Topological Studies of Monomeric and Dimeric Cytochrome c Oxidase and Identification of the Copper A Site Using a Fluorescence Probe*

Joan Hall, Ali Moubarak, Patricia O'Brien, Lian Ping Pan, II Cho, and Francis Millett

From the Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701

Beef heart cytochrome c oxidase was labeled at a single sulfhydryl group by treatment with 5 mM N-iodoacetylaminodithio-1-aminonaphthalene-5-sulfonate (1,5-I-AEDANS) at pH 8.0 for 4 h. Sodium dodecyl sulfate gel electrophoresis revealed that the enzyme was exclusively labeled at subunit III, presumably at Cys-115. The high affinity phase of the electron transfer reaction with horse cytochrome c was not affected by acetylaminodithio-1-aminonaphthalene-5-sulfonate (AEDANS) labeling. Addition of horse cytochrome c to dimeric AEDANS-cytochrome c oxidase resulted in a 55% decrease in the AEDANS fluorescence due to the formation of a 1:1 complex between the two proteins. Förster energy transfer calculations indicated that the distance from the AEDANS label on subunit I to the heme group of cytochrome c was in the range 26–40 Å. In contrast to the results with the dimeric enzyme, the fluorescence of monomeric AEDANS-cytochrome c oxidase was not quenched at all by binding horse heart cytochrome c, indicating that the AEDANS label on subunit III was at least 54 Å from the heme group of cytochrome c. These results support a model in which the lysines surrounding the heme crevice of cytochrome c interact with carboxylates on subunit II of one monomer of the cytochrome c oxidase dimer and the back of the molecule is close to subunit III on the other monomer.

In order to identify the cysteine residues that ligand copper A, a new procedure was developed to specifically remove copper A from cytochrome c oxidase by incubation with 2-mercaptoethanol followed by gel chromatography. Treatment of the copper A-depleted cytochrome c oxidase preparation with 1,5-I-AEDANS resulted in labeling sulfhydryl groups on subunit II as well as on subunit III. No additional subunits were labeled. This result indicates that the copper A binding site is located at cysteines 186 and/or 200 of subunit II and that removal of copper A exposes these residues for labeling by 1,5-I-AEDANS. Alternative copper A depletion methods involving incubation with bathocuproine sulfonate (Weintraub, S. T., and Wharton, D. C. (1981) J. Biol. Chem. 256, 1669–1676) or p-(hydroxymercuri)benzoate (Li, P. M., Gelles, J., Chan, S. I., Sullivan, R. J., and Scott, R. A. (1987) Biochemistry 26, 2091–2095) were also investigated. Treatment of these preparations with 1,5-I-AEDANS resulted in labeling cysteine residues on subunits II and III. However, additional sulfhydryl residues on other subunits were also labeled, preventing a definitive assignment of the location of copper A using these depletion procedures.

Cytochrome c oxidase from mammalian mitochondria is a redox-linked proton pump responsible for the transfer of electrons from ferrocytochrome c to molecular oxygen. Electron diffraction data have indicated that the protein is shaped like the letter Y, with the two arms spanning the inner mitochondrial membrane and extending into the matrix and the base extending into the intermembrane space (1). Cytochrome c oxidase is normally isolated as a dimer of mass 300–400 kDa (2), and this was the form observed in phospholipid membranes (1). However, monomeric cytochrome c oxidase with high electron transfer activity can be prepared by treatment with detergents such as Triton X-100 (2) or lauryl maltoside (3). Cytochrome c oxidase consists of three large subunits synthesized inside the mitochondria and 6–10 smaller subunits synthesized in the cytoplasm. It is generally accepted that each molecule contains two heme groups, cytochrome a and cytochrome a₃, and two copper atoms, Cuₐ and Cuₐ₃, which are redox-active and facilitate the catalytic process. However, Einarssottir and Caughey (4, 5) have studied the metal composition of cytochrome c oxidase using inductively coupled plasma atomic emission spectrometry (ICP-AES)1 and found that the metal stoichiometry of their enzyme preparation was 5 copper:4 iron/dimer of cytochrome c oxidase. More recently, Steffens et al. (6) have carried out ICP-AES studies showing that cytochrome c oxidase has a copper to iron ratio of 3 copper:2 iron. They suggested that Cuₐ₃ the EPR-visible copper, is located in subunit I along with Cuₐ and cytochromes a and a₃. The third copper is assigned to subunit II and described as a second EPR-invisible copper locked in the reduced state in oxidized cytochrome c oxidase. Additional evidence suggesting that the heme groups and copper atoms are located within subunits I and II is discussed in Refs. 7–10. Li et al. (11) have recently reported the specific depletion of Cuₐ from cytochrome c oxidase using p-(hydroxymercuri)benzoate. A comparison of the EXAFS spectra of native and Cuₐ-depleted cytochrome c oxidase indicated that the inner coordination sphere of Cuₐ consists of two (N, O) ligands and two (S, Cl) ligands (11). This is consistent with an earlier study by Martin (12) suggesting that two cysteines ligand to Cuₐ.

