Proton nuclear magnetic resonance study of bovine cytochrome oxidase

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1. INTRODUCTION

Bovine cytochrome oxidase is a transmembrane hydrophobic enzyme in the mitochondrial inner membrane and catalyzes the four-equivalent electron transfer between ferrocytochrome *c* and dioxygen as the terminal oxidative enzyme of the electron transfer chain. In situ this process is coupled to proton pumping for energy conservation. The concentration of this enzyme in situ is, however, relatively low and coexists with other respiratory chain components so that it is necessary to solubilize this enzyme from the mitochondrial membrane, to separate it from other respiratory chain components, and to concentrate it for biochemical and biophysical studies. On the basis of extensive investigations during the past two decades [1,2], it has been widely accepted that bovine cytochrome oxidase is a multi-subunit enzyme containing 4 different metal centers per minimal catalytic unit of about 160 kDa. These metal centers are heme *a* containing cytochromes *a* and a_3, and two copper centers designated Cu_A and Cu_B. Cytochrome *a* and Cu_A are functionally located in the electron-accepting side of the enzyme. Cytochrome *a* appears to have a relatively strong redox interaction with cytochrome a_3, which in turn seems to be magnetically coupled to Cu_B [3]. This magnetic coupling has thus far prevented unequivocal determination of the chemical and electronic nature of these two metal centers and the mode of their interaction. Bovine cytochrome oxidase has been examined with nearly every conceivable method of modern spectroscopy including magnetic susceptibility, circular dichroism, magnetic circular dichroism, linear dichroism, infra-red and electronic absorption, resonance Raman scattering, Mossbauer absorption, EPR, phosphorus NMR, ENDOR, and EXAFS. However, one cannot help noting the conspicuous absence in this extensive list of proton NMR, which has proven to be one of the most powerful probes of the structure, function, and dynamics of many other hemoproteins.

Hydrodynamic studies [2,4] have indicated that detergent-solubilized bovine cytochrome oxidase exists in solution primarily as dimers having an app. M_r of about 320000. Furthermore, the molecular size of the enzyme for each redox metal center (~160 kDa) is more than 10-times larger than those of smaller hemoproteins such as cytochrome *c*, myoglobin and hemoglobin. Therefore, it is obvious that its extremely large molecular size as well as its consequentially longer rotational correlation times make it formidable to quantitatively measure its proton NMR-spectra. This paper describes preliminary NMR studies of bovine cytochrome oxidase and shows for the first time hyperfine-shifted proton NMR-spectra of this enzyme.

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2. MATERIALS AND METHODS

Cytochrome oxidase was purified from bovine hearts according to a modification of [5]. The purified enzyme was dissolved in 0.5% potassium cholate–50 mM potassium phosphate buffer (pH 7.4) and concentrated to 2–3 mM (per heme a) by ultrafiltration through Amicon centrififlo membranes. The enzyme sample in D_2O was prepared by 3 to 4 times repeated dilution with D_2O followed by concentration under reduced pressure. The enzyme was reduced anaerobically by addition of slight excess sodium dithionite and transferred into a NMR sample tube with a 5-mm inner diameter with an air-tight seal to maintain the desired gas phase. The carbon monoxide derivative of the enzyme was prepared by flushing carbon monoxide gas over the dithionite reduced sample for 10 min at 4°C.

Proton NMR-spectra were recorded at 270 MHz using a NIH NMR spectrometer, which consists of a Bruker 6.34-T magnet and a Nicolet FT-NMR system with a NIC-1180 digital computer. Paramagnetically-shifted proton resonances were observed by the super-WEFT pulse sequence technique [6]; i.e., an extremely rapid repetition of water eliminating pulses (a pulse sequence of 180°–τ–90°-observation). Typically 11-μs (90°) and 22-μs (180°) pulses were applied with 100-ms repetition of the pulse train. Under these conditions, the solvent water proton signal was completely eliminated by setting the τ-value to 70–80 ms. About 300 k–500 k transients were accumulated to obtain reasonable signal-to-noise ratios. Chemical shifts were calculated from the residual H_2O resonance and converted to the corresponding values from the internal reference, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) by adding a conversion factor of 4.8 ppm. After NMR measurements, the samples were diluted to examine their integrity by activity assay and spectrophotometry.

