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Properties of Cupric Ions in Benzylamine Oxidase from Pig Plasma as Studied by Magnetic-Resonance and Kinetic Methods

By RICHARD BARKER, NEVILLE BODEN, GEORGE CAYLEY, STEVEN C. CHARLTON, RAYMOND HENSON, MICHAEL C. HOLMES, IAIN D. KELLY and PETER F. KNOWLES
Departments of Biophysics and Physical Chemistry, University of Leeds, Leeds LS2 9JT, U.K.

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Benzylamine oxidase from pig plasma has been studied by a variety of chemical and physical techniques. 1. Analytical ultracentrifugation, gel electrophoresis and isoelectric-focusing studies suggest that the enzyme is composed of two subunits with closely similar primary structures. 2. E.s.r. and n.m.r. measurements show that the enzyme contains two well-separated (> 0.6nm) Cu^{2+} ions at chemically distinct sites. Each Cu^{2+} ion is co-ordinated by two water molecules, one 'axial' and the other 'equatorial'. Both water molecules undergo fast exchange (10^5–$10^8 s^{-1}$) with solvent and are deprotonated in the pH range 8–9, but only the equatorial water molecule is displaced by the inhibitors N_3^- and CN^-. 3. Kinetic and e.s.r. measurements show that azide and cyanide compete against O_2 binding and also make the two Cu^{2+} sites identical. It is concluded that Cu^{2+} must participate in the re-oxidation of reduced enzyme by molecular O_2.

Biogenic amines play important roles in cellular processes and are often catabolized by oxidative deamination. This process is catalysed by two groups of amine oxidases that have an intra- or extra-mitochondrial location. Benzylamine oxidase [amine–oxygen oxidoreductase (deaminating), EC 1.4.3.6] from pig plasma is one of the better characterized enzymes in the group of extramitochondrial amine oxidases. The primary amine benzylamine has been shown to be an effective substrate (Buffoni & Blaschko, 1964), though the physiological substrate is unknown.

Benzylamine oxidase catalyses oxidative deamination through a Ping Pong mechanism (Taylor *et al.*, 1972); addition of the amine substrate leads to formation of a reduced enzyme intermediate that is subsequently re-oxidized by O_2 in a rate-limiting step (Lindstrom *et al.*, 1973, 1974*a*,*b*). The purified enzyme has mol.wt. approx. 190000 (Buffoni & Blaschko, 1964); preliminary results indicate that there are two subunits each of mol.wt. 95000 (Boden *et al.*, 1973). The enzyme contains pyridoxal phosphate (Blaschko & Buffoni, 1965; Lindstrom & Pettersson, 1973) and copper (Buffoni & Blaschko, 1964; Buffoni *et al.*, 1968; Boden *et al.*, 1973). There is some controversy over the number of molecules of pyridoxal phosphate bound to the enzyme (Lindstrom & Pettersson, 1973; Buffoni & Ignesti, 1975; Neumann *et al.*, 1975); however, the extensive kinetic studies by Pettersson and his co-workers indicate that reduction of the enzyme involves Schiff-base formation between enzyme-bound pyridoxal phosphate and the amine substrate (Olsson *et al.*, 1976). It is generally agreed that there are 2mol of Cu^{2+} per mol of enzyme (Buffoni *et al.*, 1968; Boden *et al.*, 1973; Lindstrom & Pettersson, 1974), but little is known about the structure of the Cu^{2+} sites or their functional role.

We have previously presented magnetic-resonance evidence which suggests that the two Cu^{2+} sites in the enzyme are structurally distinct (Boden *et al.*, 1973) and accessible to exchanging water molecules (Boden *et al.*, 1974). The present paper gives the results of more detailed magnetic-resonance measurements together with kinetic studies that provide new information on the properties of the Cu^{2+} sites. Each Cu^{2+} ion is co-ordinated by one axial and one equatorial water molecule and by ligands from the polypeptide chain. The equatorial, but not the axial, water molecule can be displaced by azide and cyanide, which makes the Cu^{2+} sites identical. Azide and cyanide are also shown to compete against O_2 binding to the enzyme. It is concluded that Cu^{2+} participates in the re-oxidation of reduced enzyme by molecular O_2.

Abbreviation used: pipes, 1,4-Piperazinediethanesulphonic acid.

Materials and Methods

Materials

Escherichia coli β-galactosidase, bovine liver glutamate dehydrogenase and rabbit muscle aldolase were obtained from Boehringer Corporation, London W.5, U.K. Bovine serum albumin and amphetamine sulphate were purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. All other

reagents were reagent grade and obtained from BDH, Poole, Dorset, U.K., or from Hopkin and Williams, Chadwell Heath, Essex, U.K. Reagent-grade sodium azide was further purified by passing a saturated aqueous solution through a column (6cm × 1cm) of Chelex-100 resin, precipitation of the salt from the eluate by addition of ethanol and washing the precipitate with diethyl ether. All solutions were prepared by using glass-distilled deionized water and their pH values adjusted by using a type TTTIC pH-meter (Radiometer, Copenhagen, Denmark).

Benzylamine oxidase was prepared by a modification of the method of Buffoni & Blaschko (1964), which has been described by Taylor *et al.* (1972), and stored at 277K as an $(NH_4)_2SO_4$ suspension. The enzyme was judged to be homogeneous by analytical ultracentrifugation and polyacrylamide-gel disc electrophoresis by the procedure of Weber & Osborn (1969). Caeruloplasmin, the other major copper-containing protein in serum, was assayed by the procedure described by Curzon (1966) and shown to be absent from the purified benzylamine oxidase. The enzyme used for all the experiments had a specific activity <0.097unit/mg of protein, a unit being defined as the amount of enzyme required to catalyse the production of 1μmol of benzaldehyde/min at 298K under the standard assay conditions of Tabor *et al.* (1954). Protein concentrations were determined by the method of Warburg & Christian (1941) corrected by a factor of 0.75 obtained by comparison with gravimetric measurements (Taylor *et al.*, 1972).

Methods

Copper analysis. Quantitative determination of the copper content of the native enzyme was made by using the e.s.r. integration procedure of Wyard (1965) with 2mM-Cu^{2+} in 25mM-EDTA (disodium salt) as standard. The copper content was confirmed by chemical analysis (Van de Bogart & Beinert, 1967).

Analytical ultracentrifugation. Samples were prepared by dialysis of enzyme against potassium phosphate buffer (0.05M, pH7.0) containing 0.1M-KCl. For studies of dissociation conditions, the following reagents were introduced before dialysis: urea (8.0M), guanidinium chloride (6.0M), β-mercaptoethanol (0.1M), *p*-chloromercuribenzoate (1mM) or amphetamine sulphate (2mM). Attempts to prepare enzyme depleted in copper were made by dialysis at 277K for 12h against potassium phosphate buffer (0.01M, pH7.0) containing sodium diethyldithiocarbamate (0.01M) before dialysis against the phosphate buffer containing KCl.

Sedimentation-velocity and sedimentation-equilibrium measurements (Yphantis, 1964) were made on a Beckman model-E analytical ultracentrifuge at 292.0K. The value of the partial specific volume, $\bar{v}$, used for calculations of molecular weight was determined by the procedure of Edelstein & Schachman (1967), which utilized comparison of the sedimentation velocity in solutions prepared with ^1H_2O and ^2H_2O respectively; $\bar{v}$ was also calculated from the amino acid composition of the enzyme (Blaschko & Buffoni, 1965) assuming a carbohydrate composition similar to that of amine oxidase from bovine plasma (Watanabe & Yasunobu, 1970).

Subunit composition. The molecular weights of the subunits were determined by the sodium dodecyl sulphate polyacrylamide-gel electrophoresis procedure of Weber & Osborn (1969); β-galactosidase, bovine serum albumin, glutamate dehydrogenase and aldolase were used as standards. Isoelectric focusing (pH range 3.5–9.5) was carried out as described by Vesterberg (1972) in the presence of 6M-urea with an LKB model 21171 Multiphor apparatus (LKB Producter AB, Droma, Sweden); the gels were run for 3h at approximately constant power, the initial and final voltage values being respectively 380 and 1000. The gels were stained in Coomassie Blue overnight and then destained in aq. 7% acetic acid.

