Exposure of Bovine Cytochrome c Oxidase to High Triton X-100 or to Alkaline Conditions Causes a Dramatic Change in the Rate of Reduction of Compound F*

Robert C. Sadoski‡, Dmitry Zaslavsky§, Robert B. Gennis§, Bill Durham‡, and Francis Millett‡†

From the ‡Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701 and the §School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

The final step in the catalytic cycle of cytochrome oxidase, the reduction of oxyferryl heme a₃ in compound F, was investigated using a binuclear polypyridine ruthenium complex ([Ru(bipyridine)₂]₁₄PF₆)₄ as a photosensitive reducing agent. In the untreated dimeric enzyme, the rate constant for reduction of compound F decreased from 700 s⁻¹ to 200 s⁻¹ as the pH was increased from 7.5 to 9.5. Incubation of dimeric enzyme at pH 10 led to an increase in the rate constant to 1650 s⁻¹, which was independent of pH between pH 7.4 and 10. This treatment resulted in a decrease in the sedimentation coefficient consistent with the irreversible conversion of the enzyme to a monomeric form. Similar results were obtained when the enzyme was incubated with Triton X-100 at pH 8.0. These treatments, which have traditionally been used to convert dimeric enzyme to monomeric form, have no effect on the steady-state activity. The data indicate that either the conversion of the bovine oxidase to a monomeric form or some structural change coincident with this conversion strongly influences the rate constant of this step in the catalytic cycle, perhaps by influencing the proton access to the heme-copper binuclear center.

Cytochrome c oxidase (CcO) is a redox-linked proton pump, which uses electrons from cytochrome c to reduce molecular oxygen to water (1, 2). The reaction begins with reduction of CuA by cytochrome c, followed by electron transfer from Cuₐ to heme a, and then to the binuclear heme a₃-Cu₃ center. Molecular oxygen binds to the two-electron reduced binuclear center and is reduced to form compound P (3). In successive one-electron reactions, compound P is reduced to compound F and then to the oxidized form of the enzyme, O, containing ferric heme a₃ (4). The availability of a method to prepare compound F reduction, indicating that no measurable amount of reduced Cuₐ accumulates in this process.

Cytochrome oxidases isolated from different organisms have different quaternary structures. For example, bovine cytochrome oxidase is normally isolated as a dimer, whereas shark CeO is a monomer (10–12). The functional significance of the quaternary structure has been studied extensively, but remains unclear (10–14). The major functions of bovine cytochrome oxidase, including dioxygen reduction and proton pumping, are retained when the dimeric enzyme is converted to monomeric form (12, 13). In the present study, it has been discovered that the kinetics of compound F reduction are strongly influenced by treatments of the enzyme that traditionally have been used to convert the bovine enzyme to the monomeric form. Two different methods were used to prepare monomeric bovine cytochrome oxidase, incubation with Triton X-100 at pH 8.0 (11) or pH 8.5 (10), and incubation with lauryl maltoside at pH 10.0 (15). When dimeric bovine cytochrome oxidase is incubated with Triton X-100 (10, 11), a fast phase of compound F reduction with a rate constant of 20,000 s⁻¹ in the bovine enzyme (9). Heme a then reduces the oxyferryl heme a₃ in compound F in a biphasic reaction with a minor fast phase with a rate constant of 1500 s⁻¹ and a major slow phase with a rate constant of 470 s⁻¹ at pH 8.0 (9). The slow phase is pH-dependent, decreasing to 220 s⁻¹ at pH 9.5. There is no apparent delay between heme a oxidation and compound F reduction, indicating that no measurable amount of reduced Cuₐ accumulates in this process.

EXPERIMENTAL PROCEDURES

Materials—Bovine CeO was purified by the method of Capaldi and Hayashi (16) and had a heme content of 9–11 nmol/mg of protein and a turnover number of 400 s⁻¹. In some experiments, crystalline bovine CeO prepared as described in Ref. 17 was used, and was a generous gift from Dr. Yoshikawa. There was no significant difference in the kinetics of compound F reduction in the two CeO preparations. CeO was equilibrated with the indicated buffers and detergents using Amicon-100 concentrators prior to kinetic experiments. Lauryl maltoside was ob-

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† To whom correspondence should be addressed. Fax: 501-575-4999; E-mail: millett@uark.edu.

