Low-Temperature Studies on Mixed-Valence Cytochrome Oxidase by using Magnetic Circular Dichroism

EVIDENCE FOR HAEM-HAEM INTERACTIONS

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The spin states of the haem components of mixed-valence cytochrome oxidase were studied at room temperature and at temperatures down to 20K by using magnetic circular dichroism. The room-temperature studies show the presence of a low-spin ferrous haem together with a low-spin ferric haem, which we attribute to haems a_3 and a respectively. At temperatures below 100K it appears that the CO of the mixed-valence CO complex may be irreversibly photolysed, and that in this case haems a and a_3 assume their high-spin states. Thus in this enzyme haem-haem interactions appear possible at temperatures below 100K.

Cytochrome c oxidase (EC 1.9.3.1) contains two spectroscopically distinguishable haems (Keilin & Hartree, 1939; Lemberg, 1969) designated a and a_3 . Ferrous cytochrome a_3 is defined as the site with which O_2 and inhibitory poisons such as CO react (Warburg & Negelein, 1929; Keilin, 1927). The characteristics of these sites have been probed by use of e.p.r. (electron paramagnetic resonance) (Hartzell & Beinert, 1974; Van Gelder & Beinert, 1969; Wilson *et al.*, 1976). As only one of the haems is e.p.r.-detectable in the fully oxidized protein and no signal is observed for the low-spin ferrous haem in e.p.r. studies, the technique suffers from some serious limitations. The use of m.c.d. (magnetic circular dichroism) is thus useful as a complementary technique, since it permits the observation of ferric and ferrous haems in both spin states. It does suffer, however, from the fact that its application to biological systems is as yet in its early stages of development; interpretation often requires a comparison to be made with model systems, the suitability of which might sometimes be questioned for a molecule such as cytochrome oxidase for which the exact chemical nature of the haem sites is not known.

Within the oxidase molecule it has been proposed that the state of one of the haems may affect the state of the other, and these haem-haem interactions have been studied by a number of diverse methods, such as titrations (Nichols & Peterson, 1974), e.p.r. (Leigh & Wilson, 1972; Leigh *et al.*, 1974) and ligand-binding reactions (Greenwood *et al.*, 1974).

With this in mind an investigation of the redox and spin states of the haems present in the mixed-valence species was undertaken. It has previously been noted that photolysis of the mixed-valence CO complex is rapid and essentially irreversible below 100K

(Yonetani, 1965; Chance *et al.*, 1965). This dissociation has been followed by Leigh & Wilson (1972) by studying the e.p.r. signal of haem a and they have concluded the presence of haem-haem interactions. The present study extends the work of Leigh & Wilson (1972) and we present data on the spin state of both haem centres.

Materials and Methods

Cytochrome c oxidase was prepared from bovine heart muscle by the method of Yonetani (1960), and the concentration of oxidase samples was established by using the extinction coefficient E_{605} (reduced) 21000 cm^{-1} given by Yonetani (1961). The mixed-valence species was prepared by incubation of the oxidized protein under 0.1 MPa (1 atm) of CO (Greenwood *et al.*, 1974). CO and O_2 -free N_2 were dispensed from cylinders and stored in glass vessels over an alkaline solution of dithionite-reduced anthraquinonesulphonate. All chemicals used were of analytical grade.

Static spectrophotometry was performed by using a Cary 118C instrument. M.c.d. studies were carried out by using a Cary 61 dichrograph fitted with a superconducting solenoid capable of generating a field of 5.1 T. For low-temperature studies the protein was saturated with sucrose and frozen in a flow cryostat as previously reported (Thomson *et al.*, 1976; Springall *et al.*, 1976).

