to simulate spectra of native and reduced benzylamine oxidase.

Spectrum	Component	$g_{\parallel}$		g_{zz}	Hyperfine splittings (mT) $A_{\parallel}$		A_{zz}	Line widths (mT) $W_{\parallel}$		W_{zz}
		g_{xx}	g_{yy}		A_{xx}	A_{yy}		W_{xx}	W_{yy}	
(A) Native	Axial	2.076		2.286	1.0		15.8	10.7		4.3
	Rhombic	2.038	2.063	2.294	1.0	1.0	14.5	3.6	5.0	4.3
(B) Reduced	Axial	2.082		2.292	1.0		15.5	12.0		4.3
	Rhombic	2.038	2.066	2.294	1.0	1.0	14.5	3.6	5.0	4.3

mediate species ES, ES* and EO are closely similar to those in the native enzyme. Moreover, the integrated signal intensities suggest that the amount of EPR detectable Cu^{2+} in these intermediate species is the same as in the native enzyme. Since the signal amplitude in 35 GHz EPR spectra has been found to be very sensitive to minor changes in instrument tuning, an independent check on this latter conclusion was performed at 9 GHz. The integrated signal intensity of 9 GHz spectra obtained from samples containing amphetamine or phenylhydrazine as well as a sample containing at least 90% of the EO intermediate are within experimental error (±5%) the same as that of native enzyme. Evidence has been presented to suggest that the enzymic complexes formed with amphetamine (2) and phenylhydrazine (10) are closely similar to the ES and ES* species, respectively.

By contrast, spectrum (d) in Fig. 1 (corresponding to the E_{red} species) is significantly different in the g_{xx}, g_{yy} region. Previous EPR studies at 9 GHz on benzylamine oxidase (11) and also on the diamine oxidase from pig kidney (12) have indicated that the Cu^{2+} site in E_{red} is chemically distinct from that in the native enzyme. The higher resolution available at 35 GHz allows this difference to be analysed. As shown in Fig. 2B, spectrum (d) can be simulated on the basis of two components present in equal proportions; the EPR parameters used in this simulation are given in Table 1. One of these components is identical with the rhombic component of the native enzyme, whilst the other has distinct EPR parameters from those of the native enzyme though retaining the axial symmetry of the site. This suggests that only one of the Cu^{2+} sites is modified during the conversion of ES* into E_{red}. The integrated signal intensities calculated from the 9 GHz spectra show that the copper present in E_{red} remains (within 5%) in the form of EPR detectable Cu^{2+}.

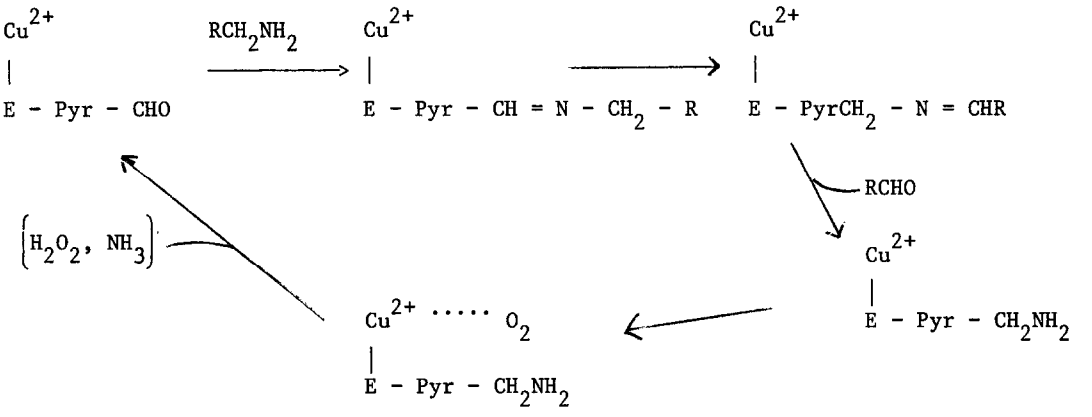
DISCUSSION

It has been well established that benzylamine oxidase from pig plasma contains two Cu^{2+} ions, both of which are fully EPR detectable (1, 2, 13,

14). The present integrations of EPR signals from native enzyme and the different intermediates formed during the catalytic action of the enzyme indicate that there is no detectable decrease in the concentration of Cu^{2+} at any stage of the catalytic process. The observation that there is no reduction of Cu^{2+} during formation of the reduced enzyme species E_{red} is consistent with previous reports (11, 14) and argues against Cu^{2+} acting as the sink for electrons donated by the amine substrate. Similarly, the present results exclude that there is any significant accumulation of enzyme species containing copper in the form of Cu^{3+}, which has been postulated to be an intermediate in the galactose oxidase reaction (15). It should, however, be noted that our rapid freeze experiments do not exclude the occurrence of either Cu^{+} or Cu^{3+} in enzyme species appearing transiently at low concentration during catalysis.

