DETERMINATION OF THE HEME SPIN STATES IN CYTOCHROME *c* OXIDASE USING MAGNETIC CIRCULAR DICHROISM

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1. Introduction

Cytochrome *c* oxidase contains two heme *a* groups and two copper ions. One of the hemes, called a_3, will bind ligands whilst the other heme termed *a* will not [1]. In spite of a great deal of investigation of the magnetic properties of these centres, mainly with the aid of low temperature electron paramagnetic resonance (e.p.r.) spectroscopy [2], there are still uncertainties about the spin states of the heme components. In the fully oxidised form of the enzyme about 50% of the heme can be detected in the e.p.r. as the low spin ferric form. A minor component, about 2.3% of the total heme, is detectable as the typical g = 6 high spin ferric signal [3]. Furthermore, although both copper ions are believed to be cupric in the fully oxidised state, only one can be detected in the e.p.r. experiments [3].

It has now been demonstrated that variable temperature magnetic circular dichroism (m.c.d.) spectroscopy can distinguish the two spin states of both ferro and ferri hemoproteins [4,5]. Thus cytochrome *c* oxidase is an excellent subject to study with this technique. In this work we have made use of the variable temperature studies to identify the spin states of the heme components, using as a guide wherever possible, our low temperature data on met- and ferro- myoglobin derivatives [5]. We have recently become aware of an apparently similar study carried out with this enzyme by Babcock et al. [6,7].

2. Materials and methods

Cytochrome oxidase (EC 1.9.3.1. ferrocytochrome *c* · oxygen oxidoreductase) was prepared by a modification of the method due to Yonetani [8], in which 10 μm EDTA was incorporated as a chelating agent, into the last fractionation steps to ensure the removal of adventitiously bound copper. The concentration of the cytochrome oxidase samples (in terms of heme *a*) was determined using $E_{605\ nm}$ = 21 000 M^{-1} cm^{-1} for the fully reduced enzyme [8].

All solutions used in this study were made up in 0.2 M potassium phosphate buffer at pH 7.2 containing 1% Tween 80. All ligands used were prepared as the neutralised solutions of the appropriate concentration. For the variable temperature studies the protein samples were saturated with sucrose prior to freezing.

M.c.d. spectra were measured with a Cary 61 dichrograph using a superconducting solenoid capable of generating a maximum field of 5.1 Tesla. The samples used in variable temperature studies were cooled in a rapid freeze flow cryostat similar to the design of Briat et al. [9]. The temperature of the sample block was monitored with a Au/Fe thermocouple and varied by controlling the cold helium gas flow rate. Solutions were made viscous by saturation with sucrose to prevent crystallisation on cooling. The cells used to hold the low temperature samples were constructed from two thin-walled glass plates separated by an 0.8 mm thick spacer. Absorption spectra were recorded using the Cary 118C instrument.

3. Results

3.1. *Fully reduced oxidase*

The m.c.d. spectrum of the fully reduced form of

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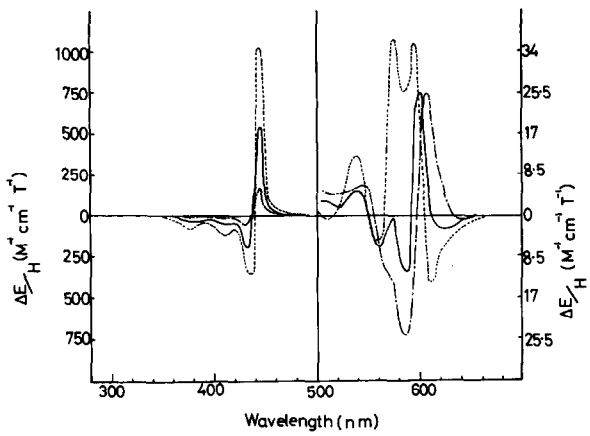

Fig.1. The effect of temperature on the m.c.d. spectrum of dithionite reduced oxidase is shown. The protein was 130 μM in the short wavelength region and 400 μM in the longer wavelength region. The temperatures shown are (- - - -) 36°K; (———) 77°K; (–·–·–·–) 293°K.

