The nature of haem a_3 in the oxidized state of cytochrome c oxidase

Evidence from low-temperature magnetic-circular-dichroism spectroscopy in the near-infrared region

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The magnetic-circular-dichroism (m.c.d.) spectra of oxidized 'resting' bovine cytochrome c oxidase and the cyanide-inhibited form are reported at 5.15 T and at 4.2 K along with m.c.d. magnetization curves plotted at selected wavelengths. In both spectra there are features at 790 nm and 1564 nm due to Cu_a and haem a respectively, the e.p.r.-detectable components of the enzyme. There is a new peak at 1946 nm only in the spectrum of the cyanide-inhibited enzyme. Arguments are advanced that assign this to low-spin ferric haem a_3 bridged to Cu_a , thereby forming a ferromagnetically coupled pair of metal ions.

Bovine heart cytochrome c oxidase (EC 1.9.3.1) is an enzyme containing four redox-active metal centres, two haem a groups and two copper ions (Malmström, 1979). Of the two haem groups only one, called haem a_3 , is able to react with substrate, O_2 , or with inhibitors such as CO and CN^- . It is e.p.r.-silent in the resting oxidized state of the enzyme and in the cyanide-inhibited form of the oxidized enzyme. The other haem group, called haem a , gives a typical low-spin ferric haem e.p.r. signal with g -values of 3.03, 2.21 and 1.45 in the oxidized enzyme. Only one of the copper centres, called Cu_a , is e.p.r.-detectable in the oxidized state of the enzyme, giving signals in the region $g = 2$ (Hartzell & Beinert, 1974; Aasa *et al.*, 1976). The other copper centre, termed Cu_b , is e.p.r.-silent. The absence of e.p.r. signals from two of the centres early led to the suggestion that they are interacting in an antiferromagnetically coupled pair (Van Gelder & Beinert, 1969). An alternative hypothesis proposes that Cu_a remains always in the cuprous state and that haem a_3 is in the ferryl Fe^{IV} form in the oxidized resting state of the enzyme and therefore both are e.p.r.-silent (Seiter & Angelos, 1980). We now report the low-temperature near-i.r. m.c.d. spectra and m.c.d. magnetization curves (Thomson & Johnson, 1980) of some derivatives of the oxidized form of the enzyme. We show that it is possible to observe separately the optical properties of at least three of the four metal centres and hence to determine the magnetic properties of each. These experiments give

strong evidence in favour of the haem a_3 and Cu_a centres being in the ferric and cupric states respectively in the cyanide-inhibited oxidized enzyme and magnetically coupled. Furthermore, the near-i.r. m.c.d. spectrum provides a characteristic optical 'fingerprint' of the haem a_3 – Cu_a pair in the cyanide-inhibited oxidized enzyme.

The m.c.d. spectra between 700 and 2000 nm at 4.2 K and 5.3 T of the oxidized and the cyanide-inhibited oxidized form of cytochrome c oxidase are given in Fig. 1. M.c.d. magnetization curves (Thomson & Johnson, 1980) have been constructed at the wavelengths of the three major features in the spectrum of the cyanide form, i.e. at 790 nm, 1564 nm and 1946 nm (Fig. 2). The curves have been normalized to the same saturation limit for comparison purposes. The initial slopes show that three different paramagnets are being detected in the m.c.d. spectrum of the cyanide-inhibited form (Thomson & Johnson, 1980). The magnetization curves of the trough at 790 nm and the peak at 1550 nm in the spectrum of the oxidized resting form of the enzyme are very similar to those in Fig. 2. The magnetization curve monitored at 1564 nm fits well to a curve simulated for an x,y -polarized electronic transition from a ground state with the g -factors of haem a , namely 3.01, 2.21 and 1.45 (Thomson *et al.*, 1981). Furthermore, the m.c.d. has the form characteristic of a low-spin ferric haem charge-transfer band, namely porphyrin $\rightarrow \text{Fe}^{\text{III}}$, in a spectral region typical of a bis-imidazole–haem a model (Stephens *et al.*, 1974; Nozawa *et al.*, 1976; Johnson *et al.*, 1981). The trough at 790 nm magnetizes

Abbreviation used: m.c.d., magnetic circular dichroism.