Preparation of samples for kinetic and magnetic-resonance experiments. The enzyme from the stock $(NH_4)_2SO_4$ suspension was dialysed at 277K for 16h against a 1000-volume excess of potassium phosphate buffer (0.05M, pH7.0 at 293K). For the kinetic investigations, fresh enzyme was dialysed daily. Samples for both e.s.r. and n.m.r. studies were passed through a column (6cm × 1cm) of Chelex-100 resin to remove any extraneously bound metal ions, then vacuum-dialysed to the desired concentration. Control experiments showed that Chelex-100 treatment had no effect on the enzyme activity. In the e.s.r. studies, the enzyme concentration was between 0.7 and 1.4mM; the titrations with azide and cyanide were carried out by thawing the frozen enzyme, adding the inhibitor from a Hamilton syringe (10μl capacity), mixing and re-freezing.

For the n.m.r. measurements, the concentrated enzyme was transferred to 7.5mm-outer-diameter glass tubes and deoxygenated by ten cycles of successive freezing, evacuation and exposure to an Ar atmosphere, after which the tube was sealed; the concentrations of enzyme used were between 0.1 and 0.7mM and were measured at the end of a series of experiments to avoid errors due to evaporation during deoxygenation.

Kinetic measurements. The inhibition of benzylamine oxidase by azide and cyanide was studied by monitoring the initial rate of benzaldehyde formation from its absorbance at 250nm with either a Unicam SP.700 or a Zeiss PMQ-3 spectrophotometer. The temperature in the cell housing was regulated by water circulated from a thermostat (Grant Instruments, Cambridge, U.K.) and measured to an accuracy of ±0.1K with a Comark type 1604 elec-

tronic thermometer (Comark Instruments, Littlehampton, West Sussex, U.K.). The kinetic measurements were carried out either at fixed saturating benzylamine concentrations (3.3mM) as functions of O_2 concentration, or at fixed saturating O_2 concentrations (1.1mM) as functions of benzylamine concentration, for various concentrations of the inhibitors. The reaction mixture (57mM potassium phosphate buffer, pH7.4, benzylamine and the inhibitor) was contained in a spectrophotometer cell fitted with a serum cap (Suba-Seal, Barnsley, Yorks., U.K.); KCl was added to the reaction mixture to maintain the ionic strength constant. The experiments with cyanide as inhibitor were carried out in a 1.0cm-light-path spectrophotometer cell, whereas those with azide were carried out in a cell with a light-path of 0.2cm, owing to the high absorbance of this ion. The gas was introduced into the cell from an Ar/O_2 gas mixer (Air Products, Rotherham, South Yorkshire, U.K.) through a stainless-steel serum needle (Thackray, Leeds, Yorks., U.K.) for 5min (the time required to saturate the solution with O_2), then the reaction was initiated by the addition of enzyme (0.10ml) from a syringe (Hamilton 705). Passage of gas through the solution was maintained for a further 30s to ensure complete mixing: the needles were then removed and the holes in the serum cap filled with Evostik (Evode, Stafford, U.K.). The absolute O_2 concentrations were determined polarographically as described previously (Taylor *et al.*, 1972). The kinetic data were assessed by using an iterative fitting procedure described by Cleland (1967).

E.s.r. spectroscopy. X-band (9GHz) spectra were run on a Decca X1 spectrometer (Decca Radar, Walton-on-Thames, Surrey, U.K.) in conjunction with a Varian 23cm magnet and Fieldial sweep unit (Varian Associates, Walton-on-Thames, Surrey, U.K.). The operating conditions used were: temperature 100K, microwave power 6.4mW and 100kHz modulation amplitude of 2.2mT. The magnetic field was calibrated with a proton magnetometer (Newport Instruments, Newport Pagnell, Bucks., U.K.) and the frequency with a wavemeter (Decca Radar). Q-band (35GHz) spectra were run on a Varian 4500 series spectrometer fitted with a low-temperature assembly for cooling the cavity. The operating conditions used were: temperature 150K (approx.), microwave power 50mW and 100kHz modulation amplitude of 2.0mT.

Computer simulation of e.s.r. spectra. The program written by Venables (1967) for polycrystalline samples of transition-metal ions in sites of rhombic or higher symmetry was modified for use on the PDP-11 visual-display system (Digital Equipment Corporation, Galway, Ireland). This modified program had facilities to mix two simulated spectra in any proportions; the calculated spectrum could be compared with the experimental one on the display.

Nuclear-magnetic-relaxation measurements. The ^1H nuclear-magnetic-relaxation measurements were made at 10, 30 and 60MHz with a Bruker B-KR 306s pulse spectrometer. Spin–lattice relaxation times (T_1) were measured by the standard 90°–τ–90° pulse sequence and the transverse relaxation times (T_2) by the Meiboom–Gill modification of the Carr–Purcell spin-echo experiment. The values of T_2 were independent of pulse spacing over the range 5×10^{-2} to 10^{-4}s. The temperature of the sample was controlled by an N_2-gas flow system and measured with a thermocouple immersed in glycerol in a standard sample tube. Temperatures are considered accurate to within ±1K. Values for the paramagnetic ion contributions T_{1p}^{-1} and T_{2p}^{-1} to T_1^{-1} and T_2^{-1} respectively were obtained by subtracting values of the corresponding rates measured in diamagnetic buffer solutions. T_{1p}^{-1} and T_{2p}^{-1} were normalized with respect to the paramagnetic ion concentration by dividing by p_m ($\approx[Cu^{2+}]/55.6$).

Results and their Interpretation

Copper content of the native enzyme

The copper content determined by integration of the 9GHz e.s.r. spectrum against a Cu^{2+}–EDTA standard was found to be 2.3mol of Cu^{2+}/mol of enzyme; the value determined by chemical analysis was 2.1±0.2mol of Cu^{2+}/mol of enzyme. Dialysis of the enzyme against sodium diethyldithiocarbonate did not decrease the copper content. The native enzyme must, therefore, contain two tightly bound Cu^{2+} ions. The similarity of the e.s.r. and chemical analysis for the concentrations of Cu^{2+} indicates the absence of Cu^{2+}–Cu^{2+} interactions.

Molecular weight of the native enzyme

Sedimentation-equilibrium measurements on the native enzyme indicated a single species (the log$c$-against-r^2 plot being linear) of mol.wt. 186000±4000. The value of $\bar{v}$ used to calculate the mol.wt. was 0.725ml/g as determined from the amino acid composition (Watanabe & Yasunobu, 1970); a similar value for $\bar{v}$ of 0.72±0.03ml/g was obtained by using the procedure of Edelstein & Schachman (1967).

Dissociation of native protein and subunit composition

The values for $s_{20,w}$ at protein concentrations of 2.5mg/ml, in solutions containing potential dissociating agents, were as follows: no addition, 8.36; 8M-urea, 3.73; 6M-guanidinium chloride, 2.78; 0.1M-β-mercaptoethanol, 8.30; 1mM-*p*-chloromercuribenzoate, 8.47; 2mM-amphetamine, 8.65. These results indicate that guanidinium chloride and, to a lesser extent, urea are able to dissociate the native enzyme,

whereas reducing or thiol-blocking reagents have no effect: amphetamine, which has been shown to be a competitive inhibitor against benzylamine binding to the enzyme (Rasmussen, 1975), also shows no effect on the quaternary structure.

The molecular weight of the subunits produced by dissociation in 6.0M-guanidinium chloride was determined by sedimentation equilibrium to be 97500± 3000, assuming a value of 0.715ml/g for $\bar{v}$. The log c-against-r^2 plot was linear, indicating a single species.

From sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the molecular weight of the subunits was estimated to be approx. 97000. Isoelectric focusing in 6.0M-urea revealed the presence of a single species.

The above results suggest that the enzyme has mol.wt. approx. 186000 and can be dissociated into two polypeptide chains with identical primary structure.

