Kinetic and e.p.r. studies of cyanide and azide binding to the copper sites of dopamine (3,4-dihydroxyphenethylamine) β -mono-oxygenase

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The kinetics of inhibition of dopamine (3,4-dihydroxyphenethylamine) β -mono-oxygenase by cyanide (CN^-) and azide (N_3^-) ions have been investigated by using steady-state methods. Both anions show complex non-competitive-inhibition patterns with respect to ascorbate, suggestive of anion binding at two different sites on the oxidized enzyme. To further investigate this finding, e.p.r. titrations of CN^- and N_3^- binding to the ^{63}Cu -reconstituted enzyme were carried out. Addition of approx. 2 equiv. of CN^- to copper elicits a new signal with $g_{\parallel} = 2.217$, $g_{\perp} = 2.025$, $A_{\parallel} = 17.0\text{ mT}$ characteristic of a copper (II)–cyano complex. Simulations show that this signal accounts for half the copper (II) in the enzyme. The remainder of the enzyme-bound copper is expressed by a signal close to, but not identical with, that of native enzyme. Further addition of CN^- induces a simultaneous decrease in intensity of both of these signals so that their 1:1 ratio is maintained. Binding of N_3^- , on the other hand, changes the e.p.r. spectrum to a form different from either that of the native or CN^- -treated enzyme, and integrates to 100% of the copper in the enzyme ($g_{\parallel} = 2.252$, $g_{\perp} = 2.050$, $A_{\parallel} = 16.5\text{ mT}$). Resolved superhyperfine structure is apparent in the $g_{\perp}$ region. N_3^- binding is also accompanied by the appearance of a broad charge-transfer band centred at 387 nm. Neither 9 nor 35 GHz e.p.r. spectra show evidence for more than one (non-interacting) species of Cu(II) in native enzyme and N_3^- derivatives. The binding and reactivity of CN^- , on the other hand, argues against independent copper sites in the enzyme.

Dopamine β -mono-oxygenase [3,4-dihydroxyphenethylamine, ascorbate: oxygen oxidoreductase (β -hydroxylating), EC 1.14.17.1] catalyses the conversion of dopamine to noradrenaline [nor-epinephrine; 4-(2-amino-1-hydroxyethyl)benzene-1,2-diol], both of which are important neurotransmitters (Skotland & Ljones, 1979a; Villafranca, 1981). An unusual feature of the co-ordination chemistry of the metal sites of this enzyme is the lability of the enzyme-bound copper ions: both Cu(II) and Cu(I) forms undergo rapid exchange with added ^{64}Cu (Skotland & Flatmark, 1983) and are readily chelated by EDTA (Skotland *et al.*, 1980), bathocuproin sulphonate (Skotland & Ljones, 1979b) or Chelex resin (Blackburn *et al.*, 1980) to yield an inactive apoenzyme that may be rapidly reconstituted to full activity by addition of a small excess of Cu^{2+} (aq.). Although these studies provide strong evidence that the enzyme contains

four functional copper atoms per tetramer, they have also identified a number of additional weak copper binding sites, and Villafranca (1981) has observed binding of eight copper atoms per tetramer by using n.m.r.-relaxation measurements. This non-specific nature of copper binding has complicated efforts to elucidate the role of copper in catalysis.

The oxidized native enzyme exhibits an e.p.r. spectrum that accounts for 100% of the protein-bound copper, and it is generally agreed that in this form the metal centres are not closely coupled (Walker *et al.*, 1977; Ljones *et al.*, 1978; Villafranca, 1981). A redox role has been established for the copper during catalysis (Skotland *et al.*, 1980), and it has been shown conclusively that ascorbate reduction occurs by a one-electron mechanism, ascorbate being oxidized to the semidehydro-ascorbate free radical (Skotland & Ljones, 1980;

Diliberto & Allen, 1981). However, there is disagreement in the literature concerning the structure of the copper sites and their detailed role in the catalytic mechanism. Blackburn *et al.* (1980) have obtained kinetic evidence for participation of two copper atoms at some stage of the catalytic mechanism, but this interpretation has been challenged by Skotland & Ljones (1983), who favour a mechanism involving four non-interacting active sites.

