Quantitative Reevaluation of the Redox Active Sites of Crystalline Bovine Heart Cytochrome c Oxidase*

(Received for publication, March 25, 1999, and in revised form, September 1, 1999)

Masao Mochizukij, Hiroshi Aoyamaq, Kyoko Shinzawa-Itohj, Toshihiro Usu, Tomitake Tsukiharaq, and Shinya Yoshikawaf

From the jDepartment of Life Science, Himeji Institute of Technology, CREST, Japan Science and Technology Corporation, Kamigohri Akoh, Hyogo 678-1297, the jInstitute for Protein Research, Osaka University, 3-2 Yamadaoka Suita, Osaka 565-0871, and the jDepartment of Applied Chemistry, Himeji Institute of Technology, 2167 Shosha Himeji, Hyogo 671-2201, Japan.

Approximately 30% of the iron contained in a bovine heart cytochrome c oxidase preparation was removed by crystallization, giving a molecular extinction coefficient 1.25-1.4 times higher than those reported thus far. Six electron equivalents provided by dithionite were required for complete reduction of the crystalline cytochrome c oxidase preparation. The fully reduced enzyme was oxidized with 4 oxidation equivalents provided by molecular oxygen, giving an absorption spectrum slightly, but significantly, different from that of the original fully oxidized form. Four electron equivalents were required for complete reduction of the O2-oxidized enzyme. The O2-oxidized form, when exposed to excess amounts of O2, was converted to the original oxidized form which required 6 electrons for complete reduction. A slow reduction of the O2-oxidized form without any external reductant added indicates the existence of internal electron donors for heme irons in the enzyme. These results suggest that the 2 extra oxidation equivalents in the original oxidized form, compared with the O2-oxidized form, are due to a bound peroxide produced by O2 and electrons from the internal donors, consistently with a peroxide at the O2 reduction site in the crystal structure of the enzyme (Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Peters Libeu, C., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) Science 280, 1723-1729).

Cytochrome c oxidase catalyzes the reduction of O2 to water as the terminal oxidase of cell respiration. The O2 reduction is coupled with proton pumping. The enzyme contains two redox active copper sites and two heme iron sites (1). The x-ray crystallographic structure of the enzyme isolated from bovine heart has been determined at 2.3Å resolution in the fully oxidized state and at 2.35Å resolution in the fully reduced state (2). The x-ray structure shows that one of the copper sites (Cuα) is dinuclear but likely to be a 1-electron accepting site and that a peroxide is bridged between iron and copper in the O2 reduction site in the fully oxidized state. The two heme A sites, the two copper sites, and the bridging peroxide may accept 6 electron equivalents in total. The prediction is not consistent with the widely accepted conclusion that 4 electron equivalents are required for complete reduction of the fully oxidized enzyme, based on metal analysis and redox titration experiments (1, 3). However, difficulties in purification and incomplete removal of O2 from the membrane protein solution are likely to decrease the accuracy in the metal analysis and in the redox titration experiment. Thus, we reexamined the metal content of the enzyme using crystalline bovine heart cytochrome c oxidase and the electron equivalents required for complete reduction of the fully oxidized form and for complete oxidation of the dithionite reduced form. The results obtained here are consistent with the enzyme structure in the fully oxidized state containing 1 equivalent of peroxide and four metal sites, each receiving 1 electron equivalent.

EXPERIMENTAL PROCEDURES

Cytochrome c oxidase was purified from bovine heart muscle by the method of Yoshikawa et al. (4). The detergent, Tween 20, was replaced with C12E8 (Nikko Chemicals, Japan), C12E23 (Pierce), or DM (Anatrace). For crystallization, the enzyme solution was concentrated with an Amicon Diaflow apparatus. Metal content was analyzed with a model Z-6100 Hitachi polarized Zeeman atomic absorption analyzer. The enzyme samples were first wet-ashed with nitric acid, hydrogen peroxide, and perchloric acid. About 20 mg of the enzyme sample was used for a single determination. Thus, at least 80 mg of the enzyme sample was required for a single set of analyses for four metals (iron, copper, magnesium, and zinc). Cytochrome c oxidase activity was determined by following the aerobic oxidation of 15 μM ferrocyanochrome c at pH 6.0.