Fig. 1. M.c.d. spectra at 4.2K and 5.15 T of oxidized cytochrome *c* oxidase resting form (-----), cyanide-inhibited form (—) and partially reduced cyanide-inhibited form (— · —)

Enzyme was dissolved in 0.1M-phosphate buffer, pD 7.4, diluted to 50% (v/v) with deuterated ethanediol. The partially reduced state was produced by a reductive titration with $\text{Na}_2\text{S}_2\text{O}_4$ solution of the cyanide-inhibited form of the enzyme until there was no further change in the m.c.d. spectrum. This corresponded to the full development of the $g = 3.58$ signal (Johnson *et al.*, 1981). Path lengths were 1.19 and 1.16 mm respectively. $\Delta\epsilon$ ($= \epsilon_L - \epsilon_R$, the extinction coefficients for left and right circularly polarized light respectively) values are given per mol of enzyme.

with the shallowest curve of the three. The curve has the form expected for a ground state with an isotropic g -factor of approx. 2.0 (Thomson & Johnson, 1980) and is therefore assigned to the e.p.r.-detectable cupric centre, namely Cu^{II} .

The peak at 1946 nm (Fig. 1) requires some special comment. In an earlier room-temperature investigation of the near-i.r. m.c.d. of cytochrome *c* oxidase, Eglinton *et al.* (1980) failed to find this peak because the intense absorption resulting from the solvent and protein vibrational overtones prevented measurement beyond about 1800 nm, although we did detect the rising background at 1800 nm. At 4.2K the electronic m.c.d. transitions of the paramagnetic centre become very intense compared with the overtone absorption. Detection of this new low-energy electronic state becomes possible. The m.c.d. spectrum between 1800 and 2000 nm has the shape expected for the porphyrin $\rightarrow \text{Fe}^{\text{III}}$ charge-transfer band of a low-spin ferric haem, namely two bands of positive sign, the one at higher energy being about half the intensity of the other (Stephens *et al.*, 1974). We take this as compelling evidence for the assignment of haem a_3 -CN to the low-spin ferric state in the oxidized form of the enzyme. However, the extremely long wavelength of the peak of this band is unique among the near-i.r. spectra of low-spin ferric haems reported (Rawlings *et al.*, 1977).

The m.c.d. peak at 1946 nm magnetizes with a much steeper initial slope than the other two paramagnets observed (Fig. 2). The form is identical with that obtained for haem a_3 -CN from detailed studies in the Soret-band region (Thomson *et al.*, 1981). Our analysis suggested a ground state of haem a_3 -CN as $S = 1$, with a predominantly axial zero-field splitting that leaves an electronic doublet, $M_S = \pm 1$, lower by approx. 10 cm^{-1} than the spin singlet state, $M_S = 0$. This characterization of the magnetic state of haem a_3 -CN is quite at variance with recent interpretations of magnetic-susceptibility data (Tweedle *et al.*, 1978).

Taking together the two characteristics of haem a_3 -CN reported here, namely the shape of the near-i.r. m.c.d. spectrum and the m.c.d. magnetization properties, we conclude that this haem, in the cyanide-inhibited oxidized form of the enzyme, is in the low-spin ferric state and ferromagnetically coupled to Cu^{II} to give an effective ground-state spin $S = 1$.

