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A ^{1}H NMR study of bovine cytochrome oxidase

Paramagnetically shifted resonances of haem *a*

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Dimeric and monomeric forms of mitochondrial cytochrome oxidase (EC 1.9.3.1) have been examined using ^{1}H NMR spectroscopy. Paramagnetically shifted resonances were detected in spectra of the monomeric protein. Studies of this protein in a number of oxidation and ligation states have assigned these resonances to ferrihaem *a*. The temperature and pH dependence of this new probe of haem *a* environment is reported.

NMR; Cytochrome oxidase; Monoamine, subunit depleted-

1. INTRODUCTION

Bovine mitochondrial cytochrome oxidase (ferrocytochrome *c*:dioxygen oxidoreductase, EC 1.9.3.1) is a dimeric (2 × 200 kDa, on the basis of amino acid sequence) metalloprotein of the mitochondrial inner membrane [1]. It catalyses the transfer of electrons from cytochrome *c* to dioxygen to form water, and links this process to energy conserving proton translocation [2]. The metal centres comprise two haem A metalloporphyrins and two, possibly three [3], copper ions per monomer. One haem group, haem *a*, and one copper ion, copper A (Cu_A), form magnetically isolated mononuclear electron transfer sites [4]. The remaining haem group, haem a_3, and another copper ion, copper B (Cu_B), form a magnetically coupled binuclear site in which the two metal ions are within 6 Å of each other [4,5]. Haem *a* and Cu_A pass electrons from cytochrome *c* to the binuclear site where oxygen is reduced and inhibitors bind. These properties form the basis of the classical distinction between the two haem groups of cytochrome oxidase, as reflected in their electronic spectra and first noted by Keilin [6].

The detergent solubilized bovine heart oxidase consists of a dimer [7], wherein each monomer comprises thirteen nonidentical subunits [8]. The cytochrome oxidase from shark (*Sphyrna lewini*) is isolated as a fully functional monomer [9]. Only two of the subunits, I and II in the nomenclature of Kadenbach, are thought to contain the redox active metal centres [10], the functions of the other subunits being at present less well defined. Several methods have been devised which produce a stable, monomeric bovine oxidase in which several subunits have been removed [11–13]. One such method employs chymotrypsin and the detergent Triton X-100 at pH 8.5 [11] to produce a monomer deficient in subunits III, VIa and VIb (nomenclature of Kadenbach). This species is fully redox competent and has a molecular mass of ~160 kDa [11].

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has been successfully employed in many studies of a variety of haem proteins [14], but only one such study of cytochrome oxidase has been reported [15]. This paper describes preliminary ^{1}H NMR studies of dimeric and monomeric bovine heart cytochrome oxidase, and shows the detection of paramagnetically shifted resonances from haem *a*.

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2. MATERIALS AND METHODS

Cytochrome oxidase was prepared from bovine hearts by the method of Yonetani [16] with minor modifications. For NMR spectroscopy the dimeric oxidase was dissolved directly into 20 mM phosphate buffer, pH 7.4, containing 0.3 M NaCl and 0.1% Triton X-100. This buffer was prepared using 99.8% D_2O.

Monomeric (subunit-III-depleted) bovine cytochrome oxidase was prepared by the method of Puettner et al. [11], except that following application to the DEAE-cellulose column, the enzyme was washed and finally eluted with the D_2O buffer described; for washing the 0.3 M NaCl was omitted.

The monomeric enzyme, in the reduced state, displayed an optical spectrum completely free of contributions from cytochromes *b* and c_1, and with an absorbance ratio of $A_{443}/A_{420} = 2.5$, which also indicates high purity. The λ_{max} of the α and γ bands of the reduced protein were 603 nm and 443 nm, respectively.

The cyanide derivative was prepared by incubating the oxidised enzyme with 1–2 mM cyanide for 18 h at 4°C. Reduced enzyme was produced by the addition of a 2–3-fold molar excess of a solution of

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sodium dithionite in D_2O buffer to the oxidised enzyme under nitrogen.

The oxidation and ligation states of the various oxidase derivatives were confirmed by optical spectroscopy using a Hitachi model 557 spectrophotometer. Enzyme was dispensed from NMR tubes into 1 mm pathlength cuvettes and optical spectra were thereby obtained with minimal dilution of NMR samples.