The redox titration of cytochrome c oxidase was performed under anaerobic conditions, using sodium dithionite or NADH-PMS system for reductive titration and O2 for oxidative titration, using a modified anaerobic titration system (Fig. 1A, b). A vacuum system for exchanging the gas phase in the cuvette with O2-free N2 (Fig. 1C), and a flask for dithionite solution (data not shown). The Thumberg type cuvette was made of Pyrex glass (Fig. 1, A and B) with three ports. The port for titrant consisted of a male joint stoppered with a rubber septum (Fig. 1A, b). A female joint, fused to a male joint (Fig. 1A, c) stopped with a silicon rubber septum (Fig. 1A, d) was attached to the male joint of the titrant port (Fig. 1A, o). A female joint mounted with a gas-tight syringe (fitted with a needle) was attached to the male joint stoppered with the silicon rubber septum (Fig. 1, A, d, and B). The brim of the silicon rubber septum was trimmed for effective sealing between the two joints. The space inside the male-female joint attached to the titrant port was filled with N2-saturated water. The rubber septum for sealing the titrant-port (Fig. 1A, b) had been degassed by keeping it under vacuum overnight. The port for connecting the cuvette to the

* This work was supported in part by Grants-in-aid for Scientific Research on Priority Area: Molecular Science on the Specific Roles of Metal Ions in Biological Functions (to S. Y.) and Grant-in-aid for Scientific Research 40068119 (to S. Y.) from the Ministry of Education and Culture of Japan. The costs of publication of this article were defrayed by page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 81-7915-8-0190; Fax: 81-7915-8-0132; E-mail: yoshi@sci.himeji-tech.ac.jp.

1 The abbreviations used are: C12E8, CH3(CH2)8(OCH2CH2)8OH; C12E23, CH3(CH2)11(OCH2CH2)23OH; C12E23, CH3(CH2)11(OCH2CH2)23OH; DM, n-decyl-β-D-maltoside; PMS, phenazine methosulfate.
vacuum system was a two-way stopcock with a male joint at the end (Fig. 1A, g). The stopper of the two-way stopcock was especially ground for close fitting and had its connecting channel running from the bottom through the center in order to minimize leakage. A fairly large space was provided between the optical cuvette and the port for connection to the vacuum system for foaming from the protein solution in the cuvette during evacuation. The last port (Fig. 1A, e) was used for introducing the enzyme solution and was sealed with a glass stopper. All of the tapered ground joints were sealed with Apiezon L high vacuum grease, whereas Apiezon N was used for the two-way stopcock. A gas-tight syringe with a screw-driven piston (Hamilton, no. 1750) was used to add titrant to the enzyme preparation in the Thumberg type cuvette (Fig. 1B). The syringe had a long stainless steel needle fixed permanently for gas-tightness with epoxy resin. A sketch of the cuvette mounted with the titrant syringe is given in Fig. 1B.

The vacuum system (Fig. 1C) consisted of three stainless steel tubing lines (3 mm in diameter; Takahama, Himeji), for connecting the Thumberg type cuvette, for supplying O2-free N2, and for evacuation with a vacuum pump. The three stainless steel tubes were connected with a T-shape (Fig. 1C, c) to a vacuum pump (Fig. 1C, h) with a liquid nitrogen trap (Fig. 1C, g) in between. The third branch was connected in tandem so that the buffer solution contained a catalyst, a cytochrome oxidase, and the buffer solutions were used for complete removal of trace amounts of O2 in N2 gas, and to a N2 tank containing ultrapure N2 (99.9999%; Takahama, Himeji) (Fig. 1C, f). The second and third branches were equipped with high vacuum two-way metal stoplocks (Nupro, SS-2H) (Fig. 1C, d) for switching the connections to the Thumberg type cuvette.

Dithionite solution was prepared, and withdrawn from, a 130-ml flask having a port at the side similar in construction to the titrant port of the Thumberg type cuvette (data not shown), with the method of Burleigh et al. (5). The flask was filled with 90 ml of 0.1 M pyrophosphate buffer, pH 9.0, and after placing a magnetic stirring bar coated with glass, the flask was sealed with a glass stopper equipped with a two-way stopcock. The stopcock was connected to the vacuum system with a rubber vacuum tubing, and the buffer solution in the flask was deaerated by five cycles of evacuation-equilibration with N2. After adding dinitrogen dithionite, the buffer solution was used for direct removal of trace amounts of O2 with N2 gas, and to a N2 tank containing ultrapure N2 (99.9999%; Takahama, Himeji) (Fig. 1C, f). The second and third branches were equipped with high vacuum two-way metal stoplocks (Nupro, SS-2H) (Fig. 1C, d) for switching the connections to the Thumberg type cuvette.