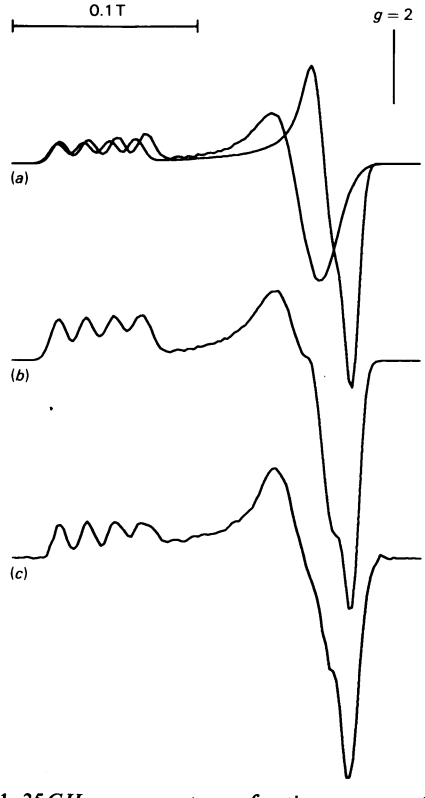

Fig. 1. *35GHz e.s.r. spectrum of native enzyme at 150K*
(*a*) Calculated axial and rhombic components; (*b*) sum of spectra in (*a*) equally weighted; (*c*) experimental spectrum. Samples were prepared in 50mM-potassium phosphate buffer, pH7.0; for other experimental conditions, see the text.

E.s.r. studies at 35GHz

(i) *Spectrum of the native enzyme.* Fig. 1 gives a comparison of observed (Fig. 1*c*) and calculated (Fig. 1*b*) spectra of the native enzyme; Fig. 1(*b*) was obtained by mixing equal proportions of axially symmetric and rhombic components (the parameters used are given in Table 1) as illustrated in Fig. 1(*a*). It is apparent that the two Cu^{2+} ions are located in non-identical chemical environments, one of axial and the other of rhombic symmetry. Attempts to fit the spectra assuming identical sites of rhombic symmetry or non-identical sites of axial symmetry were unsuccessful. The 1:1 stoicheiometry of the axial and rhombic components and the similar e.s.r. spectrum observed when the non-complexing buffer Pipes (2mM, pH7.0 at 293K) was used in place of phosphate buffer eliminates the possibility that the dissimilar Cu^{2+} sites are due to complexing with buffer.

Comparison of the g_{zz} and $g_{\parallel}$ values of the rhombic and axial components respectively, given in Table 1, with those of model Cu^{2+} complexes (Barbucci & Campbell, 1976) tentatively suggests that both Cu^{2+}

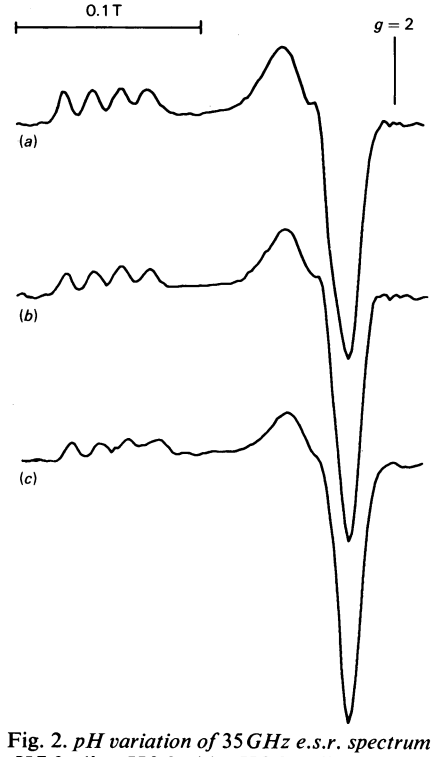

Fig. 2. *pH variation of 35GHz e.s.r. spectrum*
(*a*) pH7.0; (*b*) pH8.0; (*c*) pH8.9. All samples were prepared in 50mM-potassium phosphate buffer at a constant ionic strength of 0.15M by addition of KCl.

sites have two co-ordinated nitrogen and four co-ordinated oxygen ligands.

(ii) *Dependence of the spectrum on pH.* Fig. 2 shows the experimental spectra obtained at pH 7.0, 8.0 and 8.9. The spectra could be simulated by having equal proportions of two components with the e.s.r. parameters given in Table 1. The results in Table 1 indicate that both copper centres are affected by pH. There is a shift to lower values for both the $g_{\perp}$ (of the axial component) and g_{yy} (of the rhombic component) as the pH is increased. Furthermore, the x and y direction linewidths of the rhombic component decrease with increasing pH. The changes are small, however, and are difficult to interpret.

(iii) *Dependence of the spectrum on ionic strength.* Figs. 3(*a*) and 3(*b*) show the e.s.r. spectra recorded respectively at ionic strengths of 0.09M and 0.16M. There are small inexplicable decreases in $g_{\parallel}$ and linewidth with increase in ionic strength, Fig. 3(*c*) represents a simulation of Fig. 3(*b*) assuming equal contributions of axial and rhombic forms having the spectral parameters given in Table 1 (section iii).

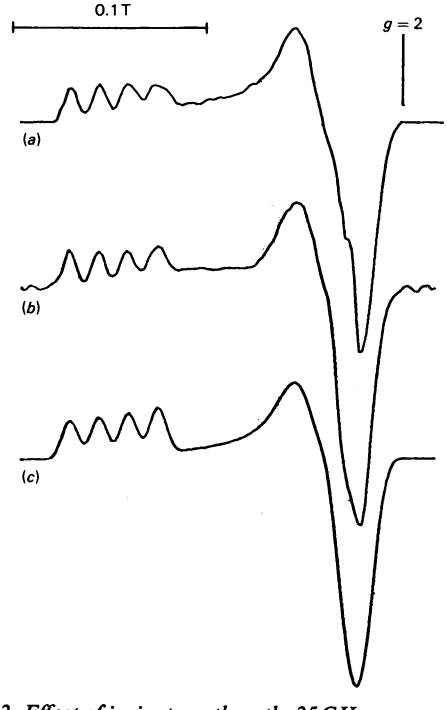

Fig. 3. *Effect of ionic strength on the 35 GHz e.s.r. spectrum*
(*a*) Ionic strength 0.09M; (*b*) ionic strength 0.16M; (*c*) simulation of spectrum (*b*) assuming equal contributions from axial and rhombic forms with the spectral parameters given in Table 1 (section iii). All samples were prepared in 50mM-potassium phosphate buffer and the ionic strength was adjusted with KCl.

(iv) *Titration with azide.* Figs. 4(*a*), 4(*b*) and 4(*c*) show the experimental spectra obtained at azide concentrations of 0.27 and 23mM respectively. Fig. 4(*d*) shows the simulation of Fig. 4(*c*) assuming a single component with axial symmetry; the e.s.r. parameters used are given in Table 1. Intermediate spectra (illustrated by Fig. 4*b*) could be simulated by

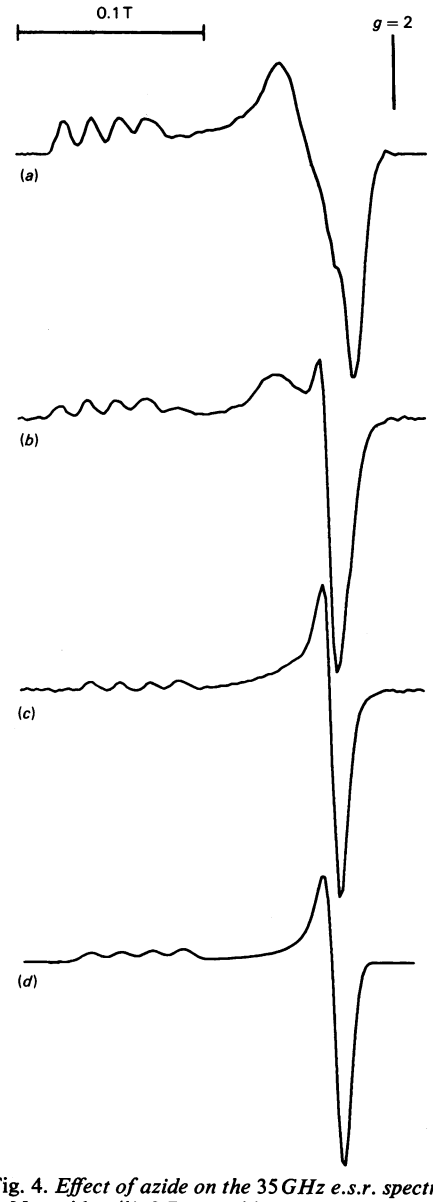

Fig. 4. *Effect of azide on the 35 GHz e.s.r. spectrum*
(*a*) No azide; (*b*) 2.7mM-azide; (*c*) 23mM-azide; (*d*) simulation of spectrum (*c*). All samples were prepared in 50mM-potassium phosphate buffer, pH 7.0.

