STRUCTURAL FEATURES OF THE COPPER-DEPLETED CYTOCHROME OXIDASE FROM BEEF HEART: IRON EXAFS

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Received 13 January 1982

1. Introduction

The role of copper in cytochrome oxidase has been a controversial one when it was reported by Keilin and Hartree [1] and by Wharton et al. [2] and has remained so because of the difficulties in identifying the chemical structure of the EPR-detectable copper (designated Cu_a) and in obtaining any structural information at all from the EPR undetectable copper (designated Cu_{a_3}). Optical studies have been similarly difficult to interpret, the main absorption band in the near infrared region being attributed to Cu_a [3] which is closely linked to the $g = 2$ EPR signal [3,4]. The optical transition, attributable to EPR undetectable copper, has been regarded by some to be scarcely detectable [3] but has been observed by us under several experimental conditions [5,6]. The near infrared band can be divided into 2 portions as shown by intermediates in the low temperature reaction with O_2 and by studies of the compound formed in the presence of formate, termed mixed valence compound [7], which consists of oxidized Cu_a and reduced Cu_{a_3}. The short wave portion of the near infrared band can be attributed to the Cu_{a_3} and a longer wave portion of the Cu_a [5–8]. The 655 nm band, characteristic of the fully oxidized or resting oxidase is not present in the Cu-depleted oxidase measured under similar conditions [9].

In [5] X-ray synchrotron radiation was used to observe the Kα edges for copper, together with both iron and copper EXAFS studies [10] and the distance in the fully oxidized resting state separating iron and copper in the active site ($Fe^{+3}_{a_3}$–$Cu^{+2}_{a_3}$) of cytochrome oxidase was found to be 3.75 ± 0.05 Å, and furthermore, that the 2 atoms were bridged by a sulfur atom. This structural determination explained the slow reactivity with inhibitors [11]. The structure also affords the anti-ferromagnetic coupling in the active site that was postulated [12] on the basis of EPR signals.

Further support for the sulfur bridge structure of the resting enzyme [10] comes from techniques which remove the copper. Such approaches have been made by a variety of workers; in [13] dialysis against cyanide or treatment with batho-cuproin-sulfonate under acidic conditions, promoted a significant removal of the copper. In the copper-depleted preparation, both the absorbance at 830 nm and the EPR signal attributed to detectable copper are diminished, as indeed is the catalytic activity. Similar work was done in [14]. We present here EXAFS data on the copper-depleted form prepared as in [9]; this shows the loss of special structural features of the EXAFS data attributable to the Cu and to the briding S atom and the formation of the sulfhemoglobin type of compound at the cytochrome a_3 site.

2. Methods

2.1. *Preparation, characterization and monitoring*

The preparations used in these studies were those in which exhaustive depletion of the copper could be made by 2 h dialysis against 0.8 M KCN; this procedure removes ≥90% of the copper (see legend of fig.1 and tables 1 and 2 in [9]). The 830 nm band was absent, the heme bands were characteristically shifted

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Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

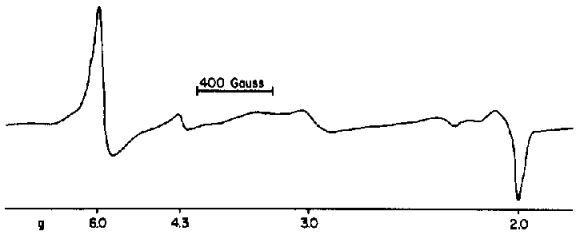

Fig.1. EPR spectrum of the oxidized resting copper-depleted cytochrome oxidase used for EXAFS studies: 10 mW power at 9 GHz; modulator amplitude 1 mT; 10 K. Copper was removed from purified beef heart cytochrome oxidase by dialysis for 2 h against 0.8 M KCN (see [9]).

to shorter wavelengths. The EPR signal at $g = 2$ showed <10% of the normal copper (fig.1). This spectrum may be compared with that of fig.12 in [5] where the $g = 6$ signal is a small fraction of the $g = 2$ signal. However, since EPR does not assay the EPR invisible Cu_{a_3}, we have used the amplitude of the EXAFS Cu-edge for Cu determination; this showed <5% Cu present compared to the native enzyme and this measurement refers to both species of copper. Samples were concentrated to ~1 mM by the techniques in [10] and stored in liquid nitrogen before and after measurements.

The properties of the cytochrome oxidase prepared in this way were monitored by continuous optical measurements and by intermittent EPR studies during the course of data collection. No deviation from the control exceeding 10% was observed.

2.2. *The acquisition of EXAFS data*

Measurements were carried out on beam line II-3 (4 X 11 mm focussed line) at the Stanford synchrotron radiation laboratory in which intensities of $\sim 10^{11}$ photons/s impinged upon the sample during dedicated beam operation (3.0 GeV, 40–80 mA). The mirror was raised for these studies to produce a resolution of ~3 eV.