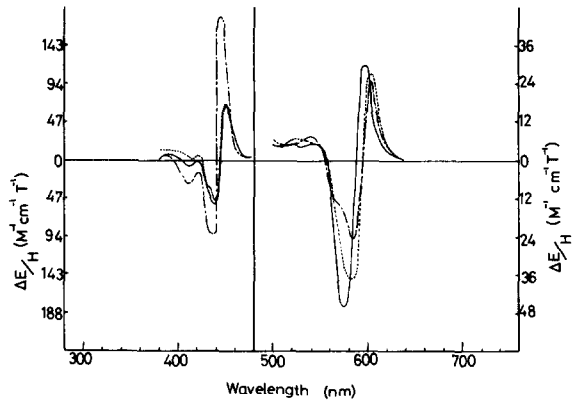

Fig.3. M.c.d. spectra of dithionite reduced oxidase (·–·–) and its carbon monoxide (- - -) and cyanide (———) derivatives. The protein was 10 μM in the short wavelength region and 40 μM in the longer wavelength region. Carbon monoxide was present at 1 atmosphere pressure and cyanide at 20 mM. Temperature was 20°C.

the enzyme is shown in fig.1. The spectra were recorded at three different temperatures. Clearly the enzyme is strongly paramagnetic, showing in the Soret region a spectrum remarkably similar to that of the high spin ferromyoglobin. Fig.2 shows the m.c.d. spectrum of the cyano derivative at three different temperatures. There is considerable sharpening of the spectrum on cooling but this does not lead to any significant intensity changes. Indeed, the spectra measured at the two lower temperatures are virtually superimposable. Hence both hemes are diamagnetic in the cyano derivative. The room temperature m.c.d. spectra of the reduced enzyme, the carbonmonoxy- and cyano complexes, are given in fig.3. The similarity of the latter two spectra shows that both the hemes are low spin in the carbonmonoxy form.

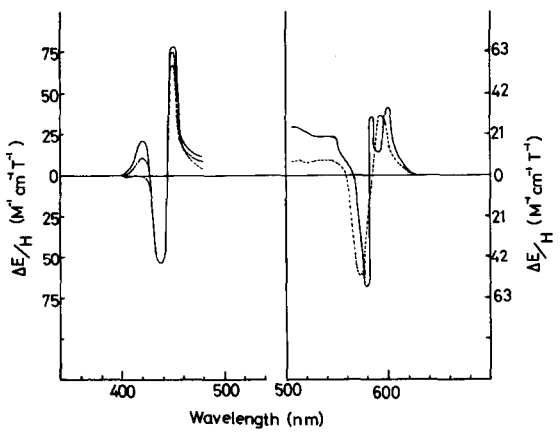

Fig.2. The effect of temperature on the m.c.d. spectrum of the cyanide derivative of reduced cytochrome oxidase. The protein was 130 μM in the short wavelength region and 400 μM in the longer wavelength region. The temperatures shown are (————) 40°K; (–·–·–) 100°K and (- - -) 293°K. Cyanide was present at a concentration of 50 mM.

The question arises as to whether one or both hemes are high spin in the reduced form of the enzyme. There is remarkably good agreement between the ΔE values, expressed per mole of heme *a*, in the Soret region of the reduced oxidase m.c.d. spectrum and the deoxymyoglobin m.c.d. spectrum [5]. Since the ΔE values of the low spin oxidase are low compared with the high spin form, a mixture of one high and one low spin heme is expected to produce a value of ΔE, expressed per heme, of approximately half this value. This seems to suggest that both the hemes are high spin in the reduced oxidase. The intensity of the Soret band of the reduced oxidase, seen in m.c.d. experiments, is found to be very temperature dependent, whereas that of deoxymyoglobin is found to be much less so. Also the fact that the deoxymyoglobin temperature dependence is non-linear [5], makes its use as a high spin model for the

oxidase system insecure. It is thus not possible to decide conclusively on the proposed fully high spin character of the reduced oxidase. Babcock et al. [6] have reported a shoulder at 452 nm in the m.c.d. spectrum of the reduced oxidase which they ascribe to the presence of one low spin ferrous heme. We have searched for this feature in the room temperature spectrum using a spectral band pass of 0.375 nm resolution but are unable to detect it unambiguously. At present we are therefore unable to reach a conclusion about the number of high spin ferrous hemes in the reduced oxidase.

