HEME SPIN-STATES OF CYTOCHROME c OXIDASE DERIVED FROM ROOM TEMPERATURE MAGNETIC SUSCEPTIBILITY MEASUREMENTS

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1. Introduction

Cytochrome c oxidase (cytochrome c: O₂ oxidoreductase EC 1.9.3.1) the terminal enzyme of the respiratory chain, contains two hemes and two copper ions per functional unit. Their role has been investigated in a number of ways, mainly by different kinds of spectroscopy, but there still remain ambiguities concerning their magnetic properties. Knowledge of the spin-states of the four electron-acceptors in the oxidized and reduced protein and also in the partially reduced enzyme is vital for an understanding of the catalytic mechanism.

The EPR signal of the resting enzyme [1,2] shows one low-spin ferric heme and a signal around g 2, usually associated with one of the copper ions. It has been suggested [3] that a coupling between the other heme and Cu make them EPR-undetectable. Furthermore, during reductive titrations several different high- and low-spin signals appear with a total maximum intensity of 0.25–0.5 heme/functional unit.

Both Mössbauer [4] and MCD [5–7] spectroscopy yield information on the heme spin-states. The former technique suffers from low sensitivity in absence of ⁵⁷Fe-enriched samples and the latter is difficult to make quantitative and relies on a comparison with models. Magnetic susceptibility measurements give a more direct measure of the spin-states. However, the published data are incomplete in that they deal with not fully characterized preparations [8,9] or with low-temperature only [10].

In the present work the NMR technique for measuring magnetic susceptibility has been applied. The availability of super-conducting magnets has increased the sensitivity of this method so that weakly paramagnetic protein solutions can be used. Also, the method requires very little material and is fast compared to other techniques.

The results suggest that both the oxidized and reduced enzyme contain one high-spin heme and that this heme is antiferromagnetically coupled to a copper ion in the oxidized state. There is no substantial increase in susceptibility at intermediate levels of reduction.

2. Materials and methods

Cytochrome oxidase was prepared from beef heart mitochondria [11]. The ratio between the absorbances was less than 2.9 for the oxidized enzyme at 280 nm and 420 nm and was larger than 2.3 nm at 444 nm and 420 nm for the reduced enzyme, which indicates that the preparations used contained very little non-reducible heme a and were without contaminating cytochromes. Extraneous copper was estimated from EPR to be less than 10% of the total amount of copper. Cytochrome c (Sigma Type VI) was further purified by ion-exchange chromatography [12]. The concentrations of cytochrome oxidase and cytochrome c were determined from the difference in absorbance between the reduced and oxidized forms using extinction coefficients of 24 mM⁻¹ cm⁻¹ at 605 nm and 21 mM⁻¹ cm⁻¹ at 550 nm, respectively. Other chemicals used were of analytical grade.

Magnetic susceptibility measurements were made with a Bruker 270 MHz NMR spectrometer with a super-conducting magnet giving a field of 6.3 T.
parallel to the sample tube. The theoretical treatment has been given by Dickinson [13]. The 'internal/external reference method' with two coaxial tubes was employed [14]. The shift-difference for an inert reference substance due to different bulk susceptibilities in the inner and outer tube is measured. The shift $\Delta \nu$ is given by

$$\frac{\Delta \nu}{\nu_0} = \Delta \chi (\frac{1}{3} - q)$$

(1)

where $\nu_0$ is the spectrometer frequency and $\Delta \chi$ the difference in volume susceptibility (here and in the following SI units are used). The term $q$ was included by Dickinson to account for effects of contact and/or pseudo-contact shifts. As discussed below (see Discussion) $q$ is negligible in our experiments and is not included in the following analysis. The molar susceptibility $\chi_{\text{mol}}$ is obtainable from

$$\Delta \chi_{\text{mol}} = \frac{3 \Delta \nu}{c \nu_0}$$

(2)

where $c$ is the concentration. Theoretical values are given by

$$\chi_{\text{mol}} = \frac{N_A \mu_0 \mu_B^2 S(S+1)}{3kT} = 5.363 \times 10^{-6} g^2 S(S+1)$$

(3)

at 293 K, where $g$ is the root-mean-square value, $\mu_B$ is the Bohr magneton and $\mu_0$ is the permeability in vacuo ($4 \pi \times 10^{-7}$ N A$^{-1}$).

