Structure of the Copper Sites in Membrane-bound Cytochrome c Oxidase*

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The structures of membrane proteins are difficult to obtain by crystallography and may be altered by the detergents used in their extraction. X-ray absorption spectroscopy has been used to identify the structures of the copper atoms of the membrane-bound enzyme in mitochondria and in submitochondrial particles at respective concentrations of 100 and 200 μm of molar copper. To within the experimental error, the x-ray absorption spectra of the copper atoms of the membrane-bound and the Yonetani (Yonetani, T. (1961) J. Biol. Chem. 236, 1680–1688) purified oxidase are identical; all detectable shells of the active site indicate no alteration of structural parameters. Significant differences are found when compared to the Hartzell-Beinert (Hartzell, R. C., and Beinert, H. (1974) Biochim. Biophys. Acta 368, 318–338) preparation. Extended x-ray absorption fine structure technology is now adequate for the direct studies of membrane proteins in situ in their natural environment.

Enzyme activities are historically employed to assay enzyme purity. In simple enzyme systems such as invertase, where the second substrate is in excess, the enzyme activity test measures the first order breakdown of the enzyme substrate complex (1), whereas in the case of catalase, the rate of combination with H₂O₂ is measured (2). In cytochrome oxidase, several steps intervene between cytochrome c oxidation and oxygen reduction, and assignment of a reaction velocity constant to the active site (cytochrome c₃ − copper) oxygen reaction from the overall kinetics of cytochrome c oxidation is dubious at best. Here we have employed a structural approach to determine purity and integrity. Thus, the integrity of the cytochrome oxidase reaction center is best assayed by directly measuring its reaction with O₂ (3). An alternative approach for integrity evaluation is to measure the structure of the active site in soluble and membrane-bound cytochrome oxidase as determined by x-ray absorption spectroscopy, and this can be done with studies of the unique copper sites. Indeed, since structure and reactivity are related, this affords a new and complementary criterion of enzyme integrity. Questions of the integrity of purified cytochrome oxidase prepared by different methods have been raised by our kinetic studies of the reaction center with cyanide, together with iron and copper x-ray absorption studies (4). Not only do the cyanide binding kinetics indicate the presence of large amounts of at least three forms in some preparations, but the local structure around the metal atoms as observed by x-ray absorption spectroscopy is significantly different among the preparations by different methods (4–6). These differences in local structure are maintained in the mixed valence and reduced states as well (7, 8). These kinetic and structural differences in the oxidized state could not be explained as a mixture of the resting oxidized form (5) containing a sulfur bridged binuclear active site and the peroxidatic (pulsed)¹ form which lacks the sulfur bridge (9).

In order to identify the authentic cytochrome oxidase, if indeed any of the purified preparations are such, we report here the results of x-ray absorption studies of copper sites for membrane-bound preparations and compare them to those obtained for purified preparations by different methods. Submitochondrial particles (SMP)¹ were derived from beef heart mitochondria according to the procedures of Lee (10), since these exhibit functional similarities to intact mitochondria (11, 12) but also contain a significantly higher concentration of cytochromes. In addition, the membrane-bound cytochrome oxidase forms fully occupied intermediates in its reaction with O₂ (13), and these intermediates are different from those formed with the purified oxidase which gave more complicated patterns of oxygen intermediates (14).

The membrane-bound cytochrome oxidase contains an environment of the copper sites and a separation of the copper and iron of the binuclear active site that is identical within the experimental error to that observed in the purified preparation by the method of Yonetani (5, 15). The first coordination shell of membrane-bound cytochrome oxidase differs significantly from that for the ethanol-treated Hartzell-Beinert purified method (4, 5, 16).

MATERIALS AND METHODS

Preparations—Submitochondrial particles derived from beef heart mitochondria by sonication were prepared according to the method of Lee (10). A typical pellet after ultracentrifugation contained 2.76 nmol of copper/mg of protein and 18.15 nmol of iron/mg of protein, which includes the entire complement of iron compounds in the mitochondrial membrane. The contents of copper and iron were determined by atomic absorption spectroscopy. Protein was determined by the method of Lowry et al. (16).