For the reductive titration with NADH-PMS system, NADH (Sigma, fast atom bombardment peptide mass) and PMS (2.5 mg per milliliter) were used for the complete removal of trace amounts of O2 in the NADH solution. The buffer solution is too low in the gas phase not to readily react with the enzyme. Therefore, the titration results were not corrected for O2 introduced by the NADH solution. The maximal amount of O2 carried by PMS solution corresponds to 0.01 oxidation equivalent to the enzyme in the cuvette. The method for the reductive titration of highly concentrated enzyme solution was described previously (7).

RESULTS

Metal Analysis—The effect of repeated crystallization on the metal content of cytochrome c oxidase was examined. The purified preparation, stabilized with C12E8 before crystallization, was washed with 2 m M EDTA in 50 m M sodium phosphate buffer, pH 7.4, using an Amicon Diaflow apparatus five times and wet-ashed for the determination of iron, copper, magnesium, and zinc atoms by atomic absorption spectrometry. The amount of enzyme in the sample was evaluated by the a-band absorption of the dithionite reduced form. The contents of copper, magnesium, and zinc were not significantly affected by the crystallization, whereas the iron content decreased significantly on repeated crystallization (Table I). The atomic ratio of iron to copper, which was 1.0 before crystallization, decreased to 0.69 after the second crystallization. Further crystallization did not affect the iron content, indicating absence of contaminant iron. In Table I, the sample crystallized three times is presumed to be free from contaminant iron and contains 2 iron atoms per enzyme molecule. Closely similar results were obtained for the preparation stabilized with C12E23. Averaged metal composition for seven different preparations of twice crystallized enzyme, stabilized with C12E8 or C12E23 (Table I), indicates the atomic ratio of Fe:Cu:Mg:Zn in the enzyme preparation to be 2:3:1:1. The iron content gives a molecular extinction coefficient (per two hemes) for the absorption of the a-band region of the fully reduced form (310 ε red = 6040–630 nm) of 46.6 m M⁻¹ cm⁻¹, with a standard error of 1.16 m M⁻¹ cm⁻¹ (Table II). The wet-ashing and the large quantity of the sample (20 mg of protein for each determination) are critical for the metal analysis at this high accuracy. The spectra of the fully oxidized and reduced forms of the enzyme free from contaminant metal are given in Fig. 2.

A preparation stabilized with DM allows for more effective purification by crystallization. The metal content of the once-crystallized preparation was about the same as that for the twice-crystallized sample stabilized with C12E8 or C12E23. The iron contaminant is likely to be associated with a contaminant protein, since repeated crystallization effectively removes contaminant peptide bands found in SDS-polyacyr-
The spectral difference between a noncrystallized preparation and a once-crystallized preparation stabilized with C12E8 reveals that about 40% of the contaminant iron removed by the initial crystallization is due to contamination by cytochrome bc1 complex. On the other hand, the contaminant iron left in the once-crystallized sample does not have strong absorption in the visible region.

The enzyme preparations described herein were highly active (250 s⁻¹ in terms of molecular activity), and no significant effect on the enzymic activity was detectable by the crystallization.
Titration with Dithionite—The once crystallized preparation stabilized with C12E8 was titrated with dithionite under the anaerobic conditions as described above. The absorbance change was completed within 20 min. The spectrum was also taken at 30 min to confirm that no further change had taken place.

Fig. 3 shows the spectral changes in the Soret, α-band, and near infrared regions during reductive titration using the once crystallized enzyme solution stabilized with C12E8 in the fully oxidized state as prepared (a) and in the fully reduced (b) state in 0.1 mM sodium phosphate buffer, pH 7.4. A slight excess amount of solid dithionite was used for complete reduction of the enzyme.

**TABLE I**

| Preparation                  | Atoms/enzyme molecule | Iron (mM) | Copper (mM) | Magnesium (mM) | Zinc (mM) | e604–630 nm |
|------------------------------|-----------------------|-----------|-------------|----------------|-----------|-------------|
| Before crystallizationa      |                       | 2.90      | 2.90        | 1.04           | 0.97      | 32.2        |
| Once-crystallizeda           |                       | 2.21      | 3.04        | 1.07           | 1.01      | 42.2        |
| Twice-crystallizeda          |                       | 2.06      | 3.00        | 1.06           | 1.00      | 45.6        |
| Three times-crystallizeda    |                       | 2.00      | 2.95        | 1.04           | 1.04      | 46.6        |

Twice-crystallized, averagedb  2.00 (0.07)  2.95 (0.12)  1.02 (0.08)  1.04 (0.04)  46.6 (1.16)

a Enzyme stabilized with C12E8 was used.
b Averaged values with (S.D.) for 3 and 4 enzyme preparations stabilized with C12E8 and C12E23, respectively.