In this paper we present kinetic and e.p.r. results on the reaction of the inhibitors CN^- and N_3^- with the enzyme. These results provide further evidence that the catalytic centres are not independent, but that ligand binding at one site induces changes in the structural and electronic properties of another.

Materials and methods

Enzyme isolation and assay

Dopamine β -mono-oxygenase was isolated by using a modification of the method of Ljones *et al.* (1976) in which high-performance anion-exchange chromatography on DEAE (LKB; TSK Blue column) was introduced as a final purification step. This procedure resulted in electrophoretically pure enzyme with a specific activity comparable with that of the highest-purity preparations obtained previously (Blackburn *et al.*, 1980). The enzyme was assayed with tyramine [4-(2-aminoethyl)phenol] as substrate by monitoring oxygen uptake at 298 K with a Clark-type oxygen-sensitive electrode as described previously (Blackburn *et al.*, 1980), except that a trace amount of Cu^{2+} (aq.) ($1.0 \times 10^{-7} \text{ mol} \cdot \text{dm}^{-3}$) was included to ensure complete reconstitution of the enzyme.

Inhibition by CN^- and N_3^-

Inhibition by CN^- and N_3^- was measured in the above assay system by varying one substrate while keeping the other fixed at the saturating value. These experiments were repeated over a range of inhibitor concentrations. Slopes and intercepts of the double-reciprocal plots were obtained by linear regression on a Hewlett-Packard 9810 computer.

Reconstitution of the enzyme with ^{63}Cu

The isolation procedure routinely produced samples containing a high proportion of apo-enzyme. It was also desirable to use a pure isotope of copper for e.p.r. experiments, and hence reconstitution with ^{63}Cu was carried out. The protein as isolated after chromatography contained about 1.5 mol of copper per tetramer present as the Cu(I) form. The copper was removed by dialysis against EDTA ($1 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$) and the resulting apo-

enzyme was exhaustively dialysed against acetate buffer ($0.10 \text{ mol} \cdot \text{dm}^{-3}$), pH 5.5. A final exhaustive dialysis against the same buffer yielded a preparation containing $4.4 \pm 0.4 \text{ g-atoms}$ of copper per tetramer, which was then concentrated to approx. 80 mg/cm^3 . The concentrated enzyme was stored frozen in liquid N_2 . Subsequent quantitative e.p.r. measurements against Cu(II)-EDTA as standard gave a value of 3.95 g-atoms of Cu(II) per tetramer, indicating essentially complete reconstitution of the enzyme into the oxidized Cu^{2+} form.

E.p.r. studies

E.p.r. spectra were recorded by using a Varian 112 spectrometer operating at either 9 or 35 GHz. X-band spectra were recorded as frozen glasses at 77 K, whereas with Q-band spectra, temperatures close to 105 K were employed. CN^- and N_3^- titrations were performed by addition of 0.002 cm^3 aliquots of a concentrated solution of Chelex-treated ligand to 0.1 cm^3 of a solution of the enzyme in a calibrated e.p.r. tube at room temperature and freezing in liquid N_2 after mixing. After the spectrum was recorded, the solution was thawed and a further aliquot added. For each spectrum, the double integral was determined by using the routine INTE on the Varian E900 data-acquisition system and the Cu(II) concentration calculated by using Cu(II)-EDTA in aq. 10% (v/v) glycerol, pH 3, as standard. The Cu(II)-EDTA system was found to give a reproducible linear relationship between second integral and Cu(II) concentration over the range $(0.02\text{--}2.0) \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$, and was used to construct a calibration curve from which enzyme Cu(II) concentrations were obtained.

Protein and copper analysis

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Total copper was determined with a Perkin-Elmer atomic-absorption spectrometer.

Reagents

All reagents were analytical grade. ^{63}Cu was obtained from the U.K. Atomic Energy Authority, Harwell, Didcot, Oxon., U.K. KCN and NaN_3 solutions were made up in sodium acetate buffer ($0.10 \text{ mol} \cdot \text{dm}^{-3}$), pH 5.5, and passed through Chelex resin (Sigma) before e.p.r. titrations.