The electron transfer reaction between cytochrome c and

---

1 The abbreviations used are: ICP-AES, inductively coupled plasma atomic emission spectroscopy; 1,5-I-AEDANS, N-iodoacetylamidoethyl-1-aminonaphthalene-5-sulfonate; AEDANS, acetylamidoethyl-1-aminonaphthalene-5-sulfonate; AF, aminodithio-1-aminonaphthalene-5-sulfonate; APS, acetamidofluorescein; SDS, sodium dodecyl sulfate; TMPD, tetramethylphenylenediamine dihydrochloride; EXAFS, extended x-ray absorption fine structure; Bicine, N.N-bis(2-hydroxyethyl)glycine.
cytochrome c oxidase involves the formation of a 1:1 complex stabilized by electrostatic interactions. Extensive chemical modification studies have shown that seven or eight highly conserved lysine amino groups surrounding the heme crevice of cytochrome c are involved in the interaction with cytochrome c oxidase (13-16). We have recently used a water-soluble carbodiimide to identify four of the specific carboxylate groups on cytochrome c oxidase that are involved in binding cytochrome c (17). They are all located on subunit II at residues Asp-112, Glu-114, Asp-115, and Glu-198. Glu-198 is located between the conserved cysteines 196 and 200 which have been proposed to serve as ligands to the EPR-visible copper atom, suggesting that CuA is close to the cytochrome c binding site. The major contribution to the high-affinity binding site, therefore, involves the formation of electrostatic charge pairs between lysine amino groups surrounding the heme crevice of cytochrome c and carboxylate groups on subunit II of cytochrome c oxidase. However, yeast cytochrome c modified with thionitrobenzoate at Cys-107 on the back of the molecule was found to covalently cross-link to Cys-115 of bovine and beef cytochrome c oxidase (18, 19). Cross-linking to dimeric cytochrome c oxidase inhibited the reaction with added ferrocytochrome c, but cross-linking to the monomeric enzyme had no effect on activity (20). This led to the suggestion that the high-affinity binding site for cytochrome c is located at a cleft in the interface between monomers in the cytochrome c oxidase dimer. The lysines surrounding the heme crevice of cytochrome c would interact with the carboxylates on subunit II, while the back of cytochrome c would be close to subunit III of the other monomer.

In the present study we have specifically labeled Cys-115 of subunit III with the fluorescent reagent 1,5-I-AEDANS (21). Fluorescence energy transfer experiments indicated that the AEDANS label is close to the cytochrome c binding site on dimeric cytochrome c oxidase, but more than 50 Å away in the monomeric enzyme. The 1,5-I-AEDANS fluorescent reagent was found to label cysteines on both subunit II and III after removal of CuA from cytochrome c oxidase by incubation with 2-mercaptoethanol. This result indicates that the CuA binding site is located at cysteines 196 and/or 200 of subunit II, and removal of CuA exposes these sulfhydryl groups for labeling by 1,5-I-AEDANS.

**EXPERIMENTAL PROCEDURES**

**Materials**—Beef heart cytochrome c oxidase was prepared by the method of Capaldi and Hayashi (22). Enzyme preparations had a heme content of 9-11 nmol/mg protein and contained about 100 μg of phospholipid/mg of protein. Horse heart cytochrome c (Type VI), bathocuproine sulfate, 2-mercaptoethanol, Tris, sodium deoxycholate, and lauryl maltoside were obtained from Sigma and used without further purification. 5-Iodoacetamidofluorescein was obtained from Molecular Probes. 1,5-I-AEDANS was obtained from Sigma.

**Labeling Cytochrome c Oxidase with 1,5-I-AEDANS**—Cytochrome c oxidase (50 μM) was reduced anaerobically with 4 mM dithiothreitol in a buffer composed of 0.1 M Tris-Cl, pH 8.0, and 0.1% sodium deoxycholate, and lauryl maltoside were obtained from Sigma and used without further purification. 5-Iodoacetamidofluorescein was obtained from Molecular Probes. 1,5-I-AEDANS was obtained from Sigma.

**Depletion Cytochrome c Oxidase with 1,5-I-AEDANS**—Cytochrome c oxidase was then concentrated using Amicon CF50A membrane cones. For the CuA depletion studies, 20 μM cytochrome c oxidase was treated with 1,5-I-AEDANS as described above, and after 12 h of incubation at room temperature the solution was chromatographed on a 1 x 50-cm column of Bio-Gel A-0.5 equilibrated with 100 mM sodium phosphate, 0.1% lauryl maltoside, pH 7.4, to remove excess reagent. The appropriate fractions were pooled and concentrated using Amicon CF50A membrane cones.

**Depletion of CuA Using 2-Mercaptoethanol**—Cytochrome c oxidase (20 μM) in 50 mM potassium phosphate, 1% sodium deoxycholate, 10 mM EDTA, pH 7.4, was treated for 15 min in a constant temperature bath at 27°C with 60-180 mM 2-mercaptoethanol. The sample was passed through a 1 x 14-cm Bio-Gel agarose A-0.5 column equilibrated with 100 mM Tris, 0.1% sodium deoxycholate, pH 8.0, to remove copper and excess deoxycholate, and recrystallize the enzyme.

**Depletion of CuA Using Bathocuproine Sulfate**—Cytochrome c oxidase (50 μM) was treated for 5 min with 5 mM bathocuproine and acetic acid (pH 4.7) as described by Weintraub and Wharton (23). The pH was then raised to 8.0 with Tris, and the sample was passed through a 1 x 10-cm Sephadex G-15 column equilibrated with 50 mM sodium phosphate, 0.1% sodium deoxycholate, pH 7.2, to remove bathocuproine and copper. The copper content of the sample was measured using the bathocuproine assay of Weintraub and Wharton (23). The extent of depletion of CuA was estimated from the position of the α peak to cytochrome c oxidase as described by Weintraub and Wharton (23).

**Depletion of CuA Using p-(Hydroxymercuribenzoate)**—Cytochrome c oxidase (30 μM) in 50 mM Tris-Cl, 50 mM NaCl, 0.5% Tween 20, pH 7.7, was treated with 1 mg of p-(hydroxymercuribenzoate) at 20°C for 24 h as described by Li et al. (11). The p-(hydroxymercuribenzoate) crystals were removed by centrifugation. The sample was passed through a Bio-Gel P-2 column equilibrated with 100 mM Tris-Cl, 1 mM EDTA, 0.1% sodium deoxycholate, pH 8.0, treated with 10 mM diethiothreitol under anaerobic conditions at 20°C for 15 min, and then passed through a second Bio-Gel P-2 column equilibrated with the same buffer. The copper content was measured using an Amicon CF50A membrane cone before treatment with 1,5-I-AEDANS.