**Titration with Dithionite**—The once crystallized preparation stabilized with C12E8 was titrated with dithionite under the anaerobic conditions as described above. The absorbance change was completed within 20 min. The spectrum was also taken at 30 min to confirm that no further change had taken place.

Fig. 3 shows the spectral changes in the Soret, α-band, and near infrared regions during reductive titration using the once crystallized enzyme solution stabilized with C12E8. The fractional changes in absorption spectra due to reduction are essentially constant regardless of the wavelength region as indicated by the titration curves (Fig. 3, insets) and show that 6.2 electron equivalents are required for complete reduction of the enzyme. The slope of each titration curve in the initial 1–2 electron equivalents is about 1/3 of that in the latter part of the titration curve. As given in Table II, the average of the end point values for eight determinations is 6.3 electron equivalents, with a standard error of 0.27. The smaller absorbance changes in the initial part of the reductive titration are not due to incomplete anaerobiosis of the titration system, since the titration curves were independent of the number of evacuation-N2 saturation cycles between 3 and 10 times. On the other hand, when the cycle of evacuation and N2 saturation was applied only once or twice, both slopes of the biphasic titration curve decreased to give higher end point value.

After the reductive titration, the fully reduced enzyme was reoxidized with air-saturated water (Fig. 4A). The oxidative titration curve showed an initial lag phase where no oxidation of the enzyme was detectable. The oxidation equivalent consumed for the lag phase corresponded to the excess dithionite in the initial reductive titration. The oxidation proceeded monophasically with increasing amounts of O2 added, in contrast to reductive titration. Compared with the spectrum before the reductive titration, the final reoxidized spectrum had a slightly weaker Soret band with a slightly higher absorbance in the region from 440 nm to 460 nm and a slightly higher α-band, giving peaks at 605 nm and 444 nm and a trough at 420 nm in the difference spectrum (Fig. 4C). The O2-oxidized enzyme was fully reduced monophasically with 5.2 electron equivalents of dithionite (Fig. 4B, Table II). The end point value was significantly smaller (about 1 electron equivalent) than that of the initial reductive titration. Titration curves for the Soret region (Δλ444–480 nm) and near infrared region (Δλ750–805 nm) coincided with that of the α-band spectrum given in Fig. 4, within the experimental accuracy.

The O2-oxidized form as well as partially reduced form during the oxidative titration showed a slow and small spectral change indicating reduction of the enzyme, which corresponded to about a 3% reduction in 1 h under anaerobic conditions. The slow reduction proceeded further, up to a 15% reduction in 24 h. The spectral change on addition of O2 in the oxidative titration was significantly faster than that on addition of dithionite in the reductive titration. A stable spectrum was obtained within 10 min after each addition of O2-saturated water, so that it took about 1 h to obtain the whole oxidative titration.

**FIG. 2. Absorbance spectra of bovine heart cytochrome c oxidase.** Absorbance spectra of the twice-crystallized preparation stabilized with C12E8 in the fully oxidized state as prepared (a) and in the fully reduced (b) state in 0.1 mM sodium phosphate buffer, pH 7.4. A slight excess amount of solid dithionite was used for complete reduction of the enzyme.
curve. Thus, the “autoreduction” was negligible in the oxidative titration.

When the O2-oxidized sample was equilibrated with air by opening the port used to introduce the enzyme solution (Fig. 1A, c), the spectrum gradually moved toward the original spectrum preceding the initial reductive titration. The spectral change was essentially completed within 30 min after addition of excess O2. The resulting spectrum in the visible-Soret region closely resembled that of the enzyme before the initial reductive titration. The O2-oxidized enzyme, exposed to air for 2 h, provided a reductive titration curve with dithionite identical with that in the reductive titration of the fully oxidized form as prepared. As is well known, bovine heart cytochrome c oxidase as prepared is in the fully oxidized form, which shows significantly lower reactivities to ferrocyanochrome c and cyanide compared with the fully oxidized form under turnover conditions. This fully oxidized form is alternatively called “resting oxidized form,” and the oxidized form under turnover conditions is called “oxygen-pulsed form” (8). In this paper, the fully oxidized form as prepared denotes the resting oxidized form.

The difference spectrum between the O2-oxidized form and the oxidized form as prepared (Fig. 4C) is similar to the redox difference spectrum of heme α, which is characterized by a stronger α band versus Soret band compared with that for heme αg (9). However, the O2-oxidized form is also in a fully oxidized state since the form does not react with O2 in the time scale for the enzymic turnover. Thus, this spectral difference must be due to a difference in the coordination structure of the O2 reduction site.