‡ The abbreviations used are: CCO, cytochrome c oxidase; Ru₃C, [Ru(bipyridine)₂]₁₄PF₆; 3-CP, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy radical.
tained from Calbiochem, and Triton X-100 and Tris-HCl were obtained from Sigma. Ru,C was a generous gift from Russell H. Schmehl (Department of Chemistry, Tulane University, New Orleans, LA), prepared as described by Baba et al. (18).

Preparation of Monomeric Bovine Cytochrome c Oxidase—Monomeric bovine cytochrome c oxidase was prepared as described by Georgevich et al. (10). 25 µM CcO was incubated in 320 mM Tris-Cl (pH 8.5) containing 5% Triton X-100 for 2 h at 4 °C. The sample was then diluted 10-fold and applied to a 0.5 × 5-cm DEAE-Sepharose column that was equilibrated with 10 mM Tris-Cl (pH 8.5) containing 0.1% Triton X-100. After washing with 10 mM Tris-Cl (pH 8.5) containing 0.1% Triton X-100, the sample was eluted with 10 mM Tris-Cl containing 0.1% Triton X-100 and 200 mM NaCl. Monomeric CcO was also prepared using Triton X-100 at pH 8.0 as described by Robinson et al. (11).

Preparation of Oxysperyl Cytochrome c Oxidase—The oxyferyl (F) state was prepared as described previously by incubating oxidized bovine CcO for 5 min with 4 mM hydrogen peroxide (9). The ferryl state was prepared as described previously by incubating oxidized bovine CcO in 5 mM Tris-Cl (pH 8.0), 10 mM aniline, 1 mM 3-CP, and 0.1% lauryl maltoside in the appropriate buffer, and determined using an extinction coefficient of 1.5 × 10^5 M^-1 cm^-1 and relative amplitude of 82%, and a minor phase with rate constant (1.5 ± 0.3) × 10^5 s^-1 and relative amplitude 18%.

Results

Electron Transfer between Heme a and Oxyferyl Heme a3 in Bovine Cytochrome c Oxidase in Lauryl Maltoside—The electron transfer reaction between heme a and the binuclear center was studied by rapidly injecting a single electron from the photoexcited ruthenium dimer, Ru2C, into CcO, as previously described by Zaslavsky et al. (9). The rate constant for electron transfer between CuA and heme a in bovine CcO in the resting O state was (2.0 ± 0.5) × 10^4 s^-1, monitored at 605 nm and 830 nm (9). No further electron transfer from heme a to the binuclear center was observed in the resting O state. The rate constant for electron transfer from CuA to heme a was also (2.0 ± 0.5) × 10^4 s^-1 in the F state of the enzyme. This was followed by reoxidation of heme a, monitored at 605 nm, and reduction of oxyferyl heme a3, monitored at 580 nm (Fig. 1). The transients at both wavelengths were biphasic for bovine CcO in 5 mM Tris-Cl (pH 8) and 0.1% lauryl maltoside. The major slow phase has a rate constant of 470 s^-1 and relative amplitude of 82%, whereas the minor fast phase has a rate constant of 1500 s^-1 and relative amplitude of 18%. Using the extinction coefficients given in Refs. 3 and 21, the reoxidation of heme a and reduction of heme a3 were equimolar.

The reaction between heme a and oxyferyl heme a3 is pH-dependent (9). As the pH increases from pH 7.0 to pH 9.5, the rate constant of the major slow phase decreases from 590 s^-1 to 220 s^-1, whereas the rate constant of the minor fast phase does not change significantly (Table I, Fig. 2). However, when the enzyme is incubated for 2 h at pH 10.0 prior to formation of compound F, a major change in the kinetics is observed (Fig. 3, Table I). Electron transfer from heme a to oxyferyl heme a3 now consists of a major fast phase with a rate constant of 1650 s^-1 and a minor slow phase with a rate constant of 210 s^-1 and a relative amplitude of 20%. When the pH of this sample is decreased to pH 7.4, the kinetics remain essentially the same, with a major fast phase with a rate constant of 1650 s^-1 and a minor slow phase with a rate constant of 400 s^-1 (Table I, Fig. 2). It thus appears that the pH 10.0 incubation led to an essentially irreversible change in the kinetics of the enzyme. Since incubation at pH 10.0 has been reported to lead to conversion of dimeric CcO to monomeric CcO (15), it is possible that this change in quaternary structure, or structural changes resulting from this treatment of the enzyme, causes the change...
Incubation at pH 8.0 (Table I). This is consistent with the report by Robinson et al. at this low Triton X-100 concentration. Raising the Triton X-100 concentration in the fast phase had a rate constant of 440 s\(^{-1}\) with 0.1% Triton X-100, with a rate constant of 450 s\(^{-1}\) at pH 8.5.