**Preparation of Monomeric Cytochrome c Oxidase**—AEDANS-cytochrome c oxidase was incubated in 5% Triton X-100, 320 mM Tris-Cl, pH 8.5, for 2 h at 4°C as described by Georgevich et al. (2). The sample was then applied to a 1 x 6-cm DEAE-agarose column and washed with 10 mM Tris-Cl, pH 6.0, 0.1% Triton X-100. The sample was eluted with 200 mM NaCl, 10 mM Tris-Cl, pH 8.5, 0.1% Triton X-100.

**Preparation of 5-AP- Yeast Cytochrome c**—Yeast cytochrome c (13 mg) was dissolved in 1 ml of 50 mM Tris phosphate, pH 8.0, and reduced with 1 ml of 1 M sodium dithiothreitol under anaerobic conditions. 5-Iodoacetamidofluorescein (3 mM) was added, and the mixture was incubated for 12 h at 4°C in the dark. Excess reagent was removed by gel filtration on a 1.5 x 25-cm Bio-Gel P-2 column equilibrated with 20 mM sodium phosphate, pH 7.0. The AF-cytochrome c derivative was found to contain 1.0 ± 0.1 mol of AF/mol of cytochrome c, using extinction coefficients of 77 μM M⁻¹ cm⁻¹ for AF at 496 nm (24) and 106 μM M⁻¹ cm⁻¹ for cytochrome c at 410 nm (25). To verify that the site labeled with AF was the single cysteine at residue 107, a sample of AF-cytochrome c (500 μg) was treated with 25 μg of trypsin in 500 μl of 0.1 M Bicine, pH 8.0, for 3 h. The digest was then separated on a gel and the RP-300 Resolve high pressure liquid chromatography column using a gradient from 5 mM phosphate, pH 7.0 to 500 mM methanol in 2 h. The eluent was simultaneously detected at both 210 and 500 nm. Only a single peptide absorbing at 500 nm eluted from the column and corresponded to the peptide AF-100-108.

**Cytochrome c Oxidase Activity Using an Oxygen Electrode**—The activity of cytochrome c oxidase was measured polarographically with a Gilson model KM Clark electrode cell in 50 mM sodium phosphate, pH 7.4, containing 12 mM ascorbate, 1 mM TMPD, and 0.02% lauryl maltoside. The concentrations of cytochrome c oxidase and cytochrome c used in the assay were 30-50 nM and 0.5-20 nM, respectively. Under these conditions only the high affinity phase of the reaction with cytochrome c was observed (16). The turnover rate of untreated cytochrome c oxidase was typically about 100/s without any type of activation. Cytochrome c oxidase concentrations are expressed in terms of a complex containing two heme a groups using an extinction coefficient of 33.0 μM⁻¹ cm⁻¹ for the 605 - 650-nm absorbance of the reduced protein (26).

**Cytochrome c Oxidase Activity Measured Spectrophotometrically**—The activity of cytochrome c oxidase was measured spectrophotometrically using a Hewlett-Packard 8452A diode array spectrophotometer. Assays were performed by following the oxidation of 20 μM cytochrome c at 550 nm in 50 mM sodium phosphate, 0.1% lauryl maltoside, pH 6.0. The concentration of cytochrome c oxidase was 2-40 nM. The turnover number of untreated cytochrome c oxidase was typically about 500/s.

**SDS-Polyacrylamide Gel Electrophoresis**—Cytochrome c oxidase samples were dissociated for 1 h in 8 M urea, 5% SDS, and 3% 2-mercaptoethanol at room temperature. Slab gels were run as described by Darley-Usmar et al. (27) using a 7% polyacrylamide stacking gel.
and a 14% running gel, both containing 6 M urea. The gel was then illuminated with UV light and photographed to observe the AEDANS fluorescence. The gels were then stained with Coomassie Blue.

Fluorescence Spectroscopy—Fluorescence spectra of AEDANS-cytochrome c oxidase were recorded on a Perkin-Elmer 650-40 instrument in the presence of an excitation wavelength of 340 nm and slit widths of 2 and 5 nm for excitation and emission, respectively. The quantum yield was measured using quinine sulfate in 0.1 M H2SO4 as standard (25). The absorbance of the solutions used in these measurements were lower than 0.05 to avoid inner filter effects, and the quantum yields were corrected for inner filter effects (29). The concentration of AEDANS was determined from the difference spectrum of AEDANS-cytochrome c oxidase relative to the same concentration of unlabeled cytochrome c oxidase using an AEDANS extinction coefficient of 6.1 mM⁻¹ cm⁻³ at 337 nm (21). Both samples were dissociated in 5% SDS to minimize light scattering. The fluorescence polarization of AEDANS-cytochrome c oxidase was measured using a polarization accessory corrected for the differential transmission of vertical and horizontal polarized light through the monochromers. Quenching curves were obtained by measuring the fluorescence of AEDANS-cytochrome c oxidase before and immediately after addition of native horse heart cytochrome c. The small decrease in fluorescence caused by the inner filter effect was corrected using the equation: 

\[ \text{I} = \frac{I_o}{1 + \frac{f}{\alpha}} \]

\[ \alpha = \frac{I_o - I}{I_o} \]

where \( I_o \) is the corrected fluorescence and \( I \) is the absorbance at the excitation and emission wavelengths. These corrections were always less than 10%.