Table 1. *Parameters used in the simulation of the e.s.r. spectra of benzylamine oxidase*
For further details see the text and Figs. 1–4. The values given between the x and y columns correspond to the ⊥ values in the cases of axial symmetry.

	Component	g_{xx}	g_{yy}	g_{zz}	Hyperfine splittings (mT) A_{xx}	A_{yy}	A_{zz}	Linewidths (mT) W_{xx}	W_{yy}	W_{zz}
(i) Native enzyme	Axial	2.078		2.286	1.0		15.5	10.7		4.0
	Rhombic	2.039	2.065	2.294			14.0	3.6	5.0	4.0
(ii) pH-dependence										
pH7.0	Axial	2.075		2.284			15.5	10.7		4.3
	Rhombic	2.040	2.063	2.288			15.5	4.8	5.5	4.3
pH8.0	Axial	2.074		2.284			15.5	10.7		4.3
	Rhombic	2.042	2.062	2.288			15.5	4.5	5.5	4.3
pH8.9	Axial	2.071		2.284			15.5	10.7		4.3
	Rhombic	2.039	2.057	2.288			15.5	4.1	5.5	4.3
(iii) Ionic-strength-dependence	Axial	2.075		2.284			15.5	10.0		4.0
	Rhombic	2.040	2.063	2.288			15.5	5.5	5.5	4.0
(iv) Enzyme with 23mM-azide	Axial	2.055		2.257			16.5	4.5		5.0
(v) Enzyme with 52mM-cyanide	Axial	2.054		2.218			15.8	9.0		4.0

mixing the native spectrum (Fig. 4*a*) and final inhibited spectrum (Fig. 4*c*) in different proportions.

The data are consistent with azide binding to both Cu^{2+} ions. The 35GHz spectra indicate only one type of Cu^{2+} with axial symmetry. There is a significant decrease in g_{zz} from the native to the inhibited state, which correlates with replacement of an oxygen ligand by nitrogen (Barbucci & Campbell, 1976). The simplest explanation is that azide replaces a water molecule co-ordinated to each Cu^{2+}.

The dissociation constant, K_D, for the azide–enzyme complex calculated from the e.s.r. measurements is 3.2mM; this value is in apparent agreement with the value similarly determined by Lindstrom *et al.* (1974*b*). However, these values are not considered to be reliable, owing to the phase and pH changes on freezing (Williams-Smith *et al.*, 1977). K_D also depends on temperature, so that the values determined by e.s.r. studies at 150K may differ from those obtained at 298K.

(v) *Titration with cyanide.* Figs. 5(*a*), 5(*b*) and 5(*c*) show the experimental spectra obtained at cyanide concentrations of 0, 4.5 and 52mM respectively. Fig. 5(*d*) shows the simulation of Fig. 5(*c*) assuming a single component with axial symmetry; the e.s.r. parameters are given in Table 1. Intermediate spectra, illustrated by Fig. 5(*b*), were simulated by mixing the native spectrum (Fig. 5*a*) and final inhibited spectrum (Fig. 5*c*) in different proportions. The interpretation of these results is similar to that given for the azide experiments.

The dissociation constant for the cyanide–enzyme complex has been calculated to be 2.3mM. This value is considered to be incorrect for the same reasons discussed above with regard to the azide–enzyme complex.

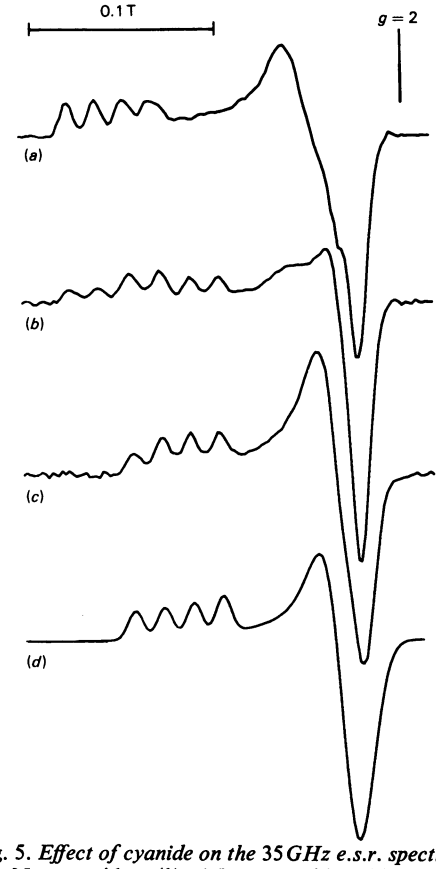

Fig. 5. *Effect of cyanide on the 35GHz e.s.r. spectrum* (*a*) No cyanide; (*b*) 4.5mM-cyanide; (*c*) 52mM-cyanide; (*d*) simulation of spectrum (*c*). All samples were prepared in 50mM-potassium phosphate buffer, pH7.0.

Kinetic studies of inhibition by azide and cyanide

The results of inhibition studies with azide and cyanide are shown in Figs. 6, 7 and 8. Table 2 summarizes the types of inhibition observed and the values obtained for the inhibition constants. The nomenclature of Cleland (1963, 1970) is used in the discussion of these results.

It is well established that pig plasma benzylamine oxidase operates through a Ping Pong mechanism; this conclusion was drawn from initial-velocity studies (Taylor *et al.*, 1972), and has been confirmed by spectrophotometric titration studies (Lindstrom *et al.*, 1973), which showed two stable enzyme forms. The enzyme shuttles between an oxidized form that is reduced by the amine substrate and a form re-oxidized via a pathway involving molecular O_2. There is some dispute over the order of product release (Taylor *et al.*, 1972; Lindstrom *et al.*, 1974*a*,*b*), though this does not influence the validity of the

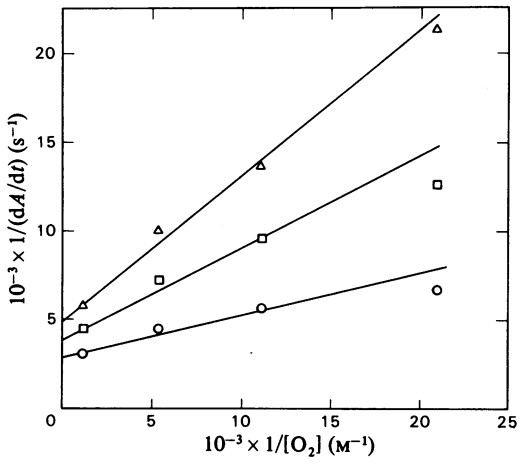

Fig. 7. *Inhibition of benzylamine oxidase by cyanide with benzylamine saturating* (3.33 mM) *and oxygen as variable substrate at* 310 ± 0.1 *K and ionic strength* 380 mM
○, No cyanide; □, 1 mM-cyanide; △, 2 mM-cyanide.

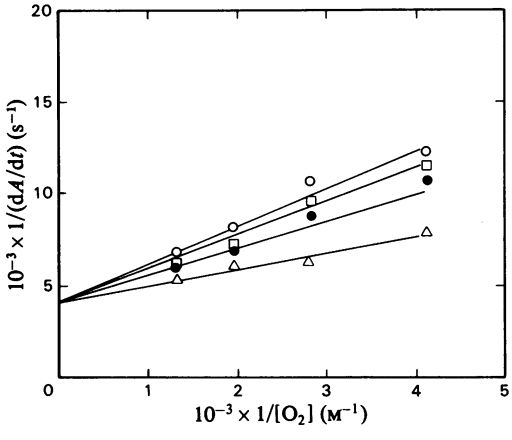

Fig. 6. *Inhibition of benzylamine oxidase by azide with benzylamine saturating* (3.33 mM) *and oxygen as variable substrate at* 298 ± 0.1 *K and ionic strength* 380 mM
△, No azide; ●, 49 mM-azide; □, 82 mM-azide; ○, 98 mM-azide.