^{1}H NMR spectra were obtained at 399.65 MHz using a Jeol GSX 400 FT NMR spectrometer. A spectral width of 80654 Hz was acquired using 32K data points. Temperature control was achieved using liquid nitrogen boil-off gas regulated by a Jeol variable temperature unit and heater. Solvent suppression was effected using the SUPER WEFT pulse sequence [17,18], 180°-τ-90°-Acquire-D where the two time periods, τ and Acquire + D, were set at 90 ms and 213 ms, respectively, at 6°C. The pulse sequence was optimised at each temperature in variable temperature experiments by variation of τ. Baseline flatness was improved by the use of a four pole Bessel filter. Sample volumes were 2.5–3 ml in 10 mm o.d. Wilmad NMR tubes. Chemical shifts are referenced relative to external 2,2-dimethyl 2-silapentane 5-sulphonate (DSS). Measurement of pH within the NMR tube was achieved using a radiometer PHM 82 pH meter connected to a Russel CMAWL combination electrode; NaOD and DCL were employed to adjust the pH.

3. RESULTS AND DISCUSSION

The two haem groups of cytochrome oxidase may be distinguished by their chemical and magnetic properties [4,6]. Haem *a* is low spin in the oxidised (ferric, $S = 1/2$) state and the reduced (ferrous, $S = 0$) state [3]. It does not bind exogenous ligands. Haem a_3 is high spin in the ferric ($S = 5/2$) and ferrous ($S = 2$) states, but some inhibitors of oxidase activity, such as cyanide or carbon monoxide, bind to this haem making it low spin [5,6]. Many proteins which contain haem groups in paramagnetic ($S \neq 0$) states give rise to paramagnetically shifted resonances in ^{1}H NMR spectra [14]. The spectra of proteins containing isolated copper ions do not show such features; usually resonances of nuclei close to cupric centres are considerably broadened due to the magnetic properties of the metal ion [19].

Fig.1 illustrates the ^{1}H NMR spectra of dimeric (a) and monomeric (b–e) cytochrome oxidase over the chemical shift range 22 ppm to 50 ppm. Only resonances which experience a hyperfine shift from a paramagnetic species are to be found in this region of the spectrum [19,20]. The spectrum of the oxidised (resting) dimeric protein is devoid of resonances in this region (fig.1a), but the oxidised (resting) monomeric protein shows two broad resonances centred at 30.5 and 24.8 ppm (fig.1b). The 'pulsed' (i.e. reduced then reoxidised with oxygen) monomeric enzyme also gives rise to such resonances (fig.1c), but the fully reduced monomeric enzyme (fig.1d) does not. Following the argument above, these resonances must arise from a redox active centre in the protein other than Cu_A. The formation of the oxidase-cyanide complex does not perturb these resonances (fig.1e). Cyanide binds at the binuclear site and while this action does not remove the antiferromagnetic coupling between haem a_3 and Cu_B [5], it does perturb the spin state of haem a_3 [5].

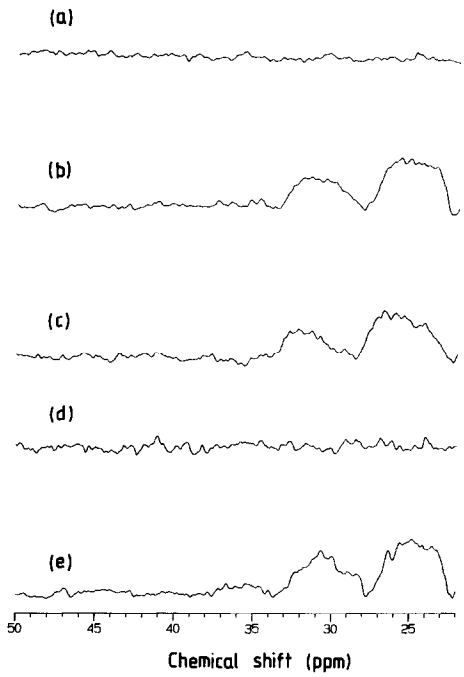

Fig.1. ^{1}H NMR spectra of cytochrome oxidase at 220 μM in 20 mM phosphate, pH 7.4, containing 0.1% Triton X-100 and 0.3 M NaCl. (a) Dimer; (b) oxidised (resting) monomer; (c) pulsed monomer; (d) dithionite (1.5 mM) reduced monomer; (e) cyanide bound oxidised monomer. Each spectrum is the Fourier transform of 30000 co-added transients, premultiplied by an exponential function equivalent to 65 Hz linebroadening. Temperature was 6°C for all spectra.

Therefore the observed resonances do not arise from haem a_3 and thus we associate these resonances with ferrihaem *a*. Since the monomeric oxidase has the same redox properties and metal centre structures as the dimer [11], we attribute the lack of resonances in the dimer spectra to the expected increase in the rotational correlation time of this protein relative to that of the monomer [21].

Comparison of the spectra of the three ferrihaem *a* containing species (fig.1b,c and e) shows that in all these species the environment of haem *a* is the same within the limits of the technique. This suggests, firstly that the differences in optical and kinetic properties between the pulsed and resting oxidase [11,22] do not arise from differences in the environment of haem *a*; and secondly that binding of a ligand to the binuclear site does not perturb the environment of haem *a* in the fully oxidised protein.