Ultracentrifugation Studies—Sedimentation velocity measurements were carried out on a Beckman model E analytical ultracentrifuge using schlieren optics as described by Robinson and Talbert (30). Samples of monomeric and dimeric AEDANS-cytochrome c oxidase were centrifuged at 40,000 rpm and 20°C in a buffer containing either 100 mM sodium phosphate, pH 7.4, 0.1% Triton X-100, or 20 mM Tris-HCl, pH 8.0, 90 mM NaCl, 0.1 mM EDTA, and 1% Triton X-100. Schlieren photographs were taken at 16-min intervals, and the apparent sedimentation coefficients were calculated as described by Chervenka (31).

EPR Spectroscopy—EPR spectra were recorded at 100 K with a Varian E-4 spectrometer using 200-milliwatt power, 10-G modulation amplitude, and 100-kHz modulation frequency. Inductively Coupled Plasma Atomic Emission Spectroscopy—Measurements of the iron and copper content of the cytochrome c oxidase sample were made using an Analytical Systems Instrumentation Laboratory Plasma-200 ICP-AES spectrophotometer and calibrated against standards prepared from known standard solutions purchased from Sigma.

RESULTS

Specific Labeling of Cytochrome c Oxidase Subunit III by 1,5-I-AEDANS—Treatment of dimeric cytochrome oxidase with 5 mM 1,5-I-AEDANS under anaerobic conditions at pH 8 was found to specifically label subunit III, as shown in Fig. 1A. Very weak fluorescent bands were observed in the gel at the positions of the other sulphydryl-containing subunits, but the sum of these bands was estimated to be less than 5% of the fluorescence of the subunit III band. The AEDANS-cytochrome c oxidase preparation contained 0.9 ± 0.2 mol of AEDANS/mol of cytochrome c oxidase, and the high affinity phase of the reaction with cytochrome c was the same as that of native cytochrome c oxidase (Fig. 2).

Effect of CuA Depletion on the Labeling of Cytochrome c Oxidase by 1,5-I-AEDANS—Three different techniques were used for the removal of CuA from cytochrome c oxidase. In a new procedure, cytochrome c oxidase was treated for 15 min with 114 mM 2-mercaptoethanol in 50 mM potassium phosphate, 1% sodium deoxycholate, 10 mM EDTA, pH 7.4, and then passed through a Bio-Gel A-50 0.5 column to remove excess deoxycholate and copper, and reoxidize the enzyme. The sample was found to be 45% depleted in total copper using the bathocuproine sulfate assay developed by Tsudzuki et al. (32). In addition, spectral shifts of both the Soret and \( \alpha \) bands which have been associated with the removal of CuA (23) were observed. Electron transfer activity was more than 90% inhibited compared to an untreated control which had a turnover of 500 s⁻¹. As shown in Fig. 3, treatment with 2-mercaptoethanol resulted in the disappearance of the CuA EPR signal of cytochrome c oxidase. Approximately 80% of the EPR-copper signal was removed when 114 mM 2-mercaptoethanol was used (Fig. 3C, Table I). The [copper] /[iron] ratio of this sample was found to be 0.6 ± 0.1 by ICP-AES spectroscopy, compared to 1.1 ± 0.1 for an untreated control. Table I shows how incubation with varying concentrations of 2-mercaptoethanol affected the electron transfer activity, CuA, EPR signal, and [copper] /[iron] ratio of cytochrome c oxidase.

The CuA-depleted cytochrome c oxidase was then treated with 4 mM dithiothreitol and 5 mM 1,5-I-AEDANS as described above. SDS gel electrophoresis revealed that both subunits II and III were labeled, as shown in Fig. 1B. The intensity of the AEDANS label on subunit II was approximately two times that of subunit III, indicating that two cysteines surrounding CuA were modified. A control that went through the same treatment but without 2-mercaptoethanol was labeled exclusively at subunit III (same as Fig. 1A).

The Weintraub and Wharton method (23) involving incubation with bathocuproine sulfate and acetic acid at pH 4.7 was also used to remove CuA. This procedure resulted in 70% depletion of CuA and 70% inhibition of electron transfer activity compared to an untreated control. Fig. 1C shows that 1,5-I-AEDANS labeled all the subunits that contain cysteine residues: I, II, III, a, V, c, and VII (Fig. 1C). Samples that were treated with less bathocuproine to give lower levels of CuA depletion were labeled to a proportionally smaller extent, but in the same overall pattern. A control that went through the same acetic acid treatment but without bathocuproine was labeled exclusively at subunit III. It is apparent that the bathocuproine method of copper depletion results in general exposure of all cysteine residues and cannot be used to assign the location of CuA.

The third method of CuA depletion involved incubation with high levels of pHMB as described by Li et al. (11). After chromatographic removal of excess pHMB and CuA, the sample had an electron transport activity of 18% relative to an untreated control. Fig. 1D shows that the major sites labeled by 1,5-I-AEDANS were subunits II and III, but several weak fluorescent bands were also observed at positions corresponding to other cysteine-containing subunits. These additional subunits were not labeled as heavily as in the case of the bathocuproine-treated sample, however.

Fluorescence Energy Transfer Studies—The fluorescence spectrum of AEDANS-cytochrome c oxidase labeled at subunit III was similar to that of other proteins labeled with AEDANS, with an emission maximum at 480 nm and an excitation maximum at 340 nm (Fig. 4). The quantum yield of the AEDANS fluorescence was measured to be 0.34 ± 0.06, and the polarization was 0.21 ± 0.02. There was no change in the fluorescence emission spectrum of AEDANS when cytochromes a and \( \alpha \) were reduced, indicating that the local environment of the AEDANS label was unaffected by the redox state of the protein. When horse heart cytochrome c was added to AEDANS-cytochrome c oxidase in 10 mM sodium phosphate, pH 7.4, the fluorescence intensity decreased in a manner consistent with the formation of a 1:1 complex between the two proteins (Figs. 4 and 6). There were no changes in the wavelength maxima of the excitation and emission spectra of AEDANS-cytochrome c oxidase, indicating that the fluorescence was quenched by energy transfer from the AEDANS label on Cys-115 of subunit III to the heme group in cytochrome c. The theoretical expression for
fluorescence quenching by formation of a 1:1 complex is
\[
1 - F/F_0 = (1 - F_c/F_0)2C_c/(A_c + C_c + K) + \sqrt{(A_c + C_c + K)^2 - 4A_cC_c}
\]