Results

Kinetics of inhibition by N_3^- and CN^-

CN^- and N_3^- act as inhibitors of dopamine β -mono-oxygenase. The results of inhibition studies with these anions are shown in Figs. 1–4. Inhibition by CN^- with respect to ascorbate as variable

Fig. 1. Inhibition of dopamine β -mono-oxygenase by CN^- with tyramine saturating ($2.0 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$) and ascorbic acid as variable substrate at $298 \pm 0.1 \text{ K}$. $10^6 \times [\text{CN}^-] (\text{mol} \cdot \text{dm}^{-3})$: $\circ$, 0; ∇ , 0.1; $\diamond$, 1.0; $\square$, 1.25; $\blacktriangle$, 2.0. Rates were measured by monitoring O_2 consumption with an oxygen-sensitive electrode as described in the text.

Fig. 3. Inhibition of dopamine β -mono-oxygenase by CN^- with ascorbic acid saturating ($1.0 \times 10^{-2} \text{ mol} \cdot \text{dm}^{-3}$) and tyramine as variable substrate at $298 \pm 0.1 \text{ K}$. $10^6 \times [\text{CN}^-] (\text{mol} \cdot \text{dm}^{-3})$: $\circ$, 0; ∇ , 1.0; $\square$, 2.5; $\blacktriangledown$, 5.0. Rates were measured by using an oxygen-sensitive electrode as described in the text.

Fig. 2. Inhibition of dopamine β -mono-oxygenase by N_3^- with tyramine saturating ($2.0 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$) and ascorbate as variable substrate at $298 \pm 0.1 \text{ K}$. $10^3 \times [\text{N}_3^-] (\text{mol} \cdot \text{dm}^{-3})$: $\blacktriangledown$, 0; $\circ$, 0.6; $\square$, 1.0; ∇ , 2.0; $\diamond$, 3.0; $\bullet$, 6.0. Rates were measured by using an oxygen-sensitive electrode as described in the text.

Fig. 4. Inhibition of dopamine β -mono-oxygenase by N_3^- with ascorbic acid saturating ($1.0 \times 10^{-2} \text{ mol} \cdot \text{dm}^{-3}$) and tyramine as variable substrate at $298 \pm 0.1 \text{ K}$. $10^3 \times [\text{N}_3^-] (\text{mol} \cdot \text{dm}^{-3})$: $\circ$, 0; ∇ , 6.0; $\square$, 15.0; $\bullet$, 60.0. Rates were measured by using an oxygen-sensitive electrode as described in the text.

substrate approximates to the competitive type at low $[\text{CN}^-]$, but the presence of a small intercept effect at higher $[\text{CN}^-]$ suggests more complex behaviour. With N_3^- this complex pattern involving both slope and intercept effects is much more pronounced. With the nomenclature of Cleland (1963), K_i (slope) and K_i (intercept) for inhibition by N_3^- have been determined from replots of slopes and intercepts versus $[\text{N}_3^-]$ and yield values of $(1.2 \pm 0.2) \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ and $(1.4 \pm 0.4) \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ respectively. An important feature of the replots for inhibition by CN^- is that positive deviations from linearity occur at high $[\text{CN}^-]$. This implies, as discussed by

Cleland (1963), that further inhibition occurs as the result of combination of one or more molecules of inhibitor with the EI complex, thereby introducing terms in $[\text{I}]^2$ and higher orders. The non-linearity has prevented determination of accurate K_i values from the data relating to ascorbate as variable substrate, since the data is insufficient to apply meaningful polynomial curve-fitting procedures (Bardsley & Childs, 1974), but extrapolation of the linear portion of the curves as $[\text{I}] \rightarrow 0$ gives estimates of K_i (slope) = $9.4 \times 10^{-7} \text{ mol} \cdot \text{dm}^{-3}$ and K_i (intercept) = $6.9 \times 10^{-6} \text{ mol} \cdot \text{dm}^{-3}$. Both anions show uncompetitive inhibition and exhibit linear replots with respect to tyramine as variable

substrate, yielding K_i values of $(2.0 \pm 0.6) \times 10^{-6} \text{ mol} \cdot \text{dm}^{-3}$ and $(5.8 \pm 2.3) \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ for CN^- and N_3^- respectively.