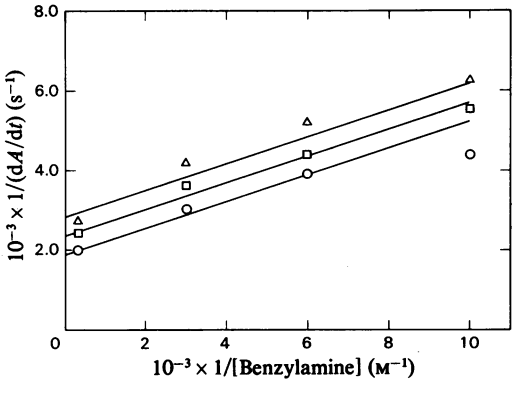

Fig. 8. *Inhibition of benzylamine oxidase by cyanide with oxygen saturating* (1.1 mM) *and benzylamine as variable substrate at* 310 ± 0.1 *K and ionic strength* 380 mM
○, No cyanide; □, 0.5 mM-cyanide; △, 1.0 mM-cyanide.

Table 2. *Kinetic-inhibition patterns and constants for azide and cyanide*

Inhibitor	Variable substrate: Benzylamine	Variable substrate: Oxygen
Azide	Uncompetitive* K_i (intercept) 40 mM	Competitive K_i (slope) 84 ± 16 mM
Cyanide	Uncompetitive K_i (intercept) 2.17 ± 0.39 mM	Non-competitive K_i (slope) 0.76 ± 0.21 mM K_i (intercept) 2.90 ± 0.74 mM

* Data of Lindstrom *et al.* (1974*a*,*b*).

following conclusions based on initial-velocity studies with the inhibitors.

The inhibition patterns for azide of uncompetitive inhibition with benzylamine as variable substrate and competitive inhibition with O_2 as variable substrate indicate that azide binds to the stable enzyme form that binds O_2, thus preventing re-oxidation of the reduced enzyme. The stopped-flow studies by Lindstrom *et al.* (1974*b*), which showed that azide concentrations up to 100mM had no effect on the rate of reduction of enzyme by benzylamine or on the reactivity of the carbonyl grouping believed to be involved in this reduction step, are also consistent with this conclusion. The value of K_i determined from the experiments with O_2 as the variable substrate (84±16mM) is the dissociation constant for azide binding to the enzyme (Cleland, 1963).

The cyanide-inhibition patterns are more complex. However, the existence of a slope effect when O_2 is the variable substrate and benzylamine is saturating would only occur if the cyanide is either binding to the same enzyme form that binds O_2 or to an enzyme form that is reversibly connected to the form of the enzyme that binds O_2. The evidence for a Ping Pong mechanism discussed above rules out the latter alternative of reversible connections between enzyme forms in the absence of products and leads to the conclusion that the K_i (slope), with O_2 varied and benzylamine saturating, is the dissociation constant for cyanide binding to the reduced form of the enzyme. The intercept effect observed under these experimental conditions can only be explained by binding of cyanide to a second enzyme form not reversibly connected to the reduced form of the enzyme. There will be no oxidized form of the enzyme under these conditions, since benzylamine is present at saturating concentrations. Cyanide must be binding to a transitory form of the enzyme. This dual mode of action of cyanide explains the observed inhibition behaviour, and is consistent with the diverse chemical properties of cyanide. Thus cyanide can act as a ligand for binding to metal ions as well as reacting with carbonyl groupings; both types of site are present in benzylamine oxidase.

Water-proton nuclear-magnetic-relaxation studies

1. *Spin relaxation in aqueous solutions of the native protein.* The normalized paramagnetic contributions $(p_mT_{1p})^{-1}$ and $(p_mT_{2p})^{-1}$ to the water proton spin-relaxation rates measured as a function of temperature and frequency in aqueous solutions of native benzylamine oxidase at pH7.0 are summarized in Fig. 9.

$(p_mT_{1p})^{-1}$ may be interpreted by using the Luz–Meiboom (1964) equation, which, in view of the marked frequency-dependence of $(p_mT_{1p})^{-1}$, which implies $\tau_m < T_{1m}$, reduces to:

$$(p_mT_{1p})^{-1} = q/(\tau_m+T_{1m}) \approx q/T_{1m}. \quad (1)$$

Here q is the number of co-ordinated water molecules per Cu^{2+} ion, τ_m is the mean lifetime of a co-ordinated water molecule and T_{1m} is the spin–lattice relaxation time of the bound water protons. We will assume T_{1m}^{-1} is given by the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957), which in this case takes the form:

$$\frac{1}{T_{1m}} = \frac{2}{15}\frac{\mu_{eff.}^2\gamma_I^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (2)$$

where ω_I and γ_I are respectively the angular Larmor precession frequency and the magnetogyric ratio of the proton, r is the ion–proton distance and $\mu_{eff.}$ is the effective electronic magnetic moment of Cu^{2+}. (Terms in $\omega_s\tau_c$ have been omitted, since $\tau_c > 10^{-12}$s, which makes $\omega_s^2\tau_c \gg 1$. Values of $\tau_c < 10^{-12}$s may be excluded, since they would necessitate unrealistic values for r. Terms in $\omega_s\tau_e$ have been omitted for similar reasons.) τ_c, the correlation time for the dipolar interaction, is given by:

$$\tau_c^{-1} = \tau_r^{-1}+\tau_m^{-1}+\tau_s^{-1} \quad (3)$$

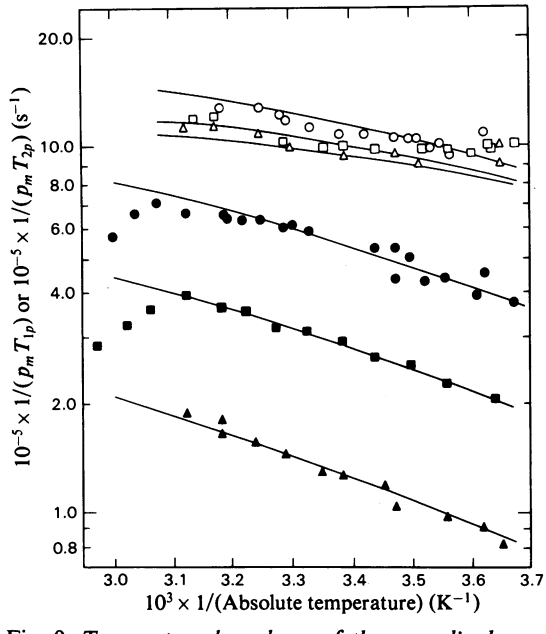

Fig. 9. *Temperature-dependence of the normalized paramagnetic contributions to the water-proton spin-relaxation rates measured in aqueous solutions of native benzylamine oxidase, pH7.0*

The solid lines were calculated by using eqns. (1)–(6) with the results given in Table 3. ▲, $1/(p_mT_{1p})$ at 60MHz; ■, $1/(p_mT_{1p})$ at 30mHz; ●, $1/(p_mT_{1p})$ at 10MHz; △, $1/(p_mT_{2p})$ at 60MHz; □, $1/(p_mT_{2p})$ at 30MHz; ○, $1/(p_mT_{2p})$ at 10MHz.

where τ_r is the reorientational correlation time that, in common with τ_m, is assumed to have an Arrhenius temperature-dependence. τ_s may be identified with the electronic spin–lattice relaxation time in solutions of macromolecules (Koenig, 1972); it will here be treated empirically.

The following two qualitative explanations for the behaviour of $(p_mT_{1p})^{-1}$ are suggested by eqns. (2) and (3).

(i) $\omega_I\tau_c<1$; $\tau_c=\tau_s$, *where* τ_s *increases as the temperature is increased.* At higher temperatures we might expect a change to τ_r or τ_m domination of τ_c, leading to a change from a negative to a positive activation energy with a maximum in $(p_mT_{1p})^{-1}$ and a loss of frequency-dependence.

(ii) $\omega_I\tau_c>1$; *with* τ_c *containing contributions from* τ_r, τ_s *or* τ_m. At higher temperatures $\omega_I\tau_c<1$, giving a maximum in $(p_mT_{1p})^{-1}$.