where \(F_c\) is the fluorescence in the absence of cytochrome \(c\), \(F_0\) is the fluorescence of the fully formed complex, \(A_c\) and \(C_c\) are the concentrations of cytochrome oxidase and cytochrome \(c\), respectively, and \(K\) is the dissociation constant (33). This equation was fitted to the fluorescence data using nonlinear regression analysis (34) to give \(K = 0.13\ \mu M\) and \((1 - F_c/F_0) = 0.55\) (Fig. 6). The measured dissociation constant is in good agreement with the Michaelis constant for the high affinity phase of the reaction between cytochrome \(c\) and cytochrome oxidase (Fig. 2), indicating that the two labels did not come close enough to cause any steric or electrostatic repulsion.

AEDANS-cytochrome \(c\) oxidase was subjected to the Triton X-100 treatment of Georgevich et al. (2) to give monomeric cytochrome \(c\) oxidase. This treatment did not remove a significant amount of subunit III and did not affect the kinetics of the high affinity phase of the reaction with cytochrome \(c\). In contrast to the results with the dimeric protein, the fluorescence of AEDANS was not quenched by addition of either horse heart cytochrome \(c\) or AF-yeast cytochrome \(c\) to monomeric AEDANS-cytochrome \(c\) oxidase (Fig. 6). This indicates that Cys-115 of subunit III is considerably farther away from the cytochrome \(c\) binding site in the monomer than in the dimer.

**Ultracentrifugation Studies**—Sedimentation velocity studies of untreated AEDANS-cytochrome \(c\) oxidase indicated that the preparation contained a major component with an apparent sedimentation coefficient of 13–14 in buffer containing 0.1% Triton X-100. No other bands were observed in the schlieren photographs, but an upper limit of 10% heterogeneous material of high molecular weight would not have been detected. The monomeric AEDANS-cytochrome \(c\) oxidase prepared as described above sedimented as a single homogeneous band with an apparent sedimentation coefficient of 8.0 in buffer containing 1% Triton X-100.

**DISCUSSION**

In the present study we have developed conditions to specifically label subunit III of beef cytochrome \(c\) oxidase with...
Fluorescence Studies of Cytochrome c Oxidase

1,5-I-AEDANS. The specific sulphydryl group labeled is presumably Cys-115, since this was the residue labeled by iodoacetamide (27) and thionitrobenzoate-yeast cytochrome c (20, 35). The other sulphydryl residue in subunit III, Cys-218, appears to be deeply buried in the membrane and is not reactive with water-soluble sulphydryl reagents (35). Subunits I, II, a, V, c, and VII also contain cysteine residues, but they were not labeled when highly purified cytochrome oxidase was treated with 1,5-I-AEDANS. Previous reported studies provide circumstantial evidence that CuA is located in subunit II of cytochrome c oxidase. Stevens et al. (9) have used electron nuclear double resonance to show that CuA is liganded by at least one cysteine and one histidine residue. The only cysteine residues conserved in subunits from human, beef, mouse, Drosophila, yeast, maize, and Neurospora cytochrome c oxidase are Cys-196 and Cys-200 of subunit II (36). It has been noted that the subunit II sequence including Cys-196, Cys-200, and His-204 could form a tight loop similar to the one involving the copper-binding residues in azurin and plastocyanin (37). There have been two difficulties associated with assigning cysteines 196 and 200 as CuA ligands. First, Powers et al. (38) have proposed on the basis of extended EXAFS experiments that a sulphydryl group bridges the cytochrome a2 iron and CuA in resting cytochrome c oxidase. Cysteines 196 and 200 could not ligand both CuA and CuB. Second, Mann and Auer (39) have found that mercuric compounds specifically inhibit the activity of cytochrome c oxidase without affecting the CuA EPR spectrum. Following SDS gel electrophoresis, mercury was bound exclusively to subunit II. This second difficulty was cleared up by our discovery that mercury compounds specifically displace zinc from its binding site on one of the smaller subunits, possibly VIa (40). Mercury compounds are very labile under the conditions of SDS electrophoresis, however, and migrate to cysteines 196 and 200 of subunit II following dissociation (10).

Our present fluorescent probe study clearly shows that CuA is located in subunit II of cytochrome c oxidase. Exposure of sulphydryl residues following depletion of CuA with 2-mercaptoethanol allowed specific labeling to occur using the fluores-

![Fig. 2. Effect of 1,5-I-AEDANS labeling on the high affinity phase of the reaction between cytochrome c oxidase and cytochrome c. The velocity of v (in μM cytochrome c reduced per s per μM cytochrome c oxidase) was measured polarigraphically in 50 mM sodium phosphate, pH 7.4, containing 0.02% lauryl maltoside, 10 mM ascorbate, 1 mM TMPD, and 1-12 μM cytochrome c. The following cytochromes were assayed: native cytochrome c oxidase and horse heart cytochrome c ( ); AEDANS-cytochrome c oxidase and horse cytochrome c ( ); AEDANS-cytochrome c oxidase and AF-yeast cytochrome c ( ).](image)