Electronic spectrum of the N_3^- complex

Addition of azide to a concentrated solution of the enzyme leads to immediate formation of a yellow-green colour, the spectrum of which consists of a broad band, $\lambda_{\text{max}} = 327 \text{ nm}$, $\epsilon = 8550 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Spectrophotometric titration leads to an estimate of $1.8 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ for the N_3^- dissociation constant.

E.p.r. studies

Native enzyme. The X-band e.p.r. spectrum of ^{63}Cu -reconstituted native enzyme is shown in Fig. 5(a). The total analytical concentration of copper in the sample was $(1.18 \pm 0.08) \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ (4.4 ± 0.4 copper atoms per protein molecule) compared with the concentration of Cu(II) determined by double integration of the e.p.r. spectrum of $1.05 \pm 0.07 \text{ mol} \cdot \text{dm}^{-3}$ (3.95 copper atoms per protein molecule). This equates to $89 \pm 12\%$ of the copper as e.p.r.-detectable Cu(II) . Thus, within experimental error, reconstitution to 4g-atoms of $^{63}\text{Cu(II)}$ has been achieved. These results confirm the value of 4g-atoms of functional copper previously found for dopamine β -mono-oxygenase (Skotland & Ljones, 1979b, 1980).

The ^{63}Cu e.p.r. spectrum is similar to previously published mixed-isotope spectra (Walker *et al.*, 1977; Ljones *et al.*, 1978; Villafranca, 1981) with respect to g - and A -values, but exhibits better resolution of the fourth $M_I = -3/2$ $g_{\parallel}$ copper hyperfine line. A simulation of the spectrum with axially symmetric g - and A -tensors is shown in Fig. 5(b) and agrees well with the experimental spectrum. In order to achieve satisfactory simulation, particularly of the positive amplitude in the $g_{\perp}$ region, 60% Lorentzian + 40% Gaussian line shape was found to be necessary. g - and A -values are given in

Table 1. The spectrum of the same preparation of (^{63}Cu) native enzyme measured at 35 GHz is shown in Fig. 6(a). Whereas the $g_{\parallel}$ copper hyperfine is rather poorly resolved, the $g_{\perp}$ region is well-

Fig. 5. 9 GHz e.p.r. spectra of ^{63}Cu -dopamine β -mono-oxygenase and its N_3^- derivative

(a) Native enzyme with $[^{63}\text{Cu}^{2+}] = 1.05 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$, in sodium acetate buffer ($0.10 \text{ mol} \cdot \text{dm}^{-3}$), pH 5.5. (b) Simulation of native spectrum using parameters given in Table 1. (c) Enzyme as in (a), but containing NaN_3 ($6.3 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$). (d) Simulation of N_3^- spectrum using parameters given in Table 1. E.p.r. spectra were measured at 77 K. The frequency was close to 9.25 GHz. The modulation amplitude was 1.0 mT and the microwave power 40 mW.

Table 1. Parameters used to simulate the e.p.r. spectra of ^{63}Cu -substituted dopamine β -mono-oxygenase and its N_3^- and CN^- derivatives

The simulations were based on a modification of the programs described by Collison & Mabbs (1982) and Gahan & Mabbs (1983). A -values and linewidths are in mT. Conditions of measurement of experimental spectra are as described in the legends to Figs. 5–7.

