X-RAY ABSORPTION EDGE STUDIES ON CYANIDE-BOUND CYTOCHROME *c* OXIDASE

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1. Introduction

Cytochrome *c* oxidase (ferrocytochrome *c* : oxygen oxidoreductase, EC 1.9.3.1) is the terminal enzyme in the mitochondrial respiratory chain and, as such, reacts directly with molecular oxygen. The mechanism of this four-electron reduction is currently a topic of intense investigation and controversy [1,2]. Recently, there has been a great deal of interest in the chemical and physical properties of various inhibitor complexes of cytochrome *c* oxidase [3–7]. In particular, considerable effort has been spent on studying the cyanide- and carbon monoxide-bound protein with the hope of trapping one or more of the metal components in the oxidized or reduced form [8–11]. The rationale for such studies is the possibility that 'mixed valence' forms of the CN- or CO-inhibited protein might provide some clues about the mechanistic pathway of electron transfer to oxygen as well as the interaction of the four metal components in the functional protein. In fact, on the basis of spectroscopic and electrochemical properties of the CO and azide complexes of cytochrome *c* oxidase, Wilson et al. have proposed that there exists a direct heme–heme interaction in the protein [9]. On the other hand, Yong and King have postulated a heme–copper–heme interaction based on their studies of a 'half-reduced' and a fully-reduced CN complex of the oxidase [11]. To our knowledge, this discrepancy has not yet been resolved.

One of the major difficulties in the above studies has been the lack of knowledge of the redox states of all four metal atoms (2 heme Fe and 2 Cu), especially in the partially-reduced complexes of the protein. Recently, Anderson et al. reported that the CO complex of cytochrome oxidase is titratable with three electrons [3], a result which contradicts an earlier report that the formation of the CO complex involves a two-electron reduction [6]. Part of the confusion here may be attributed to the fact that at least one of the coppers ('invisible' Cu) is not directly detectable by EPR or spectrophotometric techniques. Furthermore, the contribution of the remaining three components to the optical spectra is still unsettled [1,2,12]. Similarly, the partially-reduced CN complex of cytochrome oxidase obtained in the presence of reducing agents has not been definitively characterized with respect to the oxidation states of the metal atoms. Yong and King have prepared a form of the CN complex which they called 'half-reduced' in which heme *a* and one of the two copper centers are reduced while heme a_3 remains oxidized [11]. However, there is really no direct evidence for the oxidation state of the second copper atom.

Recently, we have demonstrated that X-ray absorption spectroscopy can be of great value in defining the oxidation states of the metal atoms in metalloproteins. Its usefulness arises from the fact that the

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position of the absorption edge and the absorption-edge fine structure are sensitive to the charge density and point symmetry about the atom of interest. Our studies on the oxidized and reduced forms of cytochrome oxidase provide evidence which strongly suggests that one of the coppers in the oxidized protein exists in the reduced state [13]. Thus, in view of this result, it was of interest to apply this technique to the determination of the oxidation state of the second copper in the dithionite-reduced CN complex of oxidase as well as to investigate any changes which occurred in the X-ray absorption spectra of cytochrome oxidase upon complexation with cyanide.

Preliminary results on the oxidized and partially-reduced CN complexes of cytochrome oxidase indicate that:

(i) CN has no effect on the Cu edge spectrum of the oxidized protein.

(ii) The intensity of the 1s–3d transition in the Fe edge spectrum of the oxidized protein is slightly enhanced in the presence of cyanide.

(iii) Both Cu atoms of the CN complex are reduced (i.e., Cu^{+}) in the presence of excess dithionite, a result which in conjunction with EPR data [4,14] suggests that three out of the four electron acceptors in the complex are reduced.