![Fig. 3. Effect of 2-mercaptoethanol incubation on the CuA signal of cytochrome c oxidase. Samples of cytochrome c oxidase were incubated as described in Table I with the following concentrations of 2-mercaptoethanol: A, 0 mM; B, 60 mM; C, 114 mM; D, 180 mM. The samples were then passed through a Bio-Gel A-0.5 column equilibrated with 100 mM Tris-Cl, pH 8.0, 0.1% deoxycholate to remove copper and excess reagent, and allow the enzyme to be reoxidized. EPR spectra were recorded at 100 K with a Varian E-4 spectrometer using 200-milliwatt power, 10-G modulation amplitude, and 100-kHz modulation frequency.](image)

| [2-Mercaptoethanol] | Kinetic activity | CuA EPR signal | [Copper]:[Iron] ratio |
|---------------------|----------------|----------------|---------------------|
| mM                  | % of control  | % of control  |                     |
| 0                   | 100           | 100            | 1.1 ± 0.1           |
| 60                  | 57            | 72             | 0.9 ± 0.1           |
| 114                 | 8             | 18             | 0.6 ± 0.1           |
| 180                 | 4             | 5              | 0.4 ± 0.1           |

TABLE I

Effect of 2-mercaptoethanol incubation on the electron transfer activity, CuA EPR signal, and [copper]:[iron] ratio of cytochrome c oxidase

Cytochrome c oxidase (20 μM) was treated with 2-mercaptoethanol for 15 min at 27 °C in 50 mM potassium phosphate, 10 mM EDTA, 1% sodium deoxycholate, pH 7.4. The sample was then passed through a Bio-Gel A-0.5 column equilibrated with 100 mM Tris, 0.1% sodium deoxycholate, pH 8.0, to remove copper and excess reagent, and subjected to analysis. The kinetic activity of the control was 500 s⁻¹, and the [copper]:[iron] ratio was determined by ICP-AES.

FIG. 2. Effect of 1,5-I-AEDANS labeling on the high affinity phase of the reaction between cytochrome c oxidase and cytochrome c. The velocity of v (in μM cytochrome c reduced per s per μM cytochrome c oxidase) was measured polarigraphically in 50 mM sodium phosphate, pH 7.4, containing 0.02% lauryl maltoside, 10 mM ascorbate, 1 mM TMPD, and 1-12 μM cytochrome c. The following cytochromes were assayed: native cytochrome c oxidase and horse heart cytochrome c ( ); AEDANS-cytochrome c oxidase and horse cytochrome c ( ); AEDANS-cytochrome c oxidase and AF-yeast cytochrome c ( ).
Fluorescence Studies of Cytochrome c Oxidase

Fluorescence Studies of Cytochrome c Oxidase

FIG. 4. Effect of horse heart cytochrome c binding on the fluorescence of dimeric AEDANS-cytochrome c oxidase. The fluorescence emission spectrum of 1 μM AEDANS-cytochrome c oxidase in 10 mM sodium phosphate, pH 7.4, containing 0.1% cholate was recorded at 25 °C in the presence of 0.0, 0.6, 1.1, 1.7, 2.2, and 2.8 μM horse heart cytochrome c. The excitation wavelength was 340 nm, and the excitation and emission slit widths were 2 and 5 nm, respectively.

FIG. 5. Effect of yeast AF-cytochrome c binding on the fluorescence of dimeric AEDANS-cytochrome c oxidase. The fluorescence emission spectrum of 1 μM AEDANS-cytochrome c oxidase in 10 mM sodium phosphate, pH 7.4, containing 0.1% cholate was recorded at 25 °C in the presence of 0.6, 1.0, 1.7, 2.0, and 2.3 μM AF-cytochrome c. The excitation wavelength was 360 nm.

Fluorescence energy transfer techniques have been widely used for studying the topology of large protein complexes. One of the main requirements is to specifically label a single residue on the complex with a fluorescent probe. This has been a difficult undertaking for cytochrome c oxidase, and relatively few specific labels have been reported. Dockter et al. (41) labeled one or more cysteines on subunit II of yeast cytochrome c oxidase with 1,5-I-AEDANS and studied energy transfer from the AEDANS label to the heme a groups within the protein and to the heme group of bound cytochrome c. Müller and Azzi (42) have reported the selective labeling of Cys-115 of subunit III of beef cytochrome c oxidase and eosin 5-maleimide but did not carry out energy transfer studies.

The most significant result of the present study is that binding horse heart cytochrome c quenched the fluorescence of the AEDANS label on dimeric cytochrome c oxidase but did not affect the fluorescence of the monomeric protein. The fluorescence quenching was most likely caused by Förster energy transfer to the heme and fluorescein groups on cytochrome c, since the excitation and emission curves for AEDANS fluorescence were not affected by binding. Hudson and Weber (21) have shown that environmental effects that change the quantum yield of AEDANS also cause a significant change in the wavelength for the maximum in the emission spectrum. The Förster energy transfer efficiency $E = 1 - F_o/F$ is inversely proportional to the sixth power of the distance $R$ between the donor and acceptor groups according to the following relation (43):

$$E = R_o^6/(R^6 + R_o^6)$$  \hspace{1cm} (2)

$R_o$ is defined as the distance at which the energy transfer efficiency is 50% and is given by

$$R_o = (Jx^2Q_o)^{-1/6} \times 9.7 \times 10^3$$  \hspace{1cm} (3)

where $x^2$ is the orientation factor, $Q_o$ is the fluorescence quantum yield of the energy donor in the absence of acceptor, $n$ is the refractive index of the medium between the donor and acceptor, and $J$ is the spectral overlap integral.

$$J = \int F_o \lambda^2 d\lambda / \int F_\lambda d\lambda$$  \hspace{1cm} (4)

$F_o$ is the fluorescence of the donor in arbitrary units, the wavelength $\lambda$ is in centimeters, and $\epsilon_\lambda$ is the extinction coef-
Fluorescence Studies of Cytochrome c Oxidase