Derivative	g -Values		A -Values (mT)		Linewidths (mT)		Lineshape (%)	
	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$	$A_{\perp}$	$W_{\parallel}$	$W_{\perp}$	Gaussian	Lorentzian
Native								
9 GHz	2.287	2.056	15.5	0.5	45	50	40	60
35 GHz	2.282	2.056	15.5	0.5	65	85	40	60
N_3^-	2.252	2.050	16.5	1.0	55	60	50	50
CN^-								
Component I	2.287	2.070	14.5	1.0	55	55	40	60
Component II	2.217	2.025	17.0	1.5	50	50	40	60

Fig. 6. 35 GHz e.p.r. spectrum of ^{63}Cu -dopamine β -mono-oxygenase

(a) Experimental spectrum; (b) simulation using parameters in Table 1. The sample details were as for Fig. 5(a). The spectrum was measured at 105 K at a frequency of 34.91 GHz. The modulation amplitude was 2.0 mT and the microwave power 50 mW.

developed and contains no indications of either rhombicity or the presence of more than a single species of Cu(II). The 35 GHz spectrum can be simulated (Fig. 6b) by using closely similar g - and A -values to the 9 GHz simulations of native ^{63}Cu spectra (Table 1), although the small variations, particularly in line-widths, are not unexpected, owing to the difference in the temperature of measurement. Thus the four copper ions in oxidized native dopamine β -mono-oxygenase occupy magnetically equivalent co-ordination sites and exhibit no observable distortions from axial symmetry.

N_3^- derivative. Titration with N_3^- leads to the spectral changes shown in Fig. 5(c), which are essentially complete after addition of 6 equiv. ($6 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$) of NaN_3 . The spectrum integrates to 100% Cu(II). The N_3^- derivative is of particular interest because of the appearance of partially resolved superhyperfine structure in the $g_{\perp}$ region. There has been only one previous report of superhyperfine structure in e.p.r. of dopamine mono-oxygenase when native enzyme was treated with the substrate tyramine (Walker *et al.*, 1977). Fig. 5(d) shows the simulation obtained using

Fig. 7. Titration of ^{63}Cu -dopamine β -mono-oxygenase with CN^-

Aliquots (0.002 cm^3) of KCN ($2.63 \times 10^{-2} \text{ mol} \cdot \text{dm}^{-3}$) were added to 0.10 cm^3 of native enzyme [$^{63}\text{Cu}^{2+}] = 1.05 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ in sodium acetate buffer ($0.10 \text{ mol} \cdot \text{dm}^{-3}$), pH 5.5 in an e.p.r. tube, mixed thoroughly and frozen. The contents of the tube were thawed to room temperature before addition of subsequent aliquots. Each addition took approx. 3 min. The spectra shown have CN^-/Cu ratios and percentage e.p.r.-detectable copper concentrations as follows: (a) 0, 100%; (b) 0.5, 103%; (c) 1.0, 102%; (d) 1.5, 98%; (e) 2.0, 80%; (f) 2.5, 66%; (g) simulated spectrum using equal proportions of component I to component II with parameters as given in Table 1; (h) 3.0, 51%; (i) 3.5, 39%; (j) 4.0, 31%; (k) 4.5, 28%; (l) 5.0, 21%. E.p.r. spectra were measured at 77 K at a frequency of 9.252 GHz. The modulation amplitude was 1.0 mT and the microwave frequency was 40 mW.

axially symmetric g - and A -tensors, which again requires a high proportion (50%) of Lorentzian lineshape. The e.p.r. parameters used in the simulation are given in Table 1.

CN⁻ derivative. Titration of native enzyme with CN⁻ leads to profound changes in the e.p.r. spectrum attributable to the formation of a new Cu(II) species with lower *g*-values, together with a species that resembles native enzyme. Some partially resolved superhyperfine splittings are also apparent in the *g*_⊥ region. Fig. 7 shows the spectral changes that occur as the cyanide-to-copper ratio is increased. These changes appear to be fully developed between 2.0 and 2.5 equiv. of CN⁻ per copper atom. Further addition of CN⁻ produces no significant change in the relative intensity of the two signals, but causes a simultaneous decrease in the absolute intensity of both.

It has been possible to simulate the parallel and perpendicular regions of the composite spectra of Fig. 7 by using a combination of two axially symmetric components, I and II, with *g*- and *A*-values as given in Table 1. By using 60% Lorentzian line-shape the simulated spectra still overshoot the positive amplitude of the *g*_⊥ region, suggestive of some additional component or interaction leading to broadening of this region of the experimental spectrum. A simulation using equal proportions of component I to component II is shown in Fig. 7(g), whence it may be inferred that the ratio of the two components in the fully developed experimental CN⁻ spectrum (at between 2.0 and 2.5 equiv. of CN⁻ added; Figs. 7e and 7f), is close to 1:1 and remains roughly constant at this value as the total signal intensity decreases during the later stages of the titration.