The $(p_mT_{2p})^{-1}$ measurements may be used to distinguish between possibilities (i) and (ii). $(p_mT_{2p})^{-1}$ is interpreted by using the equation of Swift & Connick (1962) that, in this case, reduces to:

$$(p_mT_{2p})^{-1}=q/(\tau_m+T_{2m}) \qquad (4)$$

where T_{2m}^{-1} is the transverse relaxation rate for bound protons. [Note that the contribution from $\Delta\omega_m$, the chemical shift between the proton resonances of the free and bound water molecules, has been neglected, since by using the value of 10^5 Hz (Luz & Shulman, 1965) for the scalar coupling constant A/h in

$$\Delta\omega_m=\frac{S(S+1)\gamma_S A\omega_I}{3kT\gamma_I h}$$

we find $\Delta\omega_m^2 \ll T_{2m}^{-2},\tau_m^{-2}$ (Bloembergen, 1957)]. For T_{2m}^{-1} we use the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957) that, in this case, is:

$$\frac{1}{T_{2m}}=\frac{1}{15}\frac{\mu_{eff.}^2\gamma_I^2}{r^6}\left(4\tau_c+\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right)+\frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\tau_e \qquad (5)$$

where τ_e is the correlation time for the process modulating the scalar interaction. The terms omitted from eqn. (2) are omitted from eqn. (5) for similar reasons. Eqn. (5) is conveniently written:

$$T_{2m}^{-1}=D\tau_c+E\tau_e \qquad (6)$$

where D and E are constant.

Fig. 9 shows the apparent activation energy for $(p_mT_{2p})^{-1}$ is less than that for $(p_mT_{1p})^{-1}$. Eqn. (4) predicts this behaviour provided condition (ii) holds and $T_{2m}\approx\tau_m$, which would give $\tau_m=1.6\times10^{-6}$ at 294K. Alternatively, eqn. (6) will also account for the observed behaviour, provided condition (ii) holds and $D\tau_c\simeq E\tau_e$; τ_c and τ_e must have opposite temperature-dependences, i.e. τ_c is dominated by τ_r and τ_e by τ_s. This latter alternative requires $1.4\times10^6<A/h<6.3\times10^7$ Hz, but, since $A/h\approx10^5$ Hz (Bloembergen, 1957) for most copper complexes, the former would appear more plausible. Nevertheless, no matter what the explanation is for the behaviour of $(p_mT_{2p})^{-1}$, condition (ii) must be valid for $(p_mT_{1p})^{-1}$.

It is not realistic to attempt to fit the experimental measurements by using a model of distinguishable Cu^{2+} sites, as this would involve 12 adjustable parameters. We have, therefore, assumed that, to a first approximation, the two sites may be treated as identical and fitted eqns. (2), (4) and (6) to the measurements given in Fig. 9. The solid lines drawn through the experimental points have been calculated from the best-fit parameters obtained in this way, and are summarized in Table 3. The sudden decrease in $(p_mT_{1p})^{-1}$ in the region of 320K is not accounted for by the above model, although it does predict a maximum at higher temperatures. This behaviour may arise from a reversible structural change at the Cu^{2+} sites that could be a precursor to the irreversible denaturation occurring at temperatures above 340K. The $(p_mT_{1p})^{-1}$ measurements in aqueous solutions of the denatured protein are characteristically different, as illustrated in Fig. 10. The parameters in Table 3 are not necessarily unique; indeed, the activation energies for both τ_c and τ_m are unrealistic. The value obtained for $\tau_m\approx2\times10^{-6}$s is, however, unequivocal.

Table 3. *Values of parameters obtained from proton spin-relaxation measurements in aqueous solutions of benzylamine oxidase at* 294K

Quantity	Frequency 10MHz	30MHz	60MHz
q/r^6 [(nm)$^{-6}$]		$(3.8\pm0.4)\times10^{-3}$	
τ_c (s)	$(8.0\pm0.8)\times10^{-8}$	$(2.6\pm0.3)\times10^{-8}$	$(1.8\pm0.2)\times10^{-8}$
$E_A(\tau_c)$ (kJ/mol)	18	13	12
τ_m (s)		$(1.6\pm0.6)\times10^{-6}$	Frequency-independent
$E_A(\tau_m)$		~8	Frequency-independent

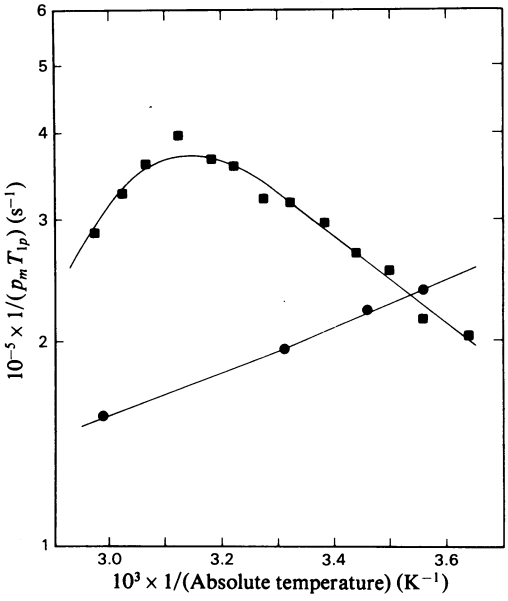

Fig. 10. *Comparison of the temperature-dependence of the normalized paramagnetic contribution to the water-proton spin–lattice-relaxation rates at 30 MHz in aqueous solutions of benzylamine oxidase, pH 7.0, before (■) and after (●) denaturation by heating to 340 K*

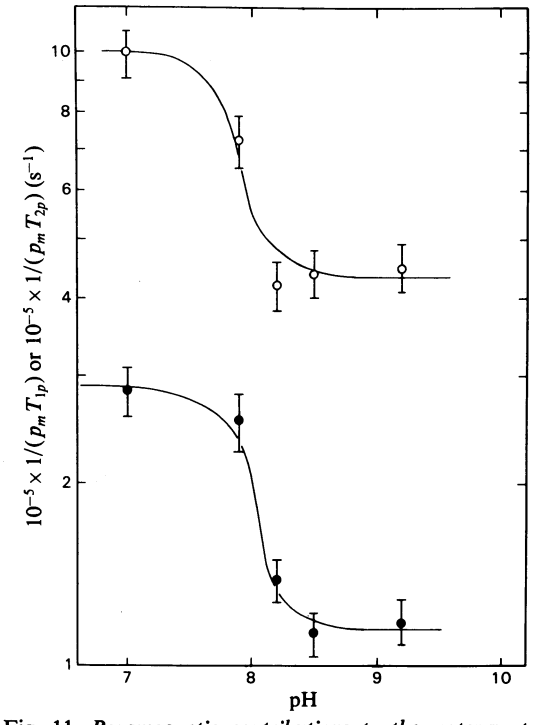

Fig. 11. *Paramagnetic contributions to the water-proton spin-relaxation rates at 30 MHz and 298 K in aqueous solutions of benzylamine oxidase as a function of pH* Potassium phosphate buffer (50 mM) at the different pH values was used. ●, $1/(p_m T_{1p}$; ○, $1/(p_m T_{2p})$.

2. *Dependence on pH.* Fig. 11 illustrates how both $(p_m T_{1p})^{-1}$ and $(p_m T_{2p})^{-1}$ vary with pH at 298 K and 30 MHz. Both rates show a sudden decrease by a factor of 2 centred on pH 8. The apparent activation energy for $(p_m T_{1p})^{-1}$ is independent of pH, as shown in Fig. 12.

3. *Effects of inhibitors.* (i) *Amphetamine sulphate.* The effect of added substrate benzylamine on the proton-relaxation rates was not investigated, owing to enzyme-catalysed substrate turnover; instead, the effect of amphetamine sulphate, a competitive inhibitor against benzylamine binding (Rasmussen, 1975), has been studied. There was no effect on $(p_m T_{1p})^{-1}$ for concentrations of amphetamine sulphate up to a 20-fold molar excess over enzyme.

(ii) *Azide and cyanide.* The addition of azide or cyanide has a marked effect on $(p_m T_{1p})^{-1}$, as can be seen in Figs. 13 and 14 respectively, which show how the ratio $(p_m T_{1p}{}^*)^{-1}/(p_m T_{1p})^{-1}$ (where the asterisk denotes the presence of added ion) varies with inhibitor concentration. These titration curves extrapolate to $T_{1p}/T_{1p}{}^* = 0.5$ at infinite inhibitor concentration, indicating binding to half the water-co-ordination sites. The possibility that the observed effects might originate from a change in τ_c may be discounted, since the profiles of the frequency-dispersion curves of T_{1p} for the native and azide-inhibited enzyme are similar (Fig. 15): this conclusion obtains from eqn. (2), which predicts that $dT_1/d\omega^2 \propto \tau_c$. Further support for the conclusion that τ_c does not change on addition of azide comes from the observation that the apparent activation energy is unaffected by the presence of azide (Fig. 16).