The sample and the reference were kept in the inner tube with an inner diameter of 1 mm. The outer tube contained D$_2$O, a small amount of H$_2$O and the reference at a concentration of approximately one-tenth of the concentration in the inner tube. We found it useful to separate the reference peaks from the inner and outer tubes by addition of a salt to the outer tube. Thus, 0.5 M K$_2$HPO$_4$ gives a separation of approx. 10 Hz for the water peaks. This procedure also changes the temperature coefficient for the water peak so that a temperature change of about 0.2°K alters the shift by 0.1 Hz.

Several references were tried for cytochrome oxidase, e.g., ethylene glycol, tetramethylammonium ion, dioxane, trimethylsilylpropionate. However, acetate was found to be the most inert one. In the presence of 0.25 mM enzyme the line-width at half-height was 0.8 Hz for acetate and 3 Hz for water. The increase in line-width for acetate on addition of the enzyme was less than 0.5 Hz. The changes in the paramagnetic susceptibility were followed with time, the starting point being cytochrome oxidase, oxygen and a catalytic amount of cytochrome c. Reduction was initiated by addition of ascorbate. For the preparation of the cyano-derivative of the fully reduced enzyme a solution of cytochrome oxidase and cytochrome c was made anaerobic, ascorbate was added and the solution was kept for 1 h at 273°K. A sample was then transferred to the NMR capillary. No shifts could be detected during 1 h, indicating that the enzyme was fully reduced. Cyanide was added to the anaerobic vessel containing the reduced enzyme and a new sample was transferred to the NMR tube.

Absorbance changes were followed at 605 nm using a dual-wavelength spectrophotometer, type DBS-I, Johnson Research Foundation. The samples were identical to those described above and were contained in the same tubes. The absorbance changes are mainly used to indicate the exhaustion of oxygen and cannot be used quantitatively. All experiments were carried out at 293°K.

3. Results

Figure 1A shows the time-course for the chemical-shift changes during reduction of cytochrome oxidase (for experimental conditions see legend of fig. 1A). As is evident from fig. 1B, where the absorbance changes at 605 nm were followed, all oxygen was exhausted within approximately 12 min. The average of the corresponding chemical-shift for acetate and water in two experiments was $-1.2$ Hz. This value fits well with the calculated shift for the disappearance of the paramagnetic oxygen ($-0.95$ Hz) plus a shift due to the addition of ascorbate, which in a separate experiment was found to be $-0.3$ Hz. The subsequent changes, $-0.8$ and $-0.9$ Hz for acetate and water, respectively, are due to the oxidase only. The shoulder occurring after exhaustion of oxygen is repro-
Fig. 1. Changes in chemical-shift (A) and absorbance at 605 nm (B) as a function of time during reduction of cytochrome oxidase. The final concentrations in the inner tube were 222 μM cytochrome oxidase, 2.0 μM cytochrome c, 0.5 M acetate and 0.5% Tween-80 in 0.1 M phosphate buffer, pH 7.0. The reduction was initiated by adding ascorbate to a concentration after mixing of 6.8 mM. Shifts for acetate (● - ●) or H₂O ( ○ - ○) are shown. The outer tube contained 0.1 M acetate and 0.5 M phosphate in H₂O : D₂O 2:100.

Table 1

<table>
<thead>
<tr>
<th>Spin-state</th>
<th>Cu</th>
<th>Fe</th>
<th>Cu</th>
<th>Fe</th>
<th>ΔXₘ₀l × 10⁶</th>
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<tbody>
<tr>
<td>Reduced + CN⁻</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Reduced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>110</td>
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<tr>
<td>Oxidized</td>
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<td></td>
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<td></td>
<td>160</td>
</tr>
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<td></td>
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<td>1/2</td>
<td>3</td>
<td></td>
<td>290</td>
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</tbody>
</table>

Observed values are given relative to the reduced CN⁻ derivative, and theoretical spin-only values are calculated under various assumptions of spin-states. SI units are used (to convert from cgs to SI units multiply by 4π × 10⁻⁶).