The *g*_{||} for component I is identical with that of native enzyme. However, changes are observed in both *g*_⊥ and *A*_{||}, which suggests that component I may represent a species different from native enzyme. The results suggest, therefore, that CN⁻ reacts with enzyme-bound Cu(II) to generate two new signals, each of which corresponds to half the copper(II) in the enzyme. This contrasts with the e.p.r. behaviour of N₃⁻, which elicits a single axial e.p.r. spectrum from all four copper atoms.

Discussion

It is generally accepted (Goldstein *et al.*, 1968) that dopamine β-mono-oxygenase operates through a Ping Pong mechanism and shuttles between an oxidized form, which is reduced by ascorbate, to a reduced form, which interacts with tyramine and O₂. The uncompetitive behaviour of both CN⁻ and N₃⁻ towards tyramine as variable substrate is indicative of inhibitor binding to an enzyme form other than, and not reversibly connected to, that which binds tyramine, and would be consistent with inhibitor binding to the oxidized form of the enzyme (Cleland, 1970).

In support of this conclusion, inhibition by CN⁻

is close to competitive with respect to ascorbate as variable substrate. However, the existence of a small intercept effect at higher concentrations of CN⁻ suggests more complex behaviour, and with N₃⁻ the complex pattern involving both slope and intercept effects is much more pronounced. The slope effects are indicative of binding of the anions to the same enzyme form or the same enzyme site which binds ascorbate, i.e. to the oxidized Cu(II). The intercept effect, however, as discussed by Barker *et al.* (1979) can only be explained by binding of the inhibitors to another oxidized form or another site on the oxidized enzyme. In the case of CN⁻ binding to plasma amine oxidase, which shows kinetic patterns similar to those shown by dopamine mono-oxygenase involving both slope and intercept effects, those authors have explained the behaviour by postulating that CN⁻ may react simultaneously as a ligand to copper and as a nucleophile towards the active carbonyl group. For dopamine β-mono-oxygenase, since there is no evidence to support the existence of prosthetic groups other than the Cu(II) centres (Villafranca, 1981), two-site action would necessitate inhibitor binding to some functionally important protein residue or co-ordination of inhibitor at a second Cu(II) ion not directly involved in ascorbate binding. This latter possibility would imply some functional inequivalence between copper centres.

CN⁻ and N₃⁻ are well-known ligands for copper proteins such as benzylamine oxidase (Barker *et al.*, 1979), galactose oxidase (Giordano *et al.*, 1974; Winkler & Bereman, 1980) and superoxide dismutase (Rotilio *et al.*, 1972; Haffner & Coleman, 1973; Boden *et al.*, 1979; Fee, 1981), where inhibition is known to result from co-ordination at the copper sites. For dopamine mono-oxygenase, the appearance of a broad absorption band centred at 387 nm on addition of N₃⁻ provides evidence for copper-N₃⁻ co-ordination (Fee & Gaber, 1972; Lindstrom *et al.*, 1974; Winkler *et al.*, 1982), whereas the close correspondence of both *K*_i (slope) and *K*_i (intercept) to the dissociation constant obtained from spectrophotometry at this wavelength is consistent with Cu(II)-N₃⁻ co-ordination being responsible for the inhibition. The e.p.r. spectral changes that are induced by addition of N₃⁻ provide further evidence for Cu(II)-N₃⁻ co-ordination, and the single component spectrum thus obtained suggests a similar co-ordinate structure at each of the four copper atoms.