Results and Discussion

The absorption and the m.c.d. spectrum of the mixed-valence CO complex obtained at room temperature (20°C) are shown in Fig. 1. A comparison

Fig. 1. Room-temperature absorption and m.c.d. spectrum of mixed-valence cytochrome oxidase

(a) shows the absorption spectrum and (b) the m.c.d. spectrum of mixed-valence cytochrome oxidase (----). The protein concentration was $10\mu\text{M}$ in the Soret region and $80\mu\text{M}$ in the α region, in both cases. In both cases the spectrum was recorded at 20°C in a 1 cm-path-length cell. The m.c.d. spectrum was recorded in the presence of a 5 T field. In (b) the spectrum produced by the addition of the spectrum of fully reduced + CO to that of the fully oxidized spectrum is shown for comparison (●).

of the m.c.d. spectrum with that of the fully reduced CO complex and the fully oxidized protein (Thomson *et al.*, 1976) shows that this spectrum contains features found in both systems. This is in line with the proposed redox state of the mixed-valence CO complex in which haem a_3 and the 'invisible' copper are reduced, whereas haem a and the other copper remain oxidized (Lindsay *et al.*, 1974). The synthesized spectrum in Fig. 1(b), produced by summing half the fully reduced CO spectrum and half the fully oxidized spectrum, shows a striking resemblance to the mixed-valence CO complex, which at least qualitatively adds weight to the assigned redox states in the mixed-valence species. The fit between the synthesized and

Fig. 2. Effect of temperature on the m.c.d. spectrum of mixed-valence cytochrome oxidase

The m.c.d. spectrum of mixed-valence cytochrome oxidase is shown at (—) 24K, (----) 41.5K and (-.-.-) 65K. The spectra were recorded at a protein concentration of $130\mu\text{M}$ in a 0.8 mm-path-length cell, in the presence of a 5 T field.

observed spectra is not expected to be exact, owing to the fact that in using the fully reduced CO complex and the fully oxidized spectra the contributions due to cytochrome a^{2+} and cytochrome a_3^{3+} would also be present, but nonetheless the comparison is still instructive.

Fig. 2 shows the marked temperature-dependence observed for the mixed-valence species below 100K. This spectrum is very similar to that observed for the fully reduced protein (Thomson *et al.*, 1976), except for a slightly more positive character between 380 nm and 420 nm. We therefore conclude the presence of a high-spin ferrous haem in the mixed-valence species, that is haem a_3^{2+} . The high-spin character of this haem shows that, under the conditions of illumination and temperature used in the dichrograph, the CO must be displaced from cytochrome a_3^{2+} (undissociated cytochrome a_3^{2+} -CO would be present as the temperature-independent low-spin form).

Considering for a moment the other haem centre, if cytochrome a^{3+} were in the low-spin form it would be expected to dominate the spectrum in the 420 nm region at low temperature (leading to a positive

$\Delta E/H$ approaching 300; Springall *et al.*, 1976), which clearly is not the case. But if cytochrome a^{3+} were in the high-spin form under these conditions, as suggested by Leigh & Wilson (1972) and Wilson *et al.* (1976), it should contribute, to the spectrum of high-spin reduced cytochrome a_3 , a small positive signal at around 420 nm and a relatively small negative signal in the 433 nm region, which is consistent with the observed data.

We thus propose that when a sample of mixed-valence CO complex is placed into the observation beam of the dichrograph, at less than 100K, the CO is irreversibly photolysed. Consequently, the spin state of the haem a_3^{2+} is transformed from low-spin to high-spin. This change then communicates itself to cytochrome a^{3+} , which also changes from its low-spin to high-spin state. In fact, Wikstrom *et al.* (1976) suggest that in the mixed-valence state, produced by titration in the absence of CO, the reduction of cytochrome a_3 induces a transition from low to high spin in the haem a by haem-haem interaction, and it is this situation that we have obtained by a different means.

The observed system changes could then be described as:

(ls) representing low-spin and (hs) high-spin states.

If this is indeed the case, then presumably, as protein mobility would be effectively zero in frozen solution at less than 100K, the communication of the change of spin state of cytochrome a_3 to cytochrome a must not require protein conformational changes to occur. The haem-haem interaction present must therefore be of some other, presumably, more direct electronic character.

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