The chemical shifts and linewidths of the haem *a* resonances are pH independent over the range pH 6.6 to 8.6 (data not shown). pH dependence of these parameters for small (9–32 kDa) cytochromes *c* [23,24] and *Escherichia coli* cytochrome *b*-562 [25] has been observed when either the haem propionic acid substituents or the axial histidine ligand undergoes ionization. Therefore the data for cytochrome oxidase indicates that the haem *a* propionate groups and

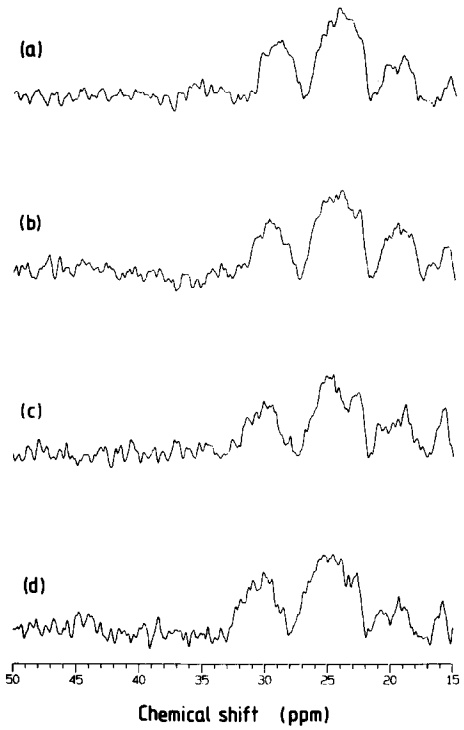

Fig.2. Temperature dependence of the ^{1}H NMR spectra of oxidised monomeric cytochrome oxidase at 220 μM in 20 mM phosphate, pH 7.4, containing 0.1% Triton X-100 and 0.3 M NaCl. (a) 20°C; (b) 15°C; (c) 10°C; (d) 5°C. Each spectrum is the Fourier transform of 15000 co-added transients, premultiplied by an exponential function equivalent to 80 Hz linebroadening.

histidine ligands do not ionize over the pH range 6.6–8.6. This lack of pH dependence argues against a direct role of these functionalities in energy conserving proton translocation.

Studies performed using the monomeric oxidase at several temperatures have revealed two more paramagnetically shifted resonances in the 15–22 ppm region of the ^{1}H NMR spectrum. Fig.2 shows the temperature dependence of the paramagnetically shifted resonances of the oxidised state over the range 6–20°C. The properties of these resonances are collected in table 1. The range of shifts displayed in these experiments is consistent with their assignment to a low spin ferric haem [14]. All the resonance shifts show a linear inverse dependence on temperature. This is typical of shifts arising through both contact and pseudocontact effects from an isolated paramagnet [26,27]. The features at 24.8 ppm and 19.7 ppm appear to show some heterogeneity in the variable temperature experiments, suggesting that they may be composed of more than one resonance. The pulsed form of the enzyme exhibits the same temperature dependence (data not shown). No further paramagnetically shifted resonances have been observed over the range −95 ppm to 105 ppm in any of the derivatives illustrated in fig.1 or in the partially reduced cyanide bound mixed valence enzyme (not shown). A previous ^{1}H NMR study of dimeric cytochrome oxidase has reported the observation of resonances arising from high spin ferrohaem a_3 and its histidine ligand. We have not observed resonances arising from high spin or low spin haem a_3 in either oxidation state, although it should be noted that under the conditions of our experiments (D_2O instead of H_2O) we do not expect to observe the ligand resonance seen by Inubushi and Yonetani [15]. The discrepancy between our observations and those of Inubushi and Yonetani [15], may arise due to Curie spin relaxation [28,29]. This effect increases with the square of the applied field and therefore it is more than twice as effective in our study relative to [15]. A variety of factors may be responsible for broadening the haem a_3 resonances. For example antiferromagnetic coupling between ferrihaem a_3 and cupric Cu_B could greatly increase the paramagnetic relaxation effects associated with this centre. Furthermore a dynamic event such as proton exchange on or close to haem a_3 could lead to exchange broadening of resonances.

Table 1

Properties of haem *a* resonances at 6°C

Chemical shift (ppm)	Linewidth (Hz)	Relative integral	Temp. dependence (Δppm/°C) × 10^{-2}	Comments
30.5	1500	3	8.6	
24.8	1900	6	2.0	heterogeneous
19.7	1100	3	2.0	heterogeneous
16.0	400	1	3.3	

We have reported here the first observation of paramagnetically shifted ^{1}H NMR resonances from haem *a* of beef heart cytochrome oxidase. This was facilitated by the use of a monomeric form of the oxidase. We intend to further these studies, using ^{1}H NMR spectroscopy to determine the effect on the haem *a* environment of a number of conditions and effectors.

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