The greatest source of uncertainty in $R_0$ is the orientation factor, which may in principle range from 0 to 4. However, if the donor and acceptor chromophores have polarizations less than the maximum of 0.5 due to local rotational diffusion or mixed transition dipole moments, then the uncertainty in $R_0$ is reduced considerably (44, 45). The polarization of the AEDANS group in cytochrome $c$ oxidase (0.21) indicates a considerable amount of local rotational diffusion. The overall rotation of the cytochrome $c$ oxidase molecule is much too slow relative to the fluorescence lifetime (16 ns) to affect the polarization. The procedure of Haas et al. (44) was used to calculate that $R_0$ will be within the extreme limits of 28–42 Å for energy transfer from AEDANS with $p = 0.21$ to the doubly degenerate heme group of cytochrome $c$ with $p = 0.14$. For energy transfer from AEDANS to the fluorescein group on yeast AF-cytochrome $c$ with $p = 0.19$, $R_0$ will be within the limits 32–51 Å. The total energy transfer to AF-cytochrome $c$ will be given by the formula, $(1 - E_{total}) = (1 - E_0)(1 - E_{AF})$, where $E_0$ is the energy transfer to the heme group and $E_{AF}$ is the energy transfer to the fluorescein group. It will be assumed that $E_H$ is the same for yeast AF-cytochrome $c$ as it is for horse heart cytochrome $c$, and $E_{AF}$ will then be obtained from $E_{total}$ using the above formula.

Since the fluorescence of monomeric AEDANS-cytochrome $c$ oxidase was not quenched by either horse heart or yeast AF-cytochrome $c$, the AEDANS label must be greater than 54 Å from the heme group of cytochrome $c$ and greater than 62 Å from the fluorescein group on Cys-107, using the minimum values of $R_0$ and the limits of 2% in the quenching measurements. Subunit III is, therefore, a considerable distance away from the cytochrome $c$ binding site on the same monomeric unit of the enzyme. The ultracentrifuge studies indicated that monomeric AEDANS-cytochrome $c$ oxidase was homogeneous and had a sedimentation coefficient similar to that of native monomeric cytochrome $c$ oxidase (2, 31).

The dimeric AEDANS-cytochrome $c$ oxidase preparation was found to contain a major dimeric component with an apparent sedimentation coefficient of 13–14 S, which is similar to that of native dimeric cytochrome $c$ oxidase (2). Although no additional components were apparent in the schlieren photographs, it was estimated that an upper limit of 10% heterogeneous aggregated material would not have been detected. Robinson and Talbert (30) found that it was not possible to isolate homogeneous dimeric cytochrome $c$ oxidase, and the preparations always contained either highly aggregated material or monomers in addition to the dimers. The effect of aggregated AEDANS-cytochrome $c$ oxidase would be to decrease the extent of quenching observed, since cytochrome $c$ would not be accessible for binding. Taking this uncertainty into account, it was estimated that the $(1 - F_s/F_0)$ value is 0.55–0.65 for quenching by horse cytochrome $c$ and 0.93–1.0 for quenching by yeast AF-cytochrome $c$.

Equation 2 was used to calculate that the distance from the AEDANS label to the heme group of cytochrome $c$ is 25–41 Å, and the distance to the fluorescein label 0–33 Å (Table II). Since the heme group is 18 Å from the back surface of cytochrome $c$, the heme measurement would place the AEDANS label 7–23 Å beyond the back surface of cytochrome $c$, which is consistent with the fluorescein measurement.

The present experiments support a model in which the lysines on the front side of cytochrome $c$ interact with carboxylate groups on subunit II of one monomer of dimeric cytochrome $c$ oxidase, and the back of the molecule is close to subunit III of the other monomer in the dimer. There is no evidence, however, that there is any direct contact between the back of cytochrome $c$ and subunit III, or any favorable contribution to the high affinity binding site. On the contrary, the bulky negatively charged fluorescein group at Cys-107 did not affect the high affinity reaction of cytochrome $c$ with either native cytochrome $c$ oxidase or AEDANS-cytochrome $c$ oxidase. In the latter case the negative charges on both labels would have repelled one another if they were closer than about 10 Å. This is in agreement with previous studies showing that modification of lysines on the back of cytochrome $c$ had a negligible effect on the reaction with dimeric cytochrome $c$ oxidase (13, 14). In addition, Muijser et al. (46) have recently found that a yeast cytochrome $c$ dimer cross-linked “back to back” at Cys-107 had nearly the same $K_a$ with dimeric oxidase as monomeric cytochrome $c$, suggesting that the putative cleft is not very deep. The cross-linked yeast cytochrome $c$-cytochrome $c$ oxidase complex prepared by Moreland and Dockter (18) and Fuller et al. (19) had only 10–15% activity in the TMPD-ascorbate assay, so the cytochrome $c$ might have been pulled somewhat out of the high affinity binding site when the cross-link was formed.

Acknowledgments—We would like to thank Susan Holt and Dr. Wayne Sabbe for help with the ICP-AES experiments, Allan Kline for his help in the ultracentrifuge studies, Dr. Kim Colvert for his help in the ultracentrifuge studies, and Jacqueline Stonehuerner for assistance in the copper depletion studies.