Samples were maintained at −140°C or below in a cryostat that permitted X-ray fluorescence detection and simultaneous optical monitoring. This cryostat, optical spectrometer and fluorescence detection array with filter are described in [5,10,15].

Data were collected under identical conditions and analysis procedures and model compounds were those used in [10]. Fig.2 shows the k^3-multiplied background-subtracted EXAFS data for the copper-depleted form that have been normalized to 1 iron

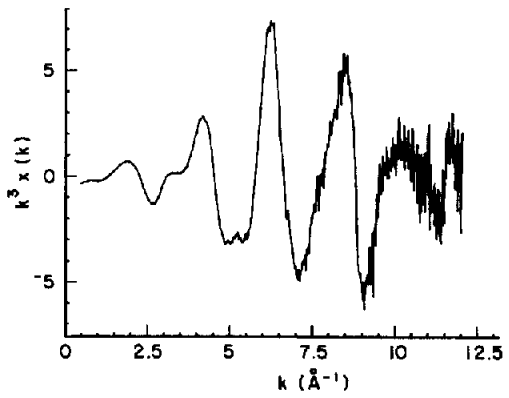

Fig.2. Cytochrome oxidase EXAFS data of oxidized resting copper-depleted form after k^3 multiplication, background subtraction, and normalization to one iron atom.

atom. The data are of similar quality to those reported for the oxidized resting state [10] and can be used for Fourier transformation without smoothing (fig.3).

3. Results

The iron EXAFS data of the copper-depleted form of cytochrome oxidase, like those of the purified enzyme [10], are complicated by the fact that they are a sum of contributions from both iron sites.

Three approaches to identifying these contributions to the first shell-filtered data were used:

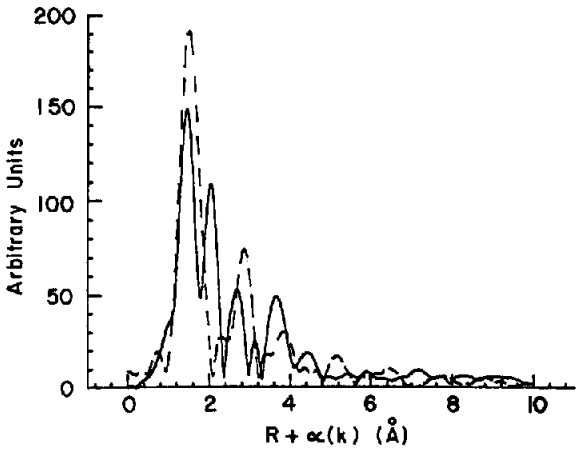

Fig.3. Fourier-transformed data for the oxidized resting native oxidase [10] (——) and the oxidized copper-depleted cytochrome oxidase (– – –); $\alpha(k)$ is the absorber–scatterer phase shift.

(i) To model each contribution with an appropriate compound of known structure: bis(imidazole)-$\alpha,\beta,\delta,\gamma$-tetraphenylporphinato-iron (III) chloride (designated Im_2FeTPP) for the *a* heme-type contribution and *c*-type cytochromes for the sulfur-ligated form.

(ii) To use a constrained fit for 2 atom types, nitrogen from Im_2FeTPP and from sulfur Holm model compounds $[Fe(S_2\text{-O-xyl})_2]^-$ or $Fe[CS_2\text{:N-}(CH_2)_4]_3$ [10,16].

(iii) To subtract the Fe_a contribution (Im_2FeTPP). This contribution of Fe_a is unlikely to be affected by copper depletion in accord with optical and EPR studies. The remainder, presumably the a_3 heme contribution, was then fitted in a constrained manner to nitrogen and sulfur atom types.

All three approaches gave the same results within their error: 25–40% of a 6 coordinate sulfur-ligated form having Fe–N = 1.99 Å* and Fe–S = 2.28 Å and similar to *c*-type cytochromes or to hemoglobin sulfide [17]. The remainder was a 6 coordinate form having Fe–N = 1.99 Å. These results are shown pictorially in fig.4. All higher shells can be attributed to nitrogen and carbon contributions from substituents of the porphyrin ring.

* Error for all measurements is ±0.03 Å (discussion in [10])

4. Discussion

It is useful to compare the results on the oxidized resting copper-depleted oxidase to those of the oxidized resting nature enzyme [10] (fig.3,4). The Fourier-transformed data clearly shows that the second of the split peaks seen in the oxidized resting state of the native enzyme (produced by the sulfur bridge) is absent in the copper-depleted preparation; while the whole first shell is similar but in both preparations it is shifted to a slightly longer distance and is more intense in the copper-depleted preparation. The optical and EPR studies [9] of the copper-depleted