Recently we reported a thorough investigation of the dithionite reduction of the cyanide-inhibited form of the enzyme (Johnson *et al.*, 1981). This leads to a partially reduced state in which all of the metal components except haem a_3 -CN are reduced and therefore diamagnetic. Haem a_3 -CN becomes e.p.r.-detectable in the partially reduced form with a resonance at $g = 3.58$. The near-i.r. m.c.d. spectrum

Fig. 2. *M.c.d. magnetization curves of cyanide-inhibited oxidized cytochrome c oxidase monitored at wavelengths of (a) 790 nm (.....), (b) 1564 nm (---) and (c) 1946 nm (—)*

Sample details are as given in the legend to Fig. 1. A total of 50 data points have been plotted on each curve, although for clarity some have been omitted at low values of magnetic field. For curves (a) and (b) the temperatures used were 1.5 K (○), 2.1 K (□), 4.2 K (▽) and 20.0 K (△); for curve (c) the 20 K data points have been omitted. Magnetic fields employed were 0.156, 0.293, 0.436, 0.579, 0.721, 0.861, 0.994, 1.128, 1.259, 1.386, 2.001, 2.594, 3.013, 3.754, 4.035, 4.325 and 5.049 T. The saturation limits of the three curves have been arbitrarily normalized to 100.

at 4.2 K of the course of reduction shows the disappearance of the characteristic peak at 1946 nm and concomitantly the appearance of a sharp positive m.c.d. peak at 1500 nm (Fig. 1). The magnetization curve of this latter peak shows it to originate from the e.p.r.-detectable haem a_3 -CN with $g_z = 3.58$. Therefore the low-spin ferric charge-transfer band of haem a_3 -CN shifts from 1946 to 1500 nm as the other redox centres in the protein are reduced and as haem a_3 becomes e.p.r.-detectable.

The energies of the near-i.r. charge-transfer bands of low-spin ferric haems are sensitively dependent on the field strength of the two axial ligands that bind the haem (Rawlings *et al.*, 1977). It is clear from a number of lines of evidence, including the Soret-region m.c.d. (Thomson *et al.*, 1981), that CN⁻ binds to haem a_3 in the oxidized state of the enzyme. This ligand remains bound to haem a_3 , preventing its reduction during the formation of the partially and

fully reduced states (Johnson *et al.*, 1981). Thus it is clearly not ligand exchange of CN⁻ at haem a_3 that accounts for the shift in energy of the band. We cannot conclusively rule out the possibility of ligand exchange at the proximal position of haem a_3 , which is certainly occupied by the imidazole group of histidine in the fully and partially reduced states of the enzyme (Stevens & Chan, 1981). However, it would be necessary to link proximal ligand exchange with the process of Cu^{II}_{*a*} reduction. We therefore prefer the following explanation, namely that CN⁻ forms a bridge between haem a_3 and Cu^{II}_{*a*}, thus: Fe^{III}-CN-Cu^{II}_{*a*}. The presence of the oxidizing ion, Cu^{II}, will cause the charge-transfer band, porphyrin → Fe^{III}, to lie at lower energy, since the Fe^{III} ion bridged to Cu^{II} will be more oxidizing. Reduction of Cu^{II}_{*a*} to Cu^I, as the enzyme is partially reduced, causes the charge-transfer band to move to higher energy. Possibly the bridge is broken

as reduction proceeds. The model compounds of Gunter *et al.* (1980) and Berry *et al.* (1980), in which a ferric porphyrin is bridged via CN^- to a cupric ion, should provide an excellent means of testing this hypothesis. The evidence given in the present paper is the first that points to the likelihood of an added ligand becoming bound between two metal centres in cytochrome *c* oxidase. We note that the bond length of the cyanide ion is 0.115 nm (1.15 Å) (Wells, 1962), not dissimilar from 0.121 nm (1.21 Å) (Wells, 1962), the interatomic distance of O_2 , the substrate of the enzyme. Furthermore, a bridged cyanide structure places an upper limit of approx. 0.55 nm (5.5 Å) on the separation between Cu_a , and the Fe^{III} ion of haem a_3 .

The preparation of cytochrome *c* oxidase and its derivatives has been given in detail elsewhere (Johnson *et al.*, 1981). The measurement of near-i.r. m.c.d. spectra and the construction of magnetization curves have also been described earlier (Eglinton *et al.*, 1980; Thomson & Johnson, 1980; Thomson *et al.*, 1981).

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