3.2. *Fully oxidised oxidase*

The m.c.d. spectra, at room temperature, of three derivatives of the fully oxidised form of the enzyme are given in fig.4. We confine our attention at this point to the Soret region of the spectrum since the visible region is not typical of that seen in other hemoproteins. M.c.d. studies of met-myoglobin derivatives show that the intensity of the high spin signal is almost negligible compared with that of the signal due to the low spin species. A correlation has been drawn between the percentage low spin character and the intensity of the m.c.d. signal [4,5]. In the oxidase spectrum fig.4, the intensities of the major peaks in the Soret region fall in the series $CN^- >$ oxidised > azide ~ fluoro. This strongly suggests that the spin state of the heme which binds the ligand is moving towards the high spin form as the ligand is changed in this series.

In order to estimate the contribution of each heme to the m.c.d. spectrum in the oxidised form it is, strictly, necessary to know the magnitudes of the m.c.d. signals of 100% low spin heme *a* and a_3 at a given temperature. Since the nature of the axial ligands in the protein is unknown it is problematical to select suitable model compounds. However, there is an alternative approach to this problem.

The low temperature e.p.r. spectra show that the proportion of low spin heme detectable remains constant in all the derivatives, oxidised, fluoro- and cyano- [13]. Since we observe in the m.c.d. spectra of the Soret region at least one heme changing its spin state on changing the axial ligand from cyanide to fluoride we conclude that this variation is solely due to the change in the spin state of the e.p.r. undetectable heme. By implication this is a_3, the ligand binding site. If it is supposed that the cyano derivative contains a_3 as the pure low-spin derivative, and similarly if it is assumed that the fluoro-derivative contains a_3 as the pure high spin and *a* totally low spin, the intensity change in the negative peak at 434 nm on changing from cyanide to fluoride indicates the spin state change of a_3. The spectra then show that the oxidised form contains at least 75% of a_3 in the high spin form.

The negative peak at 377 nm in the low temperature m.c.d. spectra of oxidised cytochrome *c* oxidase, fig.5, has a temperature dependence similar to that

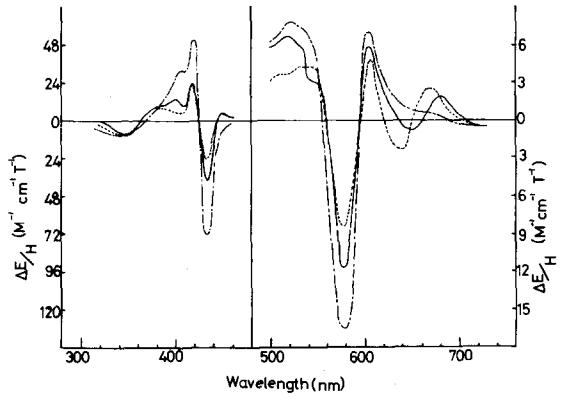

Fig.4. M.c.d. spectra of oxidised oxidase (——) and its cyanide (–·–) and fluoride (- - - -) derivatives are shown. Cyanide was used at 20 mM and fluoride at 100 mM at a temperature of 20°C.

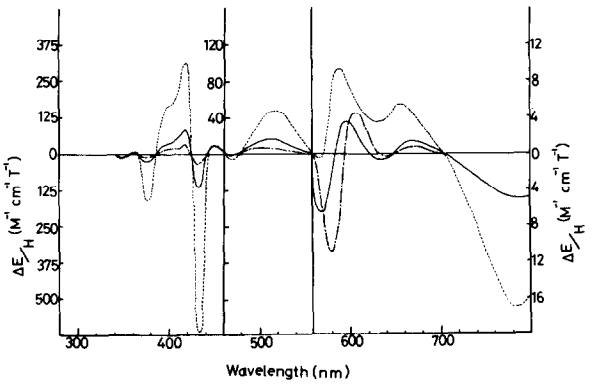

Fig.5. The effect of temperature on the m.c.d. spectrum of oxidised oxidase is shown. The temperatures are (- - - -) 15°K; (———) 80°K and (–·–·–) 293°K.

found in the m.c.d. spectrum of metmyoglobin fluoride at the same wavelength. We are uncertain of the origin of this peak but have argued [5] that it is due to adventitious ferric ion. Its intensity remains unchanged by the addition of cyanide ion to the oxidised oxidase although the intensities of the main peaks at 420 and 454 nm increases by a factor of at least two.