Quantitative addition of O2 to the fully reduced enzyme was possible only when the air-saturated water was added directly to the reduced enzyme solution without exposure to the gas phase. When the air-saturated water was dispensed on the L-shaped arm of the titrant port, followed by the addition of reduced enzyme solution to the titrant solution (about 10 μl), the reproducibility and the spectral change due to oxidation of the enzyme were definitely more reduced than when the titrant was added directly. This result indicates that a significant part of the O2 in the air-saturated water dispensed to solution of the enzyme in the O2-oxidized state on the titrant arm diffuses to the gas phase in the Thumberg type cuvette. The residual O2 in the gas phase would react slowly with any reducing equivalent introduced as in the case of the reductive titration under incomplete anaerobiosis. Thus, the second reductive titration has no initial phase for consuming the excess O2 (Fig. 4, A and B) and the residual O2 in the gas phase may interfere with progress of the reduction with dithionite. Some part of the extra O2 could react with O2-oxidized form to form the fully oxidized form, equivalent to that before initial reductive titration. These two factors are likely to yield a slightly higher end point value as opposed to the true end point value for the reductive titration of the O2-oxidized form. The number of electron equivalents required for complete reduction of the O2-oxidized form therefore should be identical with the number of oxidation equivalents required for producing the O2-oxidized form from the fully reduced form.

The apparatus for highly concentrated protein solutions in which no evacuation is required (7) was used for anaerobic titration of the preparation stabilized with DM, since extra DM added to the medium (0.2%) for stabilizing the micelles including the enzyme molecules interferes with complete removal of O2 by the above procedure. Enzyme preparations stabilized with C12E8 and C12E23 require no extra detergent in the medium, because the critical micelle concentration is low enough. The reductive titration results are fully consistent with those for the preparation stabilized with C12E6 obtained by the system including the Thumberg type cuvette (Table II). The preparation solubilized with C12E6 at 0.7 mM gave titration results identical to those at 7.5 mM. These results indicate that the redox properties of bovine heart cytochrome c oxidase are independent of the detergent species and of the protein concentration (Table II).

Absorption Spectral Changes during Redox Titration—As shown in Fig. 3, changes in the difference in the absorbance between 444 and 480 nm during reductive titration with dithionite parallels quite well the increase in absorption of the α-band and the decrease in the near infrared region. However, the fractional decrease in the absorbance at 416 nm is slightly, but significantly, larger than the fractional increase in absorbance at 604 nm at any electron equivalent added, except for the initial segment of the titration curve (Fig. 5A). The fractional increase in absorbance at 444 nm is significantly smaller than that at 605 nm in the initial half portion of the titration curve (Fig. 5A). On the other hand, the fractional changes at these
wavelengths coincide well with each other in the oxidative titration with O₂ (Fig. 5B) and in the reductive titration of the O₂-oxidized form (data not shown). The fractional absorbance changes in the near infrared region paralleled well that at 604 nm in all the above cases.

The shape of the spectral change, induced by the initial 1 electron equivalent in the reductive titration of the fully oxidized form as prepared, was significantly different from that induced by the last 1 electron equivalent before the end point was reached (Fig. 5, C and D). The most obvious difference was in the ratio of the maximum intensity at 444 nm to the minimum intensity at 420 nm. The ratios were approximately unity for the absorbance change in the initial 1 electron equivalent and 0.4 for the absorbance change in the final 1 electron equivalent. The difference was consistent with the absence of an isosbestic point in the regions near 432 and 560 nm, where the spectra in different oxidation states intersect.

The spectral changes induced by the addition of various amounts of O₂ to the fully reduced form, or of dithionite to the O₂-oxidized form were essentially the same as those induced by the addition of the last 1 electron equivalent in the reductive titration of the fully oxidized form as prepared (Fig. 5D).

**Titrations with NADH-PMS System**—The reductive titration curve using NADH and a catalytic amount of PMS indicated that 4.6 electron equivalents were required for complete reduction of the fully oxidized enzyme as prepared (Table I) without any initial decrease in slope (data not shown). The change in PMS concentration between 0.02 and 1.0 μM did not affect the titration curve. As in the case of reductive titration with dithionite, the reductive titration curve was not influenced by the