2. Materials and methods

Purified cytochrome *c* oxidase was generously provided by Drs Tsoo E. King, Chang-An Yu and Linda Yu of Suny at Albany, NY. It was suspended in 0.5% sodium cholate/50 mM phosphate buffer, pH 7.4, at a concentration of 100 mg/ml. To form the oxidized CN complex, 17 μl 0.25 M potassium cyanide solution, pH 7.2, was added to 0.4 ml of the concentrated oxidase solution and the resultant solution was left standing under argon for 20–30 min. The so-called 'half-reduced' complex was prepared by adding the oxidized cyanide complex (0.4 ml) to an excess amount (10 mg) of solid dithionite under argon. The samples were kept on ice until the X-ray absorption measurements were made.

The Cu and Fe K-edge absorption spectra were obtained using synchrotron radiation from Spear at the Stanford Linear Accelerator Center. The broad-band radiation was passed through a channel-cut silicon crystal monochromator and the Cu or Fe fluorescence Kα radiation from the target was monitored using a nine-element array of NaI scintillation counters. Details of the experimental set-up and data analyses are described [13,15,16]. Since the samples were dilute, multiple scans were recorded and later summed.

3. Results and discussion

The Cu K-edge spectrum of the oxidized CN complex showed virtually no change from that of the fully-oxidized cytochrome *c* oxidase (fig.1). On the other hand, the Fe K-edge of the oxidized CN complex shows a slight increase in the intensity of the 1s–3d transition although the position of the edge as a whole remains unchanged (fig.2). These results may be taken to mean that while cyanide does not grossly affect the charge density about either the Cu or the Fe atoms in the protein, it does enhance the probability of the 1s–3d transition of the iron chromophore.

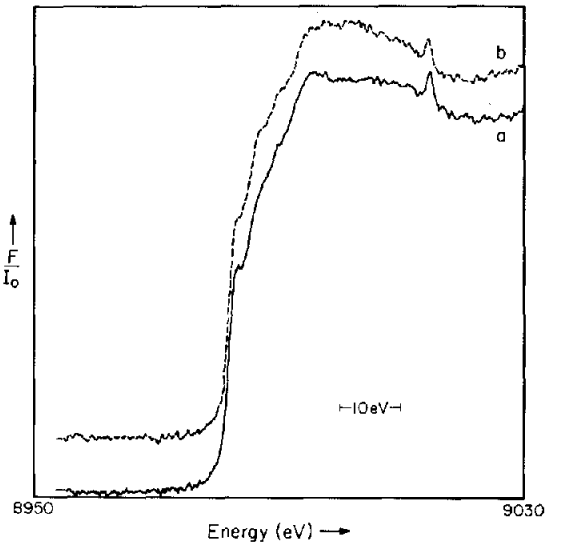

Fig.1. The copper K-edge spectra of (a) the oxidized CN complex of cytochrome *c* oxidase and (b) oxidized cytochrome *c* oxidase.

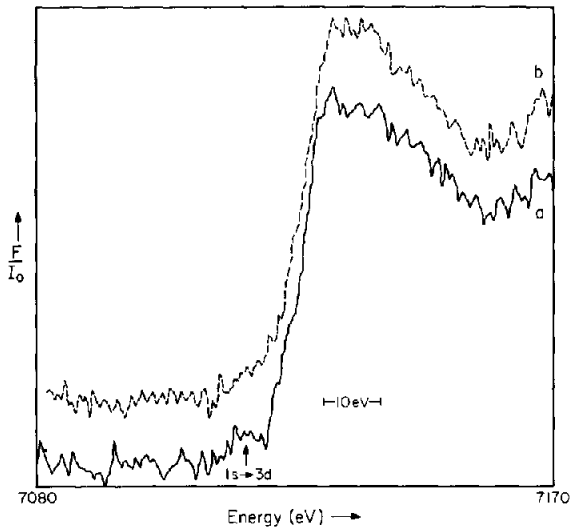

Fig.2. The iron K-edge spectra of (a) the oxidized CN complex of cytochrome *c* oxidase and (b) oxidized cytochrome *c* oxidase.