Electron Transfer in Cytochrome Oxidase Compound F

Electron Transfer between Heme a and Oxyferryl Heme a\(_3\) in Bovine Cytochrome c Oxidase in Triton X-100—The kinetics were further investigated in different concentrations of Triton X-100, which has also been shown to affect the quaternary state of bovine CcO (10, 11). Electron transfer from heme a to oxyferryl heme a\(_3\) was monophasic for bovine CcO equilibrated with 0.1% Triton X-100, with a rate constant of 450 s\(^{-1}\) at pH 8.0 (Table I). This is consistent with the report by Robinson et al. (11) that bovine CcO consists of dimers and higher aggregates at this low Triton X-100 concentration. Raising the Triton X-100 concentration to 1% resulted in biphasic electron transfer from heme a to oxyferryl heme a\(_3\) (Table I). The fast phase had a rate constant of 1600 s\(^{-1}\) and amplitude of 50%, and the slow phase had a rate constant of 440 s\(^{-1}\) and amplitude of 50%. Monomeric CcO was prepared by incubating the enzyme with 5% Triton X-100 at pH 8.5 and then exchanging the sample into 0.1% Triton X-100 as described by Georgievich et al. (10). Essentially the same results were obtained for a preparation incubated with 5% Triton X-100 at pH 8.0, as described by Robinson et al. (11). Electron transfer from heme a to oxyferryl heme a\(_3\) in compound F at pH 8.0 consisted of a major fast phase with a rate constant of 2400 s\(^{-1}\) and relative amplitude of 89%, and a minor slow phase with a rate constant of 280 s\(^{-1}\) (Table I). The rate constant of the major fast phase was independent of pH from pH 8.0 to pH 10.0 (Fig. 2). The rate constant for electron transfer from CcO to heme a was (2.4 ± 0.5) \times 10^4 s\(^{-1}\) for the Triton X-100-treated preparation, which is the same as that previously in the presence of 0.1% lauryl maltoside (9). There was also no change in the equilibrium electron distribution between CcO and heme a as a result of the treatment with Triton X-100. Furthermore, the steady-state cytochrome c oxidase activity was not affected by the 5% Triton X-100 treatment, when the assay was carried out in buffer containing 0.1% lauryl maltoside.

The reaction between heme a and oxyferryl heme a\(_3\) in CcO incubated with Triton X-100 was also studied in 96% D\(_2\)O with a pH meter reading of 7.6, which is equivalent to pD 8.0 (23). The 605- and 580-nm transients each consist of a major phase with a rate constant of 300 s\(^{-1}\). This rate constant is also independent of pH from pH 8.0 to pH 9.5 (Fig. 4). The major phase of electron transfer from heme a to oxyferryl heme a\(_3\) in the Triton-treated enzyme, thus, has a very substantial deuterium isotope effect of 8.0.

Ultradeuteron Studies of Bovine Cytochrome c Oxidase—The quaternary structure of the cytochrome c oxidase preparations was studied using sedimentation velocity ultracentrifugation. The apparent sedimentation coefficient of untreated bovine CcO in 5 mM Tris-Cl (pH 8.0) containing 0.1% lauryl maltoside was 11.7 S. The sedimentation coefficient of the CcO preparation incubated for 2 h in 0.1% lauryl maltoside at pH 10.0 in 5 mM Tris-Cl was 9.5 S, whereas that of the preparation treated with 5% Triton X-100 was 9.0 S. The sedimentation coefficient of 11.7 S for the untreated CcO is consistent with previous studies of the dimeric protein (10). Robinson et al. (11) measured a sedimentation coefficient of 8.7 S for monomeric CcO treated with Triton X-100, which is consistent with the value of 9.0 S obtained in the present study.