**TABLE II**

Redox titration of bovine heart cytochrome c oxidase

| Enzyme form                      | Concentration | Titrant           | End point (S.D.) | No. of determination |
|----------------------------------|---------------|-------------------|------------------|----------------------|
| Fully oxidized as prepared       | 7.5 μM        | Dithionite        | 6.3 (0.27)       | 8                    |
| Fully reduced                    | 7.5 μM        | O₂                | 4.5 (0.12)       | 7                    |
| O₂-oxidized                      | 7.5 μM        | Dithionite        | 5.2              | 2                    |
| O₂-oxidized, exposed to air      | 7.5 μM        | Dithionite        | 6.4              | 2                    |
| Fully oxidized as prepared       | 7.5 μM        | NADH-PMS          | 4.6 (0.20)       | 6                    |
| Fully oxidized as prepared*      | 0.7 mM        | Dithionite        | 6.0              | 1                    |
| Fully oxidized as prepared       | 0.7 mM        | Dithionite        | 6.4              | 1                    |
| Fully oxidized as prepared       | 0.7 mM        | NADH-PMS          | 4.5              | 1                    |
| Fully oxidized as prepared*      | 0.7 mM        | NADH-PMS          | 5.1              | 1                    |

*The enzyme preparation was stabilized with DM. The other enzyme samples were stabilized with C₁₂E₈.

**FIG. 4.** Oxidative titration of the fully reduced form and reductive titration of the O₂-oxidized form. A, oxidative titration of the fully reduced form with O₂; B, reductive titration of the O₂-oxidized form. The ordinate is increase in absorbance difference between 604 and 630 nm, normalized by the maximum increase in the initial reductive titration (data not shown). In panel A, loss of the enzyme protein during the evacuation are corrected, assuming that the shape of the spectrum of the fully reduced form is not influenced by the titration procedure. C, spectra of the O₂ oxidized form (---) and the fully oxidized form as prepared (- - -). The inset shows the difference spectrum of the O₂ oxidized form versus the fully oxidized form as prepared. The enzyme concentration is 7.5 μM. The medium conditions are as given in Fig. 3.
Redox Sites of Crystalline Bovine Heart Cytochrome c Oxidase

The fully reduced form with O2 (m form reacted with 200 μM cyanide for the fully oxidized form as prepared (a), for the fully oxidized form treated 2 h with 1 μM PMS (b), and for the O2-oxidized form (c). All the measurements have been done under strictly anaerobic conditions in the Thumberg type cuvette, containing 7.5 μM enzyme stabilized with C12E8 in 0.1 M sodium phosphate buffer, pH 7.4.

The enzyme solution in 77% oxidized state in the oxidative titration reacted just as fast with cyanide at 200 μM as the O2-oxidized form, indicating that the reducing equivalent (1 electron/enzyme) does not influence the reactivity of the cyanide binding site, i.e. the O2 reduction site.

Effect of Contaminant Metals on Redox Titration—As seen from Table I, the once crystallized enzyme contains contaminant iron at a level of 10% of total iron. However, the twice crystallized enzyme, which is free from contaminant iron, gave the same reductive titration curve with dithionite as the once crystallized enzyme which was used for the present titration experiments. The kinetics of the spectral change after each addition of titrant was not affected by the removal of the residual contaminant iron. However, the kinetics of the spectral change was significantly influenced by the contaminant iron in the non-crystalline preparation. A non-crystalline sample stabilized with C12E8 showed a biphasic absorbance increase in the α-band, with a rapid initial increase within 10 min followed by a slow increase in absorbance. No stable spectrum was obtained even 1 h after the addition of dithionite. An approximate reductive titration curve, drawn from the spectrum obtained at 70 min after each addition of dithionite solution was monophasic without any initial lag phase (data not shown). The end point was significantly (1.0 electron equivalent) higher than that of the crystalline preparation. These results indicate that at least part of the contaminant iron is redox active, which is consistent with a spectrum of the contaminant fraction showing the presence of cytochrome bc1 complex, as described above. Furthermore, fractional spectral change in near infrared region showed a sigmoidal titration curve, giving a definitely smaller percentage reduction in the initial half of the reductive titration (at most, in 16% reduction) than those in α and Soret regions. Spectral changes in the visible-Soret region were essentially parallel with each other. The delay in reduction of CuA is likely to be due to a redox interaction between the contaminant metalloproteins and the enzyme.

**DISCUSSION**

**Purity and Molecular Extinction Coefficient of Cytochrome c Oxidase**—Table III summarizes the extinction coefficients reported thus far. The extinction coefficients based on iron content of non-crystalline preparations of the enzyme (4, 9–13) are significantly lower than the value determined in the present

![Graph of absorbance changes at 604 nm, 444 nm, and 416 nm in the titrations of the fully oxidized form as prepared with dithionite and of the fully reduced form with O2.](image)

![Graph of absorbance change at 420 nm induced by 200 μM PMS treated just as fast with cyanide at 200 μM as the O2-oxidized form, indicating that the reducing equivalent (1 electron/enzyme) does not influence the reactivity of the cyanide binding site, i.e. the O2 reduction site.](image)
from the protein moiety. Thus, a possible physiological role of the stable $O_2^-$-bound form is to prevent modification of amino acid residues when electron transfer is limited from the upstream of this enzyme in the respiratory chain. The electrons for forming $O_2^-$ under physiological conditions could come from an external reductant, in which no chemical modification would be caused in the protein.