Values for the Cu^{2+}–inhibitor dissociation constant, K_D, have been obtained from the measurements in Figs. 13 and 14 by using the expression (Dwek, 1973):

$$\frac{T_{1p}}{T_{1p}{}^*} = \frac{1+(T_{1p}/T_{1p}{}^*)_\infty [L]/K_D}{1+[L]/K_D} \qquad (7)$$

where $(T_{1p}/T_{1p}{}^*)_\infty$ is the value of $(T_{1p}/T_{1p}{}^*)$ at infinite ligand concentration [L]. The values of K_D obtained for azide and cyanide are 48 ± 15 mM and 0.7 ± 0.3 mM respectively. These values are compared with the kinetic inhibitor constants in Table 5.

(iii) *Other potential copper-complexing ligands.* In contrast with the behaviour of CN^- and N_3^- ions, saturation of the solutions with SCN^- or F^- had no effect on either the enzymic activity or the proton spin-relaxation rates.

4. *Implications of the effects of pH and inhibitors.* The empirical effects of pH and inhibitors on the

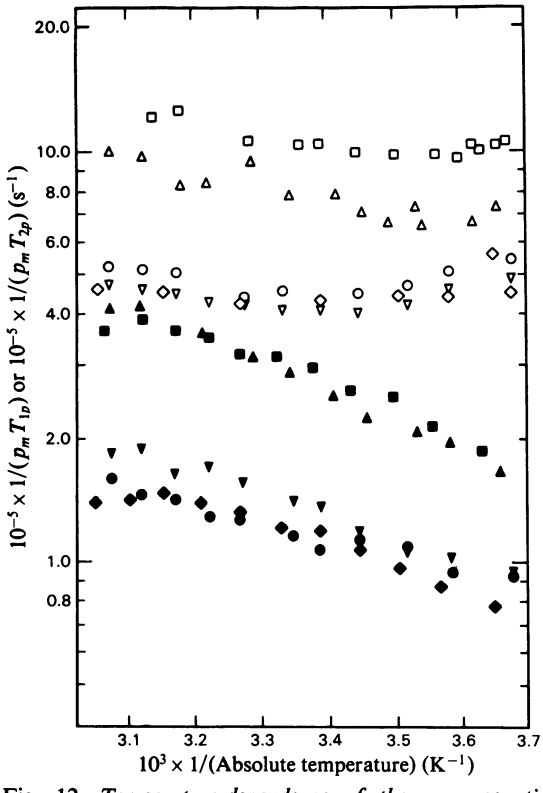

Fig. 12. *Temperature-dependence of the paramagnetic contributions to the water-proton spin-relaxation rates at 30 MHz as a function of pH in aqueous solutions of benzylamine oxidase*

Potassium phosphate buffer (50mM) at the different pH values was used. ■, $1/(p_m T_{1p})$ at pH7.0; ▲, $1/(p_m T_{1p})$ at pH7.9; ▼, $1/(p_m T_{1p})$ at pH8.2; ●, $1/(p_m T_{1p})$ at pH8.5; ◆, $1/(p_m T_{1p})$ at pH9.2; □, $1/(p_m T_{2p})$ at pH7.0; △, $1/(p_m T_{2p})$ at pH7.9; ▽, $1/(p_m T_{2p})$ at pH8.2; ○, $1/(p_m T_{2p})$ at pH8.5; ◇, $1/(p_m T_{2p})$ at pH9.2.

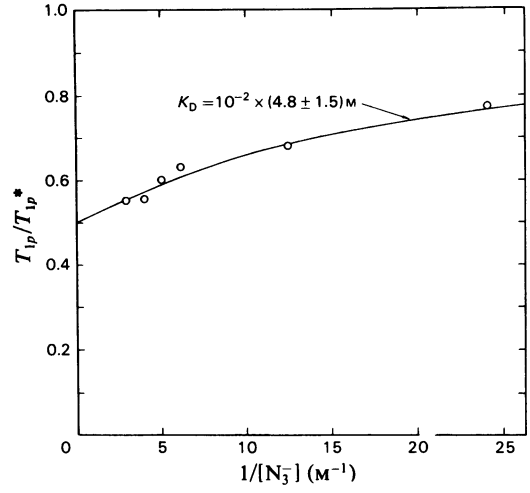

Fig. 13. *Attenuation of the water-proton spin–lattice-relaxation rate T_{1p}/T_{1p}^* at 30 mHz, pH7.0 and 298 K as a function of N_3^-*

The solid line represents the best fit of the experimental measurements to eqn. (7).

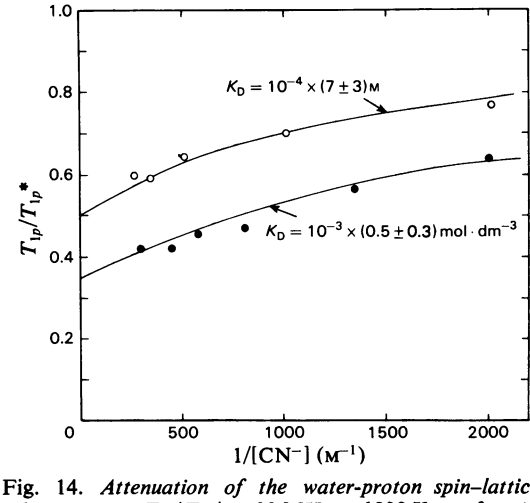

Fig. 14. *Attenuation of the water-proton spin–lattice-relaxation rate T_{1p}/T_{1p}^* at 30 MHz and 298 K as a function of CN^-*

The solid line represents the best fit of the experimental measurements to eqn. (7). ○, 50mM-Potassium phosphate buffer, pH7.0; ●, 50mM-potassium phosphate buffer, pH9.2.

relaxation behaviour may be used to infer models for the Cu^{2+} sites in the enzyme. These models are not restricted by the assumptions invoked in fitting the experimental measurements for the native enzyme. The observed effects may consistently be rationalized in terms of changes in the number of bound water protons: (*a*) by displacement of one half of the bound water molecules by a neighbouring group in the protein, or (*b*) by deprotonation of all bound water molecules to OH^-.

These possibilities can be distinguished through the results of the titration experiments with cyanide at pH7.0 and 9.0 (Fig. 14). In the case of (*a*), the addition of CN^- will either decrease $(p_m T_{1p})^{-1}$ to zero or leave it unaffected, whereas if (*b*) is correct $(p_m T_{1p})^{-1}$ will be decreased by a factor of 2 at both pH7.0 and 9.0. The results are clearly consistent with model (*b*).

The changes observed in T_{1p} and T_{2p}, when inhibitors are added to the enzyme, can be similarly explained in terms of a decrease in the number of

Table 4. *Properties of axial and equatorial water co-ordinated to* Cu^{2+}
Abbreviation used: tren, 2,2′,2″-triaminotriethylamine.

Complex	Water binding	Ligand	k_{on} ($M^{-1}\cdot s^{-1}$)	Method used
$[Cu(tren)H_2O]^{2+}$	Equatorial	Water	2.5×10^{5}*	N.m.r. (Rablen *et al.*, 1972)
	Equatorial	Imidazole	2.0×10^{5}	Temperature jump (Cayley *et al.*, 1976)
$Cu(H_2O)_6^{2+}$	Axial	Imidazole	5.7×10^{8}	Temperature jump (Diebler & Rosen, 1972)

* First-order rate constant (s^{-1}).