REFERENCES

1. Henderson, R., Capaldi, R. A., and Leigh, J. S. (1977) J. Mol. Biol. 112, 631–648
2. Georgievich, G., Darley-Ussar, V. M., Malatesta, F., and Capaldi, R. A. (1983) Biochemistry 22, 1317–1322
3. Bolli, R., Nalcz, K., and Azzi, A. (1985) Arch. Biochem. Biophys. 240, 102–116
4. Einarsdottir, O., and Caughey, W. S. (1984) Biochem. Biophys. Res. Commun. 124, 836–842
5. Einarsdottir, O., and Caughey, W. S. (1985) Biochem. Biophys. Res. Commun. 125, 840–847
6. Steffen, G. C. M., Berwald, R., and Buse, G. (1987) Eur. J. Biochem. 164, 295–300
7. Winter, D. B., Bruyninckx, W. J., Foukle, F. G., Grinch, N. P., and Mason, H. S. (1980) J. Biol. Chem. 255, 11406–11414
8. Ludwig, B. (1980) Biochim. Biophys. Acta 589, 177–189
9. Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., and Chan, S. I. (1982) J. Biol. Chem. 257, 12106–12113
10. Stonehuerner, J., O’Brien, P. Kendrick, L., Hall, J., and Millett, F. (1986) J. Biol. Chem. 261, 11456–11460
11. Li, P. M., Gelles, J., Chen, S. J., Sullivan, R. J., and Scott, R. A. (1987) Biochemistry 26, 3091–3095
12. Martin, C. T. (1965) The Structures of CuA and Cytochrome a in Cytochrome c Oxidase. Ph.D. thesis, California Institute of Technology
13. Smith, H. T., Staudenmayer, N., and Millett, F. (1977) Biochemistry 16, 4971–4975
14. Ferguson-Miller, S., Bultjant, D. L., and Margoliash, E. (1978) J. Biol. Chem. 253, 149–159

### Table II

| Parameter | AEDANS to heme $c$ | AEDANS to fluorescein |
|-----------|---------------------|-----------------------|
| $Q$       | 0.34                | 0.34                  |
| $p$ (donor) | 0.21               | 0.21                  |
| $p$ (acceptor) | 0.14       | 0.19                  |
| $J$       | $5.3 \times 10^{-14}$ | $14.9 \times 10^{-14}$ |
| $n_{c}$   | 0.25                | 0.26                  |
| $R_0$ ($k = 2/3$) | 43 Å             | 43 Å                  |
| $R_0$ (minimum–maximum) | 28–42 Å  | 32–51 Å              |
| $E$       | 0.55–0.65           | 0.84–1.0              |
| $R$       | 25–41 Å             | 0–33 Å                |
Fluorescence Studies of Cytochrome c Oxidase

15. Rieder, R., and Bosshard, H. R. (1980) J. Biol. Chem. 255, 4732–4739
16. Smith, H. T., Ahmed, A. J., and Millett, F. (1981) J. Biol. Chem. 256, 4984–4990
17. Millett, F., de Jong, C., Paulson, L., and Capaldi, R. A. (1983) Biochemistry 22, 546–552
18. Moreland, R. B., and Dockter, M. E. (1981) Biochem. Biophys. Res. Commun. 99, 333–346
19. Fuller, S. D., Darley-Usmar, V. M., and Capaldi, R. A. (1981) Biochemistry 20, 7046–7053
20. Darley-Usmar, V. M., Georgevich, G., and Capaldi, R. A. (1984) FEBS Lett. 166, 131–135
21. Hudson, E. N., and Weber, G. (1973) Biochemistry 12, 4154–4161
22. Capaldi, R. A., and Hayashi, H. (1972) FEBS Lett. 26, 261–263
23. Weintraub, S. T., and Wharton, D. C. (1981) J. Biol. Chem. 256, 1669–1676
24. Takashi, R. (1979) Biochemistry 18, 5164–5169
25. Margoliash, E., and Frohwirt, M. (1959) Biochem. J. 71, 570–572
26. Briggs, M. M., and Capaldi, R. A. (1977) Biochemistry 16, 73–77
27. Darley-Usmar, V. M., Capaldi, R. A., and Wilson, M. T. (1981) Biochem. Biophys. Res. Commun. 103, 1223–1230
28. Scott, T. G., Spencer, R. D., Leonard, N. J., and Weber, G. (1970) J. Am. Chem. Soc. 92, 687–693
29. Rao, A., Martin, P., Reithmeier, R. A. F., and Cantley, C. C. (1979) Biochemistry 18, 4505–4516
30. Robinson, N. C., and Talbert, L. (1986) Biochemistry 25, 2328–2335
31. Chervenka, C. H. (1973) A Manual of Methods for the Analytical Ultracentrifuge, Beckman Instruments, Inc., Fullerton, CA
32. Teodori, T., Ori, Y., and Okunuki, K. (1967) J. Biochem. (Tokyo) 62, 37–45
33. Geren, L. M., and Millett, F. (1981) J. Biol. Chem. 256, 10485–10490
34. Duggleby, R. G. (1981) Anal. Biochem. 110, 9–18
35. Malatesta, F., and Capaldi, R. A. (1982) Biochem. Biophys. Res. Commun. 109, 1180–1185
36. Macino, G., and Morelli, G. (1983) J. Biol. Chem. 258, 13230–13235
37. Lundeen, M. (1983) J. Inorg. Biochem. 18, 1–9
38. Powers, L., Chance, B., Ching, Y., and Angiolillo, P. (1981) Biophys. J. 34, 465–495
39. Mann, A. J., and Auer, H. E. (1986) J. Biol. Chem. 255, 454–458
40. Moubarak, A., Pan, L. P., and Millett, F. (1987) Biochem. Biophys. Res. Commun. 143, 1090–1096
41. Dockter, M. E., Steinemann, A., and Schatz, G. (1978) J. Biol. Chem. 253, 311–318
42. Muller, M., and Azzi, A. (1985) FEBS Lett. 184, 110–114
43. Stryer, L. (1979) Annu. Rev. Biochem. 47, 819–846
44. Haas, E., Ephrussi-Katchalski-Katzir, and Steinberg, I. Z. (1978) Biochemistry 17, 5064–5070
45. Dale, R. E., Eisenger, J., and Blumberg, W. E. (1979) Biophys. J. 26, 161–194
46. Muijsers, A. O., Roelofs, Y., Sinjorgo, C. M. C., and Haukoort, T. B. M. (1985) 13th International Congress on Biochemistry, Amsterdam, Abstr. Tu-513