¹The peroxidatic state of Ref. 9 has been recently shown to be the pulsed state of Kumar et al. (30). This is discussed in detail in L. Powers, A. Naqui, C. Kumar, and B. Chance, manuscript in preparation.

¹The abbreviations used are: SMP, submitochondrial particles; EXAFS, extended x-ray absorption fine structure.
mined by the Biuret method with bovine serum albumin as standard (17). No adventitious copper was found in these preparations as judged by the line shape of the g = 2 signal. The total copper concentrations for all samples of SMP were ~200 and 100 μM for mitochondria. Optical assays of typical samples are shown in Fig. 1, and such assays were used periodically (see below). Resting beef heart cytochrome oxidase samples were prepared using the Yonetani method (15) exactly as described in Ref. 5 and characterized according to Refs. 4 and 5.

X-ray Absorption Studies—X-ray absorption data were collected at the Stanford Synchrotron Radiation Laboratory during dedicated operation of the SPEAR storage ring (3.0 gigaelectron volts, 60–20 mA) on beam line 1-5 which provides 10¹⁰ photons/s and employs a double crystal Si 220 monochromator (~1 eV resolution at 9 kiloelectron volts). Fluorescence data were collected by seven photon counters that were protected by a nickel filter so that efficient counting could be obtained; 10,000–30,000 counts/s⁻¹ were due to the copper oxidase copper (5, 18). In order to compare data and results from previous collection intervals, data were also collected for purified resting oxidized cytochrome oxidase samples (5) together with several standard model compounds as controls.

The preparations were studied at temperatures lower than ~100 °C in order to minimize the mobility of hydrated electrons and radical intermediates produced during x-ray exposure (19). This is especially important in dilute preparations, and the damage to each sample was monitored every hour of exposure by optical absorption spectroscopy. The data indicated that these 200–300 μmol copper samples could be exposed for a few hours at ~100 °C or lower before 10% damage had occurred, after which a fresh sample was used. The measured rate of reduction of cytochrome c by hydrated electrons was 2.5 nmol/liter/s (10⁻³ that of room temperature (13)).

Data Analysis—The data were analyzed as described previously using identical procedures (including model compounds and length of data) to those for the purified preparations (4–6, 9).

The edge data were normalized by subtraction of a linear background to set the absorption below the edge equal to zero and appropriately scaled (to 1.5 mma copper) for comparison with previous data (6). The energy was calibrated by comparison to CuCl₂·2H₂O and copper metal (Fig. 2).

The EXAFS modulation to the absorption is described by

\[ x(k) = - \sum_{i} \frac{N_i}{kr_i^2} |f_i(k, r)| e^{-2r_i/k}\sigma + \text{back transformation} \sin(2(kr_i + \alpha_i(k))) \]

where the sum is over \( N_i \) atoms having a backscattering amplitude of \( f_i(k, r) \).

\(^3\) T. Onishi, personal communication.
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Fig. 3. Background subtracted copper EXAFS data. Top, submitochondrial particles and bottom, resting oxidized Yonetani preparation (5) after $k^3$ (wave vector) multiplication and normalization to one copper atom.

FIG. 4. Fourier transforms of the background subtracted, $k^3$ multiplied, normalized copper EXAFS data shown in Fig. 3. Top, resting oxidized purified preparation by the Yonetani method (——) (5) and resting oxidized submitochondrial particles (——). Middle, resting oxidized purified preparation by the Hartzell-Beinert method (4). Bottom, pulsed state from purified preparation by the Yonetani method (9). $a(k)/2$ is absorber-scatterer phase shift.

Fig. 5. Fourier filtered back transformed data for the resting oxidized purified preparation by the Yonetani method (——) (5) and resting oxidized submitochondrial particles (+). Top, first coordination shell; middle, second coordination shell; bottom, third coordination shell.

These preparations have been shown to contain significant amounts (>20%) of at least three different species by cyanide binding studies, whereas the Yonetani preparation is highly homogeneous (>85%) (4). It is important to note that these differences are apparent in the edge features, and these features alone can be used as a method to distinguish the preparations and assess the heterogeneity. On reduction with CO, the purified preparations by the Yonetani (5, 6, 15) and Volpe-Caughey (20) method(s) likewise are identical within the respective error to that obtained from the membrane-bound preparation.