4. Discussion

In the NMR method employed in this study the chemical-shift difference is taken as a measure of the bulk magnetic susceptibility. This is a valid procedure provided other contributions to the shift are small. The water and acetate peaks shift with the same amount and have a constant line-width during the course of the reduction of the oxidase. This indicates that there is no appreciable interaction with the paramagnetic centres, i.e., the value of q is most likely negligible. This is further supported by the fact that the water-shift difference is independent of the presence of acetate.

Table 1 gives observed and calculated molar susceptibilities relative to the reduced CN⁻ derivative, which is considered completely diamagnetic [5,7,8]. Fe is taken to be pure high- or low-spin with no thermal mixture. This assumption is supported by the finding seen. Based on the oxidase concentration the obtained shift was −10 Hz mM⁻¹. The shift-values relative to the CN⁻ derivative are converted to molar susceptibilities according to eq. 2 and given in table 1.
that EPR signals have g-values that are perfectly well explainable with \( S = \frac{1}{2} \) or \( \frac{5}{2} \) states. For the theoretical values spin-only behaviour is assumed. Low-temperature EPR gives g-values for some of the species in table 1 but their use would introduce minor changes only. The same is true if experimental rather than spin-only values for \( S = \frac{2}{2} \) ferrohemo-proteins are used. The oxidized protein is considered to contain one isolated low-spin heme in agreement with EPR data [1,2]. The other heme is high- or low-spin either magnetically isolated or with strong \((\gg kT)\) ferro- or antiferromagnetic coupling to a \( S = \frac{1}{2} \) Cu.

Our data clearly show that the reduced oxidase is paramagnetic. The measured susceptibility allows for the presence of one and only one high-spin heme. This is in agreement with the data published by Ehrenberg and Yonetani [8] and reports from MCD measurements [5,7] that the reduced form is paramagnetic.

For the oxidized protein, where our values are somewhat lower than those reported [8,10], the only possible model includes one high-spin heme antiferromagnetically coupled to a \( S = \frac{1}{2} \) Cu in addition to magnetically isolated Cu and low-spin heme. Thus, the presence of a high-spin heme in the oxidized form has now been established by Mössbauer, MCD and magnetic susceptibility techniques and it seems highly probable that the two hemes are non-identical entities. Furthermore, our interpretation does not require an equilibrium between high- and low-spin forms as suggested earlier [10,7].

The results obtained in this study also contain information on the magnetic susceptibility of the partially reduced oxidase. Our data do not show the clear increase in the susceptibility during the reduction as suggested by Palmer [15], but the shoulder in fig.1 could indicate the presence of the same amount of uncoupled high-spin ferric heme at an intermediate reduction state as found in EPR titrations with cytochrome c [1,2]. However, the amount of intermediate high- and low-spin forms are known to depend on pH and reductant [1] and more studies are required before the redox-potential relations in the coupled unit are understood.

The findings reported in this paper give strong support to the picture of cytochrome oxidase where one heme is in the high-spin state and antiferromagnetically coupled to a copper and where one low-spin heme and one copper are magnetically isolated. The strong-coupling found requires a close proximity with electronic-overlap between two metal ions. This would facilitate a two-electron transfer to dioxygen overcoming the energetically unfavourable superoxide intermediate. In this respect cytochrome oxidase would closely resemble laccase [16], which presumably has two strongly-coupled Cu\(^{2+}\)-ions. In equilibrium titrations they behave as a two-electron acceptor, whereas such an acceptor has not yet been observed in cytochrome oxidase. It cannot be excluded, however, that in turnover conditions a pair of electrons is transferred to dioxygen also in the cytochrome oxidase case.

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References