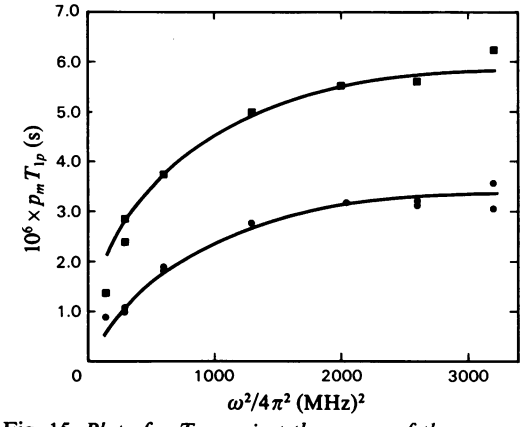

Fig. 15. *Plot of* p_mT_{1p} *against the square of the resonance frequency,* ω
●, Native enzyme in 50mM-potassium phosphate buffer, pH7.0; ■, enzyme in 50mM-potassium phosphate buffer, pH7.0, containing 200mM-sodium azide.

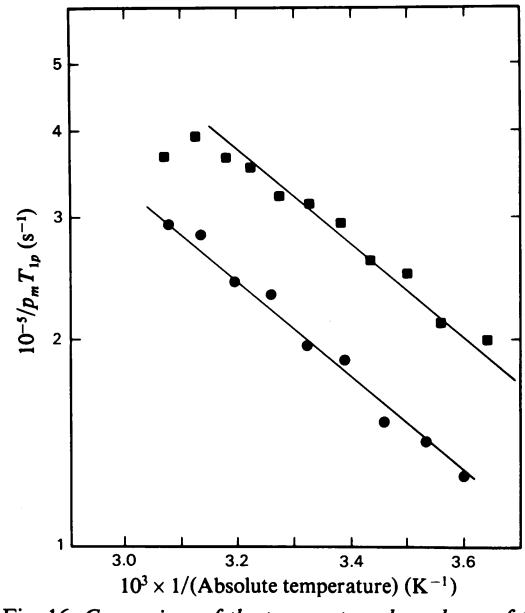

Fig. 16. *Comparison of the temperature-dependence of the paramagnetic contribution to the water-proton spin–lattice-relaxation rates at* 30*mHz in aqueous solutions of benzylamine oxidase at pH*7.0 *in the absence* (■) *and presence* (●) *of* 15*mM-sodium azide*

bound water protons. For cyanide and azide, the extrapolated value for T_{1p}/T_{1p}^* at infinite inhibitor concentration is 0.5 (see Figs. 13 and 14). Clearly, not all of the water molecules bound to the two Cu^{2+} ions are displaced by these inhibitors. There are again two possible explanations: (*a*) only water molecules co-ordinated to one of the two Cu^{2+} ions can be displaced; this is consistent with the e.s.r. measurements indicating that two Cu^{2+} sites are distinguishable, or (*b*) each Cu^{2+} ion has at least two co-ordinated water molecules differing in their reactivity with the inhibitor.

Explanation (*a*) requires that one of the two Cu^{2+} sites is accessible to solvent water but inaccessible to the inhibitors. There is no chemical basis for this assertion. The second explanation is consistent with the known properties of axial and equatorial water molecules co-ordinated to Cu^{2+}. The association constants for substitution of axially co-ordinated water molecules are unfavourable; for the successive displacement of water by ammonia in $Cu(H_2O)_6^{2+}$, only the four equatorial water molecules are readily substituted (Cotton & Wilkinson, 1966). It is, therefore, probable that only equatorially co-ordinated water molecules in benzylamine oxidase would be substituted by the various inhibitors. Postulation of one equatorial and one axial water molecule per Cu^{2+} site rationalizes the extrapolated limit of 0.5 for T_{1p}/T_{1p}^* at infinite azide and cyanide concentrations

Table 5. *Comparison of values of the dissociation constant* K_D *for inhibitor–enzyme complexes*

	K_D (mM)	
Ligand	From kinetics	From n.m.r.
Azide	84±16	48±15
Cyanide	0.76±0.21	0.7±0.3

(Figs. 13 and 14). Support for this hypothesis comes from the value of τ_m (the residence time of co-ordinated water molecules) for the native enzyme. $\tau_m^{-1} = 6.25 \times 10^5 s^{-1}$ and is of the same order as the water exchange rate constant for the Cu^{2+} complex with 2,2′,2″-triaminotriethylamine, where the single co-ordinated water molecule occupies a site with equatorial character (see Table 4). The profile of the curve in Fig. 11 is too steep to be described in terms of a single deprotonation process, but is consistent with ionization of two chemical species whose pK values are similar (Edsall & Wyman, 1958). This implies different pK values, yet within the pH range 8–9, for the axially and equatorially bound water molecules. The similarity of these pK values is less than would be expected from the behaviour of axially and equatorially bound water molecules in model Cu^{2+} complexes (Paoletti *et al.*, 1973; Brookes & Pettit, 1975).

Finally, it seems reasonable to deduce, from the absence of any effect of amphetamine, a competitive inhibitor against benzylamine, on the water proton-relaxation rate, that benzylamine does not displace water molecules co-ordinated to Cu^{2+} in the enzyme. This suggests that Cu^{2+} is probably not involved in reactions leading to reduced enzyme intermediates.

Discussion

Location and structure of copper sites

The ultracentrifugation, gel-electrophoresis and isoelectric-focusing results indicate that the two subunits in benzylamine oxidase have identical primary structure. The 35GHz e.s.r. spectrum showing that the two copper sites are different was therefore unexpected and suggests that the tertiary structure of the two subunits is distinct; there are precedents for this in other dimeric enzymes, e.g. hexokinase (Fletterick *et al.*, 1975). The 35GHz e.s.r. results also suggest that azide and cyanide binding renders the copper sites identical; this might imply that the tertiary structure of the subunits becomes identical too. The functional significance of these observations is unclear at present.

The studies into the effects of the enzyme on the nuclear spin relaxation of solvent water protons show that the copper sites are accessible to solvent and are therefore probably located on the surface of the protein. The copper sites cannot be close to each other, since spin interactions would have resulted in a smaller value for $[Cu^{2+}]$ from e.s.r. integration than determined by chemical assay. The n.m.r. measurements in conjunction with the conclusions drawn from a comparison of the e.s.r. parameters of the copper ions with those of well-defined copper complexes suggest that each copper site in the native enzyme has both nitrogen and oxygen (i.e. water) ligands; moreover, there are two water molecules per Cu^{2+} ion, one of which is co-ordinated equatorially and the other axially. The axial water molecule is not displaced by either N_3^- or CN^-, which is consistent with the absence of a dynamic Jahn–Teller effect due to the chemical inhomogeneity of the Cu^{2+} sites (Noack *et al.*, 1971; Lin *et al.*, 1975). This apparent rigidity of the Cu^{2+} sites could account for the low catalytic rate constant ($0.2 s^{-1}$) for the enzyme. In contrast, the Cu^{2+} sites in superoxide dismutase from bovine erythrocytes appear to be 'fluxional', which could explain the high catalytic rate of this enzyme (Boden *et al.*, 1979).

Function of the copper sites in the catalytic mechanism of benzylamine oxidase

The effects of azide and cyanide on the e.s.r. spectrum of the native enzyme and on the nuclear spin-relaxation measurements, taken with the effects of these reagents as competitive inhibitors to oxygen binding by the enzyme, implicate the copper sites in oxygen binding. Further support for this conclusion comes from comparison of the values of K_D for the azide and cyanide complexes as determined by n.m.r. and by steady-state kinetics (see Table 5). These measurements are in reasonable agreement, given that the conditions of temperature and pH are not identical. Our kinetic and magnetic-resonance experiments indicate that azide and cyanide inhibit benzylamine oxidase through displacement of water molecules co-ordinated to copper, a reaction that competitively inhibits reaction with oxygen. Thus the copper sites in benzylamine oxidase must be involved in the re-oxidation by oxygen of the enzyme species produced through reduction by amine.

We thank Professor A. C. T. North for use of the PDP11 computational facilities and Dr. Y. K. Levine, Dr. D. J. Lowe and Dr. T. V. Willoughby for advice on e.s.r. spectral simulation, and Dr. D. Herries for helpful discussions. The skilled assistance of Mr. R. Boyes in the preparation of the enzyme and with analytical ultracentrifugation is gratefully acknowledged. The isoelectric-focusing analysis was kindly carried out by Dr. M. J. C. Crabbe. The work has been supported by Medical Research Council and Science Research Council project grants to P. F. K. and N. B. M.R.C., S.R.C. and ICI are thanked for the award of Research Studentships to S. C. C., M. C. H. and I. D. K. and Research Fellowships to G. C. and R. H.

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