The isolated EXAFS modulations of the membrane-bound preparation (SMP) are shown in Fig. 3. These data are comparable in signal-to-noise ratios to those reported in 1979 for the Yonetani purified preparation (5, 6) and required approximately the same averaging time (6-7 h). The concentration is approximately a factor of 10 more dilute than the Yonetani purified preparation, and this enhanced fluorescence detection is largely due to improved methods of photon counting. The beam conditions and stability were significantly deteriorated from the 1979 measurements, requiring nearly four times as many scans to obtain approximately the same number of acceptable quality scans (5) as averaged for the 1979 data.

The data collected for the Yonetani preparation were identical within the signal-to-noise ratios to those published previously (4, 5). Fig. 4 (top) shows the Fourier transform of the data in Fig. 3 compared to that for the Yonetani method (5). It is easily seen that these are identical within the error. The
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**Table I**

| Model              | $\Delta\langle\bar{A}\rangle(\times10^3)$ | $\Delta N$ | $\Delta\sigma(\bar{A}^2)(\times10^3)$ | $\Sigma R^2$ |
|-------------------|-------------------------------------------|-----------|---------------------------------------|-------------|
| Yonetani resting  |                                           |           |                                       |             |
| First shell       | 0                                         | 0         | 0                                    | 1.8         |
| Second shell      | 0.5 $\pm$ 1.3                             | 0.07 $\pm$ 0.08 | $-0.82 \pm 1.0$            | 1.5         |
| Third shell       | $-1.5 \pm 1.0$                           | 0.02 $\pm$ 0.08 | $1.2 \pm 1.0$                   | 0.82        |
| Yonetani pulsed   |                                           |           |                                       |             |
| First shell       | 0                                         | 0         | 0                                    | 3.2 $\times 10^{-2}$ |
| Second shell      | $-1.5 \pm 1.5$                           | 0.03 $\pm$ 0.07 | $-1.1 \pm 1.0$                   | 0.26        |
| Third shell       |                                           |           |                                       |             |
| Hartzell-Beinert resting |                                         |           |                                       |             |
| First shell       | 0                                         | 0         | 0                                    | 52.7        |
|                    | 2.5 $\pm$ 3.5                            | 0.18 $\pm$ 0.35 | $0.1 \pm 3.0$                   | 33.8        |

$^a$ $\Delta r$ is the difference in average distance.
$^b$ $\Delta N$ is the difference in coordination number.
$^c$ $\Delta\sigma$ is the difference in Debye-Waller factor.
$^d$ $\Sigma R^2$ is the sum of residuals squared.

SMP and Yonetani purified preparations (Fig. 4, top) contain a single broad first coordination shell ($R + \alpha(k)/2 = 1.2-3.2 \bar{A}$) and two higher shells ($R + \alpha(k)/2 = 2.4-3.0$ and $3.1-4.0 \bar{A}$, respectively), whereas the Hartzell-Beinert purified preparation (Fig. 4, middle) (4, 7) and the pulsed state from the Yonetani preparation (Fig. 4, bottom) (9) contain bifurcated first coordination shells. The latter preparations differ significantly in the higher shells, with the third shell of the pulsed state of the Yonetani preparation being similar to that of the resting Yonetani purified preparation (5, 9). The structural differences, together with the optical and cyanide binding differences for the preparations by different methods, are discussed in detail in Ref. 4.

Fig. 5 compares the filtered data for each of the three coordination shells for the SMP and Yonetani purified preparation (5) and shows that the phases and amplitude are identical within the error. This same comparison of the different purified preparation methods with that of the membrane-bound preparation (SMP) was also made numerically using the single-atom type nonlinear least squares fitting procedure. (The parameters and procedure are described under "Materials and Methods.") These results are shown in Table I where the first fit is made with all parameters held fixed, which gives a numerical comparison before any of the parameters were allowed to vary. All parameters were allowed to vary in the second fit. The sum of residuals squared ($\Sigma R^2$) gives the goodness of fit, and the change in each of the parameters is listed (zero indicates that the parameter was held fixed). The comparison to that of the Yonetani purified preparation gives a sum of residuals squared that is $>10$ better than comparison to the other purified preparation methods or to the pulsed state. Even when all parameters are allowed to vary, which gives maximum fitting flexibility, no change within the experimental error is observed for any of the parameters. On the other hand, when all parameters were allowed to vary, the data from the other preparation methods or the Yonetani pulsed state could not be made to simulate those of the membrane-bound preparation or the Yonetani purified preparation.