4. Discussion

The m.c.d. spectra of the reduced form of cytochrome *c* oxidase show unambiguously that the cyanide and carbonmonoxy derivatives are completely diamagnetic, containing two low spin heme *a* units. This result bears out the magnetic susceptibility study carried out many years ago [10]. In the reduced form at least one heme becomes paramagnetic. At this stage we are unable to demonstrate conclusively whether one or two hemes are high spin. The magnetic susceptibility measurements indicated that only one heme is high spin in the reduced form [10].

In the fully oxidised form of the protein heme a_3 appears to be in equilibrium between its high and low spin forms and it is possible to estimate a ratio of high to low spin a_3 of at least 3:1. On addition of cyanide the proportion of the low spin form increases and the m.c.d. intensity at room temperature changes by a factor of two, but a study of the temperature dependencies of the two derivatives shows an increase in slope of about three times. Thus caution should be exercised in attempting to estimate the relative proportions of heme spin states by making comparisons of the intensities of m.c.d. spectra at a fixed temperature. Taken in conjunction with e.p.r. estimates of heme *a* being low spin our results suggest, for the oxidised form of oxidase, one low spin heme *a* and the ligand binding heme, a_3, in the spin equilibrium. It seems clear that in the cyano derivative both heme *a* and a_3 are contributing to the temperature dependence. This implies that both are paramagnetic although only one can apparently be detected in e.p.r. experiments [12]. It has been supposed that the signal is undetectable because of an anti-ferromagnetic interaction between the cupric ion and the heme [13]. The m.c.d. shows that this coupling if present, is not strong enough to remove the paramagnetism of the heme. Any interaction of this type can do no more than alter the relaxation rates in order to make the heme e.p.r. undetectable.

There is another aspect of these spectra which merits comment at this stage. The spectrum of the cyano derivative of the reduced enzyme, fig.2, is a most unusual hemoprotein spectrum. All diamagnetic hemes show A terms under the Soret and visible absorption bands which have positive sign (negative ΔE at lower energy). By contrast the oxidase heme bands at 422 nm and 580 nm give negative A terms. Furthermore the 605 nm peak, always taken to be a heme band apparently has no A term under it, only a B term.

What are the ways in which the sign of the A terms under the heme absorption bands might be changed? We offer one possibility. Charge transfer transitions into the lowest empty porphyrin orbital of e_g symmetry under D_{4h}, may give rise either to positive or negative A terms depending upon the nature of the orbital from which the electron is taken. Briefly this orbital must transform as a_1, a_2, b_1, or b_2 in order to give an excited state, E_u, with configurations such as $(a_1^1\, e_g^1)$ or $(b_1^1\, e_g^1)$. If the hole is left in an a_1 or an a_2 orbital a positive A term will result and if the hole resides in b_1 or b_2 then a negative A term can be obtained. Since the heme excited states are also E_u they can be mixed by interelectronic repulsion and in this way acquire A terms of the same sign as the charge transfer state. It is of interest to note that the cuprous ion could provide a donor electron occupying a b_2 orbital.

The presence of a B term under the 605 nm band is intriguing. There are two obvious ways in which this could arise. First, a charge transfer transition from an e donor orbital into the empty e_g porphyrin orbital will be Z polarised and give rise to a B term. We merely note that the highest occupied d-orbitals of the cuprous ion in a distorted tetrahedral site would transform as e and b_2. If we associate the negative A term at ~ 580 nm with the transition $Cu^I\,(b_2) \rightarrow$ porphyrin (e) and the B term at ~ 600 nm with the transition $Cu^I\,(e) \rightarrow$ porphyrin (e), we obtain a consistent interpretation of the spectrum. The second possibility is that the four-fold degeneracy of the heme responsible for the 605 nm band has been lifted so that the E state is split, resulting in oppositely signed B terms under the split components as is seen

in the metal free porphyrin m.c.d. spectrum [11].

The Soret band may also show a splitting in this case and, indeed, at low temperature the m.c.d. of the Soret region resolves three bands. We refrain at this stage from attempting an assignment of the visible m.c.d. spectrum for the derivatives since they all contain C terms. It is of interest to compare the m.c.d. spectrum of the reduced cytochrome *c* oxidase and deoxymyoglobin in the visible region. The spectra are remarkably similar except for the addition in the oxidase spectrum of two C-terms of opposite sign at ~ 593 nm and 610 nm, possibly belonging to the 605 nm band.

Acknowledgements

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