3. RESULTS AND DISCUSSION

An earlier magnetic susceptibility study [7] indicated that the carbon monoxide-binding component of bovine cytochrome oxidase, i.e., reduced cytochrome a_3, is in a ferrous high-spin ($S = 2$) state. This suggestion was supported by subsequent magnetic circular dichroism studies [8,9]. The latter studies have further indicated that cytochrome a in the reduced cytochrome oxidase is in a diamagnetic low-spin ($S = 0$) state. Since both Cu_A and Cu_B centers in reduced cytochrome oxidase are in diamagnetic cuprous states, cytochrome a_3 becomes the only paramagnetic metal center in reduced cytochrome oxidase. Therefore, any paramagnetically-shifted proton resonances observed in reduced cytochrome oxidase can be assigned exclusively to cytochrome a_3. Fig.1a and

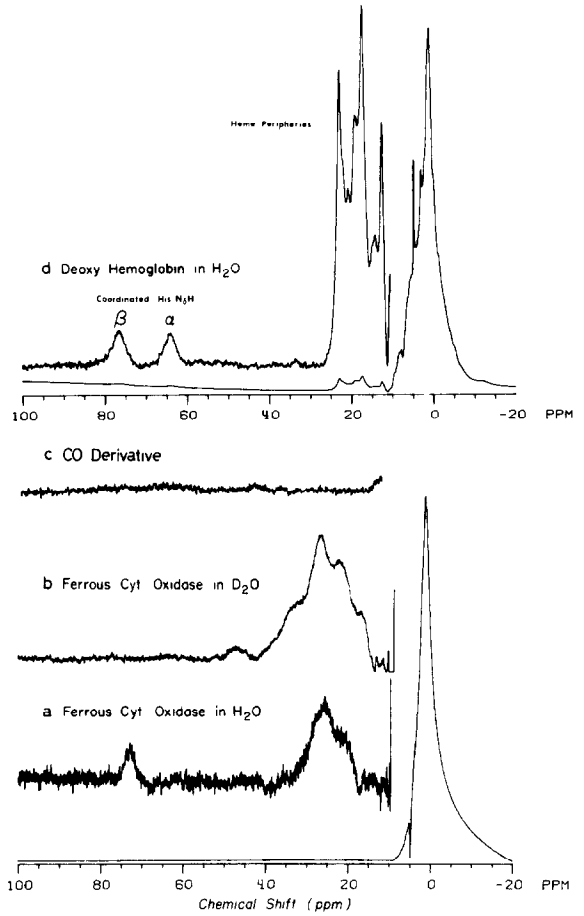

Fig.1. 270-MHz proton NMR spectra of reduced cytochrome oxidase and deoxy human hemoglobin: (a) Fully reduced cytochrome oxidase in H_2O; (b) fully reduced cytochrome oxidase in D_2O; (c) the carbon monoxide derivative of reduced cytochrome oxidase in H_2O; (d) deoxy human hemoglobin in H_2O. All the samples of cytochrome oxidase have been measured at 7°C, whereas deoxy hemoglobin has been measured at 17°C.

b illustrate proton-NMR spectra of the dithionite-reduced cytochrome oxidase in non-deuterated and deuterated buffers, respectively. Fig.1a and b include the hyperfine-shifted region (10–100 ppm) which have been vertically expanded about 100-times to obtain reasonable signal intensities. The isolated resonance at 73.5 ppm observed in the sample prepared in the non-deuterated buffer was found to be absent in the one prepared in the deuterated buffer, indicating that this resonance is H–D exchangeable. The signals between 10 and 40 ppm which remain observable in the deuterated buffer were better resolved in fig.1b, probably because a better dynamic range for the detector was achieved by better suppression of the water resonance signal. Upon addition of carbon monoxide to reduced cytochrome oxidase, these hyperfine-shifted proton resonances disappeared completely, as shown in fig.1c. Therefore, it is concluded that these resonances in the down-field region are of a paramagnetic origin and thus are derived exclusively from paramagnetic cytochrome a_3.