Thus, the resting oxidized state of cytochrome oxidase in membrane-bound preparations is identical within experimental error to the purified preparations by the Yonetani (5, 15) method and significantly different from the pulsed state (9) and from purified preparation produced by the Hartzell-Beinert method (4, 7, 16).

**DISCUSSION AND CONCLUSIONS**

Criteria for purity have historically been established with biochemical and functional parameters together with spectroscopic characteristics. For the membrane-bound enzyme cytochrome oxidase, controversies over purity and integrity have occupied many conferences and texts (23). This is not surprising since the extraction of a membrane protein from its native lipophilic environment, often by incorporation into micelles, is likely to stress and in some cases to destroy the native active site.

Initially, Gibson found Green's purified cytochrome oxidase to contain only one cytochrome $a_3$ to two cytochrome $a$ as judged by the changes at 445 and 605 nm in combination with CO but clearly showed this inequality was due to irreversible heme (24). He concluded that the ratio was 1:1, in agreement with the flash photolysis studies with intact mitochondria, particularly at 77 K, which showed 56% decrease in absorption at 444 nm on formation of CO-bound cytochrome $a_3$ (25). Morrison also found that degradation of the cytochrome $a_3$ heme prevents the reaction with CO (26).

The second order velocity constants for the reaction with oxygen, oxygen disappearance, and the reaction of cytochrome $a$ with oxygen are $5 \times 10^7$ M$^{-1}$ s$^{-1}$ for mitochondria at 23 $^\circ$C and estimated to be $2.5 \times 10^9$ M$^{-1}$ s$^{-1}$ at 37 $^\circ$C. Somewhat higher values are obtained for purified preparations (25), possibly due to increased accessibility of oxygen to cytochrome $a_3$ (27). More recently, the reaction with cyanide has exposed significant heterogeneity in oxidized cytochrome oxidase prepared by different methods (4). X-ray absorption studies of these preparations showed that the local structures around the redox centers indeed differed (4, 28) and that these structural differences were maintained in the mixed valence and fully reduced states as well (7, 8). The cyanide studies showed that only purified preparations using the Yonetani method (5, 15) were highly homogeneous, and x-ray absorption studies on more than 20 such preparations since 1976.
have shown that the local structure around the redox centers does not vary within the signal-to-noise ratios of the measurements.

Spectroscopic differences among the purified preparations by different methods are small, and the characterization of the "invisible" copper of the binuclear active site has been difficult since it contributes only a small portion to the weak 830 nm band in the oxidized state (6, 29) and is EPR-silent. It has only been with x-ray absorption studies (4–7, 22) that physical, biochemical, and structural characterizations have been made. The conflicting results for the purified preparations by different methods are resolved by the studies presented here where the various purified preparations are compared to cytochrome oxidase in the native membrane. The conclusion that the copper sites of resting oxidized and CO-reduced cytochrome oxidase isolated in detergent micelles are identical to those of cytochrome oxidase in its natural membrane is surprising, since isolation subjects the enzyme to stressful treatment and foreign environments. Why the purification method of Yonetani (5, 15) preserves these sites or allows reconstitution in detergent micelles is not clear. One explanation may involve the ethanol treatment used in the Hartzell-Beinert method (16) that is absent in the Yonetani method (5, 15). Another consideration is the beef hearts themselves. In 1965 Gibson et al. (24), in an effort to find a basis for the reported kinetic differences in Green's preparations, stated, "However, we still got different results from one preparation to another." Each preparation method was biochemically designed for hearts obtained from beef that were raised in different locales and under different nutritional conditions, programs which have been shown to produce variations in the lipid content of preparations using the same purification method.

In summary, the "invisible copper" of cytochrome oxidase in its native membrane environment has been characterized by x-ray absorption spectroscopy. This copper component is diagnostic of the integrity of the active site in purified preparations and suggests that at least one method of purification (5, 15) together with careful controls (4) can be used for study of the remarkable properties of this enzyme.

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