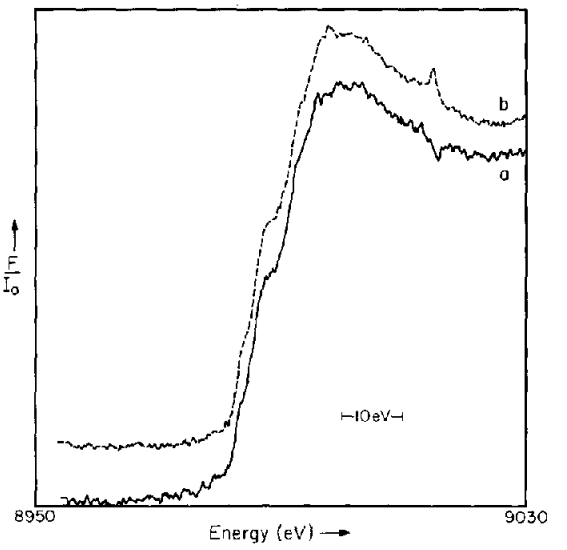

Fig.3. The copper K-edge spectra of (a) the dithionite-reduced CN complex of cytochrome *c* oxidase and (b) reduced cytochrome *c* oxidase.

This may be interpreted as evidence for binding of cyanide to iron (presumably heme a_3). The enhanced 1s–3d transition is to be expected since π-bonding of cyanide to iron introduces some p character to the iron d orbitals.

Reduction of the CN complex in the presence of excess dithionite results in the Cu K-edge absorption spectrum shown in fig.3. For comparison we also show the spectrum of the fully-reduced oxidase. There is no apparent spectral difference between the 'half-reduced' CN complex and the fully-reduced protein as far as the copper edge is concerned. This implies that both coppers are in the +1 oxidation state in the so-called 'half-reduced' complex described by Yong ad King [11]. This result is entirely consistent with the available EPR data [4,11,14]. The addition of excess dithionite to cyanide-treated cytochrome *c* oxidase results in the disappearance of the low-spin ferric heme signal at *g* 3.03, as well as a decrease in the very weak high-spin heme signal at *g* 5.86. Concomitantly, a new signal appears at *g* 3.58. The latter has been attributed to a low-spin ferric heme of the cyano complex of cytochrome a_3 [4]. In addition, Yong and King have shown that the *g* 2 signal disappears in the 'half-reduced' complex. We have confirmed this observation. Yong and King have attributed the disappearance of the *g* 2 signal to the reduction of one of the coppers [11]. However, they did not explicitly assign an oxidation state to the second copper, which is presumably EPR-silent.

Taken together, the EPR spectra of the dithionite-treated CN complex show the loss of two paramagnetic entities (centered at *g* 3.03 and *g* 2) and the gain of one new paramagnetic entity, heme a_3–CN. The latter is supposedly antiferromagnetically-coupled to the EPR-invisible Cu in the fully-oxidized protein [17] If the coupling between heme a_3 and invisible Cu is broken and heme a_3 becomes EPR-visible, the EPR-invisible Cu should also become detectable by EPR, if it were still oxidized. However, no Cu EPR signals are observed, implying that both coppers are reduced.

Recent X-ray absorption studies on oxidized and reduced cytochrome *c* oxidase provide evidence that one of the coppers in the oxidized protein is already in the +1 oxidation state [13]. This would imply that either the EPR signal at *g* 2 is due to the remaining copper (which would be in contradiction to other studies which suggest antiferromagnetic coupling between a heme and a copper in the oxidized protein) or that it is due to some other paragmagnetic species

that accepts electrons reversibly. Hu et al. have postulated that one of the four redox sites is a protein ligand perhaps coupled to Cu^+ in the oxidized protein [13]. A Cu(I)–disulfide system has been discussed as a possible electron acceptor in cytochrome *c* oxidase [18]. In addition, the possibility that the EPR signal *g* 2 is due to a sulfur radical in a particular environment has been raised [19]. However, regardless of the assignment of the *g* 2 EPR signal, the X-ray absorption and EPR studies together show that the CN complex of cytochrome *c* oxidase, in the presence of excess reducing agent, can accept three reducing equivalents.

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