E.p.r. spectral changes also accompany CN⁻ binding. In Table 2, *g*_{||} and *A*_{||} values of a number of Type 2 copper proteins are compared with those of their corresponding cyano complexes for which independent evidence on CN⁻ co-ordination exists (Haffner & Coleman, 1973; Barker *et al.*, 1979; Boden *et al.*, 1979; Winkler & Bereman, 1980). In

Table 2. Comparison of e.p.r. parameters for cyano complexes of copper proteins

The Table compares the shifts in $g_{\parallel}$ and $A_{\parallel}$ observed when the native forms of the enzymes react with CN^- . The parameters used to simulate the dopamine mono-oxygenase- CN^- spectra are included for comparison.

Enzyme	$g_{\parallel}$		$A_{\parallel}$ (mT)		Reference
	Native	Cyano	Native	Cyano	
Superoxide dismutase	2.265	2.210	13.0	18.6	Lieberman <i>et al.</i> (1981)
Human carbonic anhydrase B	2.300	2.199	13.4	18.2	Taylor & Coleman (1971)
Benzylamine oxidase	2.286	2.218	15.5	15.8	Barker <i>et al.</i> (1979)
Galactose oxidase	2.276	2.229	17.7	15.8	Giordano <i>et al.</i> (1979)
Dopamine mono-oxygenase					
Component II	2.287	2.217	15.5	17.0	The present work
Component I		2.287		14.5	The present work

all cases, binding of CN^- leads to a decrease in $g_{\parallel}$ to 2.20–2.23, whereas for superoxide dismutase and carbonic anhydrase, $A_{\parallel}$ increases to 18.0–19.0 mT. The spectral parameters observed for component II parallel those documented above for other copper-protein-cyano complexes whose e.p.r. parameters are known to be extremely diagnostic of square-planar co-ordination (Hathaway & Billing, 1970; Bertini, 1981; Van Kamp *et al.*, 1982). Thus component II may be assigned to a Cu(II)-cyano complex in a more planar environment than that of native dopamine mono-oxygenase.

The e.p.r. parameters for component I, however, do not resemble those of any of the cyano complexes in Table 2. The characteristic decrease in $g_{\parallel}$ is not observed, $A_{\parallel}$ decreases and $g_{\perp}$ increases. Component I, therefore, does not appear to contain co-ordinated CN^- , but may represent a modified form of native enzyme induced by CN^- binding at a separate copper site (component II).

It is well known that Cu(II)-cyano complexes undergo reduction to Cu(I) and cyanogen (Cotton & Wilkinson, 1972), and it is likely that the observed decrease in e.p.r. intensity during the CN^- titration is due to reduction to Cu(I). However, the data are difficult to rationalize in terms of four independent and non-interacting catalytic copper centres as advocated by Skotland & Ljones (1979a, 1983), because such a model would predict a monotonic increase in the CN^- signal at the expense of the 'native' signal. However, it is observed experimentally that, during the cyanide titration, the e.p.r. spectra remain two-component, with indications from the simulations of equal amounts of these components persisting when the CN^- -to-enzyme ratio exceeds 2.5 and the total e.p.r. intensity decreases. This suggests that loss of e.p.r. intensity results from further reaction of both components with CN^- in a concerted process. An attractive hypothesis that is also consistent with the conclusions derived from inhibition kinetics is that a second molecule of CN^- binds to the

enzyme- CN^- complex and induces concerted reduction of two copper centres according to the general scheme:

However, alternative processes involving reduction by protein-bound residues such as RS^- , or ligand-induced spin coupling between Cu(II) centres cannot be excluded as possible mechanisms for decrease in e.p.r. intensity. Whatever the detailed mechanism, the results suggest that the catalytic centres of dopamine β -mono-oxygenase do not act independently, but are located within the tetrameric molecule such that CN^- binding at one copper influences the structural electronic and/or redox properties of another. The absence of significant magnetic coupling in the native and N_3^- derivatives would seem to argue in favour of medium-to-long-range conformational effects as the mediators of site-site interaction, but it is worth noting that deviations from pure Gaussian or pure Lorentzian lineshape (as required to obtain an 'overlay' simulation of the native e.p.r. spectrum) have been attributed by others to the presence of a weak magnetic interaction between paramagnetic centres (Wyard, 1965; Abragam & Bleaney, 1970; Cusumano & Troup, 1974; Royaud & Treville, 1974; Poole, 1983).

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