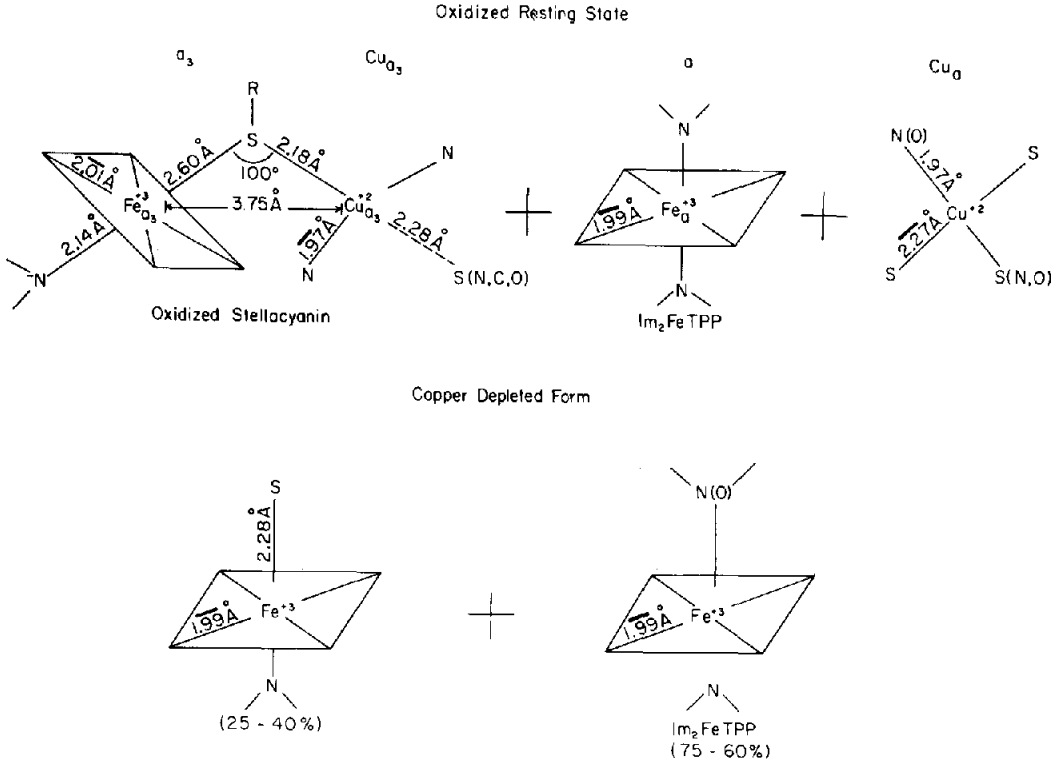

Fig.4. Pictorial comparison of the X-ray absorber results for the oxidized resting state [10] and the copper-depleted form of cytochrome oxidase. Other ligand possibilities are given in parentheses and model compounds are identified where appropriate. Bars over distances indicate average distance for all the same type of ligand and that center and error is ±0.03 Å except for the Fe_{a_3}–Cu_{a_3} distance which is 3.75 ± 0.05 Å.

cytochrome *c* oxidase have suggested that a 6 coordinate, low spin, sulfur-ligated form is present in addition to the low spin form attributed to the heme of cytochrome *a* (*a* heme). Although the source of the sulfur atom found in the copper-depleted form cannot be uniquely identified, the simplest attribution is to the bridging sulfur of the oxidized resting state. Copper depletion then, would leave the structure of Fe_a unchanged and some Fe_{a_3} would retain the sulfur from the bridge at a shorter distaince so that it contributes as a first shell ligand together with the 5 nitrogen atoms. The balance of *a* and a_3 forms remaining following Cu depletion is here identified by the fraction having a S ligand (25/75 to 40/60). This differs from the approximately 50/50 ratio of Fe_{a_3}/Fe_a in the native enzyme and we suggest that some of the cytochrome a_3 is lost in the CN^- treatment due to disruption of its binding to the protein. If, however, some Fe_{a_3} does not bind sulfur and instead has nitrogen (oxygen, carbon) as a sixth ligand, then a_3 would be indistinguishable from *a* in the oxidized state and a study of mixed valence states [5,9] would be required.

In addition to the disappearance of the sulfur bridge, the copper (Cu_{a_3}) contribution at 3.75 ± 0.05 Å is missing as well, leaving only the heme substituent carbon, nitrogen contribution diminishing and shifting the third shell peak to ±4.0 Å. In fact, all higher shells observed in the copper-depleted form are very similar to those of Im_2FeTPP and *c*-type cytochromes.

In summary, the data obtained from copper-depleted cytochrome oxidase from beef heart provides strong support for our assignments derived from the native enzyme data [10]. In the copper-depleted state, not only does the sulfur bridge assume a more commonly found structure but the remaining heme contribution to the shell containing copper in the oxidized resting state is exactly that of our assignments in [10].

Acknowledgements

The portion of this research was supported by NIH grants GM-27476, 27308, 28385, HL-16686, HL-18708 and NFS-PM-80-26684 awarded to the University of Pennsylvania. SSRL project 423B was supported by the NSF through the Division of Materials Research and the NIH through the Biotechnology Resource Program in the Division of Research Resources in cooperation with the Department of Energy.

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