Previous studies have shown that the two Cu^{2+} sites in native benzylamine oxidase are chemically distinct (2). The present results provide evidence that one of these sites is modified during catalysis and that the change occurs concomitantly with the appearance and the disappearance of the intermediate E_{red}. This observation lends direct support to previous suggestions that at least one of the copper ions in benzylamine oxidase has a catalytic function, participating in the electronic rearrangements occurring on formation and reoxidation of the species E_{red} (2, 16). Since E_{red} is prabably a pyridoxamine intermediate, the modification of a Cu^{2+} site in this species is most simply explained by Cu^{2+} being proximal to the pyridoxamine as proposed by Hamilton (17) and by Finazzi Agro *et al.* (18). The observation that only one Cu^{2+} site is modified during formation of E_{red} is consistent with the finding that there is only one active site carbonyl grouping in the enzyme (1).

Scheme 2 summarises our present knowledge of the catalytic mechanism of benzylamine oxidase. The Cu^{2+} site which is modified during the formation of E_{red} is postulated to mediate the later electron transfer



Scheme 2. A possible catalytic mechanism for benzylamine oxidase from pig plasma. Pyr stands for the enzyme-bound pyridoxal phosphate ring.

from the reduced pyridoxal system to oxygen. Our studies have not suggested a role for the second (rhombic) Cu^{2+} site; the possibility exists that this Cu^{2+} forms part of a second active site which has been chemically modified during the isolation procedure or as a result of evolutionary changes of the enzyme structure.

REFERENCES

1. Lindström, A. and Pettersson, G. (1978). Eur.J.Biochem. 83, 131-135.
2. Barker, R., Boden, N., Cayley, G., Charlton, S.C., Henson, R., Holmes, M.C., Kelly, I.D. and Knowles, P.F. (1978). Biochem.J. accepted.
3. Olsson, B., Olsson, J. and Pettersson, G. (1976). Eur.J.Biochem. 71, 375-382.
4. Olsson, B., Olsson, J. and Pettersson, G. (1976). Eur.J.Biochem. 74, 329-335.
5. Lindström, A. and Pettersson, G. (1978). Eur.J.Biochem. 84, 479-485.
6. Taylor, C.E., Taylor, R.S., Rasmussen, C. and Knowles, P.F. (1972). Biochem. J. 130, 713-728.
7. Tabor, C.W., Tabor, H. and Rosenthal, S.M. (1954). J.Biol.Chem. 208, 645-661.
8. Bray, R.C. (1961). Biochem. J. 81, 189-193.
9. Wyard, S.J. (1965). J.Sci.Inst. 42, 769-770.
10. Lindström, A., Olsson, B. and Pettersson, G. (1974). Eur.J.Biochem. 42, 177-182.
11. Lindström, A. and Pettersson, G. (1978). Eur.J.Biochem. 35, 70-77.
12. Mondovi, B., Rotilio, G., Costa, M.T., Finazzi-Agro, A., Chiancone, E., Hansen, R.E. and Beinert, H. (1967). J.Biol.Chem. 242, 1160-1167.
13. Boden, N., Charlton, S.C., Holmes, M.C. and Knowles, P.F. (1973). Biochem.Soc.Trans. 1, 1008.
14. Buffoni, F., Della Corte, L. and Knowles, P.F. (1968). Biochem.J. 106, 575-576.

15. Hamilton, G.A., Libby, R.D. and Hartzell, C.R. (1973). Biochem.Biophys. Res.Comm. 55, 333-340.
16. Olsson, B., Olsson, J. and Pettersson, G. (1978). Eur.J.Biochem. (in press).
17. Hamilton, G.A. (1968) in: Pyridoxal Catalysis: enzymes and model systems. (Snell, E.E., Braunstein, A.E., Severin, E.S. and Torchinsky, Y.M. eds.) pp.375-390, Interscience, New York.
18. Finazzi-Agro, A., Guerrieri, P., Costa, M.T. and Mondovi, B. (1977). Eur.J.Biochem. 74, 435-440.