Fig.1d illustrates hyperfine-shifted proton NMR-spectra of deoxy human hemoglobin whose hemes are in ferrous high-spin ($S = 2$) states. The down-field spectra of deoxy hemoglobin (fig.1d) are remarkably similar to those of reduced cytochrome oxidase (fig.1a). The resonance positions of hyperfine-shifted proton signals of these compounds are compared with those of other ferrous high-spin hemoproteins (table 1). Reduced cyto-

Table 1

Hyperfine-shifted proton resonances of ferrous high-spin hemoproteins

Hemoprotein	5th Axial ligand	Chemical shift (ppm)	Assignment	Ref.
Deoxy-hemoglobin	His		Exchangeable $N_\delta H$ of proximal His	
		75.8	(β-subunits)	
		63.3	(α-subunits)	
			Unassigned	
		22.3	(β-subunits)	[10]
		17.0	(α-subunits)	
		12.8	(β-subunits)	
Reduced horseradish peroxidase	His	79.0	Exchangeable $N_\delta H$ of proximal His	
		26.5		
		22.3		
		21.5	Unassigned	[11]
		19.0		
		15.5		
		13.0		
Reduced cytochrome P-450	Cys	27.2		
		20.0	Unassigned	[13]
		17.4		
Reduced cytochrome oxidase	His	73.5	Exchangeable $N_\delta H$ of heme-bound histidine	
		34.0		
		27.0		[Here]
		22.0	Unassigned	
		16.5		

chrome oxidase exhibits two types of hyperfine-shifted proton resonances: the isolated H–D exchangeable resonance at 73.5 ppm and the heavily overlapped non-exchangeable resonances between 10 and 40 ppm. The former resonance can be readily assigned to the H–D exchangeable $N_\delta H$ proton of the proximal His by comparison with the corresponding resonances of deoxy hemoglobin [10] and reduced horseradish peroxidase [11] which contains His as the 5th axial ligand of the penta-coordinated ferrous heme group. No such resonance has been observed in reduced cytochrome P-450 containing Cys as the 5th axial ligand [12,13]. It has been demonstrated by EPR studies [13,14] that the hexa-coordinated heme group of reduced cytochrome a_3–nitric oxide contains His as the 5th axial ligand. It can now be further stated that reduced cytochrome a_3 is a penta-coordinated ferrous high-spin compound with His ligation. As shown in table 1, ferrous high-spin hemoproteins such as deoxy hemoglobin, reduced horseradish peroxidase, and reduced cytochrome P-450 exhibit non-exchangeable proton resonances between 10 and 40 ppm which are derived from porphyrin ring and peripheral protons. The presence of similar proton resonances in reduced cytochrome oxidase further supports our conclusion that reduced cytochrome a_3 is a ferrous high-spin compound. The nature of the Fe–N_ϵ(His)-bonding in ferrous cytochrome a_3 appears to be remarkably similar to those observed in hydrophilic ferrous high-spin hemoproteins such as deoxy hemoglobin and reduced horseradish peroxidase. Furthermore, porphyrin peripherals in reduced cytochrome a_3 are electronically in an environment similar to those of hydrophilic hemoproteins, despite significant structural and chemical differences in the apoprotein moiety, on the basis of the chemical shifts and the linewidths of the hyperfine-shifted proton resonances. The fact that the linewidth of these hyperfine-shifted resonances, particularly that of the His $N_\delta H$ resonance, is remarkably independent of the rotational correlation times, indicates that these hyperfine-shifted resonances are derived primarily from the contact interaction. The presence of the electron-withdrawing formyl side chain in heme *a* does not appear to significantly affect the nature of the Fe–N_ϵ(His)-bonding.

We have demonstrated for the first time that paramagnetically-shifted proton NMR signals can be observed in such a large macromolecule as bovine cytochrome oxidase under appropriate conditions. We plan to extend proton NMR measurements of bovine cytochrome oxidase to the redox states other than the fully reduced state in order to characterize the electronic states of different metal centers of this enzyme and to further elucidate the molecular mechanism of oxygen activation and electron transfer in this enzyme.

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