As described above, in the presence of PMS, 4 electron equivalents from NADH are enough for complete reduction of the enzyme. This result indicates that PMS stimulates an internal electron transfer process to reduce (or remove) $O_2^-$ in the fully oxidized form as prepared, and that the 2-electron process provides a non-radical oxidative derivative of an unidentified amino acid residue which is non-reducible with NADH. Many amino acid residues in the enzyme, though unidentified, could be the 2-electron donor sites, as in the case of the formation of $O_2^-$ from $O_2$. No significant modification in the absorption spectrum as well as the enzymic function has been detected in the PMS-treated enzyme, compared with the $O_2^-$-oxidized form. Similarly, the fully oxidized form regenerated by treatment of the $O_2^-$-oxidized form with excess $O_2$ has the absorption spectrum and the function strictly identical with the fully oxidized form as prepared. Furthermore, the effects of PMS and $O_2$ appear fairly slowly (in the time scale of 30 min or so). These findings suggest that the unknown modification sites are far apart from the active center of the enzyme, at least, after the first treatment by PMS or $O_2$. This enzyme may have a pool of such amino acids for reducing radical species accidentally produced near the $O_2$ reduction sites. The direct electron donors to the $O_2$ reduction site stimulated by PMS or $O_2$ could be very near the active site. However, the radical species produced could be readily reduced with amino acids in the pool remote from the active site, for preserving the integrity of the active site.

The peroxide is most likely to be situated between Cu$_b$ and Fe$_{a3}$ as a bridging ligand, which has been shown recently in the crystal structure of the fully oxidized enzyme (2). However, the electron density at 2.3-A resolution does not exclude the possibility that $O_2$ instead of $O_2^-$ is bridged between the two metals. Thus, the present study excludes the possibility that it is $O_2$.

Absorption Spectral Changes—The fractional change in absorption in the transition between the fully reduced form and the $O_2^-$-oxidized form is independent of wavelength, i.e. two spectrally independent species are sufficient to account for the spectral transition. This result indicates the following two possibilities: (a) the enzyme system in any oxidation state between the $O_2^-$-oxidized state and the fully reduced state contains only the two extreme forms in various ratios, and (b) all of the redox active metal sites in an overall oxidation state have an identical redox potential, and the potential depends on the oxidation state. The reactivity of cyanide to the enzyme in 77% oxidized state suggests the absence of the fully oxidized form as prepared in the partially reduced state. On the other hand, it has been shown that the number of cyanide sensitive site is independent of the oxidation state between the fully oxidized state and the 3-electron-reduced state (7). This result indicates the absence of fully reduced form in the partially reduced preparation since the fully reduced form has much weaker reactivity to cyanide than those of the fully oxidized and partially reduced forms. These results indicate that possibility b given above is the case, suggesting an extremely tight negative cooperativity between the redox-active metal sites, which is consistent with the results of x-ray structure studies showing close proximity in the locations of the four redox active metal sites (14–16).

The equipotential state of the four metal sites in any overall oxidized...
tion state suggests an extremely facile electron transfer between these metals.

Comparison of the Present Results with the Redox-coupled Spectral Changes Reported thus Far—The spectral changes of bovine heart cytochrome c oxidase in relation to the oxidation state in the visible-Soret and near infrared regions, have been reported by several groups (7, 12, 17–21). All of them concluded that 4 electron (or oxidation) equivalents are required for complete reduction (or oxidation) of the fully oxidized (or reduced) form. However, using our extinction coefficient, their results give 5 equivalents in stead of 4 equivalents. Five electron equivalents by NADH-PMS system and by ferricyanide (17, 18, 21) seem slightly higher than our results: 4.6 by NADH-PMS system and 4.5 equivalents by O$_2$, respectively (Table II). The small differences may be due to contaminant iron in their non-crystalline preparation. The reported end point value of a reductive titration with dithionite (19), recalculated with our extinction coefficient, gives 5 electron equivalents, which is significantly lower than our value, 6.3 ± 0.27 equivalents. Their titration curve has no initial lag phase. The inconsistency is likely to be due to incomplete occupancy of the bridging peroxide on the O$_2$-reduction site of their enzyme preparation.

PMS provides the fully oxidized form corresponding to the O$_2$-oxidized form. Thus, redox mediators used in potentiometric titrations are also likely to give the O$_2$-oxidized form. All of the redox titrations reported thus far except for the dithionite titration (19) correspond to the present titration between the fully reduced form and the O$_2$-oxidized form. None of the reported titrations shows parallel fractional absorbance changes, in contrast to the present results, indicating weaker interactions among the four metal sites in non-crystallizable enzyme preparations than in crystalline preparation. The weaker interactions could be caused by modification of the intrinsic three-dimensional structure of the enzyme. Crystallization is effective in removing partially denatured protein, if present, from isolated cytochrome c oxidase preparation. Our preparation before crystallization showed a delay in fractional absorbance change in the near infrared region and monophasic reductive titration curves for the absorbance changes in the visible-Soret region, suggesting that contaminant metalloproteins could influence the titration curve of the integral (or crystallizable) enzyme. Thus, redox titration of cytochrome c oxidase in mitochondria or submitochondrial particles could be influenced by coexisting various metalloproteins to provide non-parallel titration curves.

It should be noted that the difference between dithionite and NADH-PMS titrations has never been recognized until this work. After the discovery by Antonini et al. (8) that the fully oxidized form as prepared is not involved in the enzymic turnover, many models for the structure of the form has been proposed. However, none of the proposed models (22–27) is consistent with the peroxide bridge between the two metals in the O$_2$ reduction site.

REFERENCES

1. Ferguson-Miller, S., and Babcock, G. T. (1996) Chem. Rev. 96, 2889–2907
2. Yoshikawa, S., Shizawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Peters Liew, C., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1995) Science 269, 1723–1729
3. Malmström, B. G. (1990) Chem. Rev. 90, 1247–1260
4. Yoshikawa, S., Choc, M. G., O’Toole, M. C., and Caughey, W. S. (1977) J. Biol. Chem. 252, 5498–5508
5. Burleigh, R. D., Jr., Foust, G. P., and Williams, C. H., Jr. (1969) Anal. Biochem. 27, 536–544
6. Washburn, E. W. (ed) (1930) International Critical Tables on Numerical Data, Physics, Chemistry and Technology: National Academy of Science U. S. A., McGraw-Hill, New York
7. Yoshikawa, S., Mochizuki, M., Zhao, X-J., and Caughey, W. S. (1995) J. Biol. Chem. 270, 4270–4279
8. Antonini, E., Brunori, M., Colosimo, A., Greenwood, C., and Wilson, M. T. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3128–3132
9. Vanneste, W. H. (1966) Biochemistry 5, 838–848
10. Okumuki, K., Sekuzu, I., Yonetani, T., and Takemori, S. (1958) J. Biochem. (Tokyo) 45, 847–854
11. Yonetani, T. (1961) J. Biol. Chem. 236, 1680–1688
12. Van Gelder, B. F. (1966) Biochim. Biophys. Acta 118, 36–46
13. Gibson, Q. H., and Greenwood, C. (1963) Biochem. J. 86, 541–555
14. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shizawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Science 269, 1069–1074
15. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–669
16. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shizawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) Science 272, 1136–1144
17. Tiesjema, R. H., Muijsers, A. O., and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 1327–1333
19. Babcock, G. T., Vickery, L. E., and Palmer, G. (1978) J. Biol. Chem. 253, 2400–2411
20. Blair, D. F., Ellis, W. R., Jr., Wang, H., Gray, H. B., and Chen, S. I. (1986) J. Biol. Chem. 261, 11524–11537
21. Steffens, G. C. M., Soulimane, T., Wolff, G., and Buse, G. (1993) Eur. J. Biochem. 213, 1149–1157
22. Landrum, J. T., Reed, C. A., Hatano, K., and Scheidt, W. R. (1978) J. Am. Chem. Soc. 100, 3232–3234
23. Seiter, C. H. A., and Angelos, S. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 77, 1806–1808
24. Dessens, S. E., Merril, C. L., Saxton, R. J., Ilaria, R. L., Jr., Lindsey, J. W., and Wilson, L. J. (1982) J. Am. Chem. Soc. 104, 4357–4361
25. Franceschi, F., Gullotti, M., Monzani, E., Casella, L., and Papaefthymiou, V. (1996) J. Chem. Soc. Chem. Com. 1645–1646
26. Scott, R. A., Schwartz, J. R., and Cramer, S. P. (1986) Biochemistry 25, 5546–5555
27. Powers, L., and Chance, B. (1985) J. Inorg. Biochem. 23, 207–217