**DISCUSSION**

The reduction of compound F is an important step in the mechanism of dioxygen reduction by cytochrome c oxidase and is coupled to proton pumping (1, 2). The single-electron reduction of compound F by photoexcited Ru\(_2\)C has been shown previously to be biphasic for bovine CcO in 0.1% lauryl maltoside at pH 8.0, with a major slow phase with a rate constant of 470 s\(^{-1}\), and a minor fast phase with a rate constant of 1500 s\(^{-1}\) (9). These results are comparable to those observed previously using time-resolved spectroscopy (7), or an electrogenic method (24, 25). The minor fast phase was observed in all three studies, but its origin was not known. The present studies demonstrate that at least one contributing factor for the observed biphasic kinetics of compound F reduction is heterogeneity of the state of aggregation of cytochrome oxidase. In the current work, CcO in 0.1% lauryl maltoside has a sedimentation coefficient of 11.7 S, which is comparable to that of previous dimeric preparations (10). This is inconsistent with the report of Suarez et al. (12) that bovine CcO is monomeric in lauryl maltoside, but it is important to note that a partially delipidated preparation of enzyme was used in this previous work. Significantly, the bovine CcO is dimeric in single crystals obtained from decyl maltoside solutions (17). Hence, it seems likely that the bovine...
CcO preparation in lauryl maltoside used in the current work is mostly in the dimeric form, and this correlates with the observed biphasic kinetics of reduction of compound F, with a dominant (>80%) slow phase.

The reduction of compound F occurs in a single slow phase with a rate constant of 450 s\(^{-1}\) for the bovine enzyme in 0.1% Triton X-100, which has been shown to consist of dimers and higher aggregates (11). Raising the Triton X-100 concentration to 1% resulted in biphasic kinetics with equal amplitudes for the fast and slow phases. This is consistent with ultracentrifuge studies showing approximately equal concentrations of monomers and dimers at this Triton X-100 concentration (11).

Incubation with 5% Triton X-100 at pH 8.0 or 8.5 has been shown to lead to nearly complete formation of monomeric CcO (10, 11). The sedimentation coefficient of 9.0 obtained for this preparation in the current work is comparable to the value of 8.7 reported previously for monomeric bovine CcO (11). The relative amplitude of the slow phase of compound F reduction is only 12% in this preparation of the enzyme. The rate constant for the fast phase of compound F reduction in this preparation, 2400 s\(^{-1}\), is somewhat larger than observed at lower concentrations of Triton X-100 or lauryl maltoside. It is known that there is some disruption of subunit-subunit interactions in CcO by high concentrations of Triton X-100. Georgevich et al. (10) reported partial loss of subunit III, as well as several small subunits in the preparation treated with 5% Triton X-100 at pH 8.5. However, we have not observed significant loss of subunit III with this procedure (26). No loss of subunits occurs in the preparation of Robinson and Talbert (11), which involves incubation with 5% Triton X-100 at pH 8.0. It should be noted that the steady-state cytochrome c oxidase activity is very low when measured in the presence of Triton X-100, but can be restored by diluting cytochrome oxidase into buffers containing other detergents such as lauryl maltoside or Tween 80 (10, 11). The steady-state activity of the preparation treated with 5% Triton X-100 was found to be the same as untreated dimeric cytochrome oxidase, in agreement with previous studies (10, 11). Furthermore, the rate constant and equilibrium constant for electron transfer from Cu\(_{a}\) to heme \(a\) is not affected by the 5% Triton X-100 treatment. These results indicate that the Triton X-100 does not significantly affect the structural integrity of the enzyme.
X-100 treatment does not cause a general denaturation of the enzyme.

Similar to the results obtained with high Triton X-100, incubation of the dimeric CeO preparation at pH 10 in 0.1% lauryl maltoside led to a major fast phase of compound F reduction with a rate constant of 1650 s⁻¹. Incubation at pH 10.0 has previously been reported to lead to conversion of dimer CeO to monomeric CeO (15). The sedimentation coefficient of 9.5 for this preparation in the current work is consistent with substantial conversion to the monomeric enzyme. The effect of the pH treatment was irreversible, since decreasing the pH back down to 7.4 did not restore a major slow phase of the kinetics.

The modest pH dependence of the rate constant of the slow phase of compound F reduction in 0.1% lauryl maltoside was previously fit to an ionization with a pKₐ of 9.0, with limiting rate constants of 525 and 120 s⁻¹ at low and high pH, respectively (9). This is comparable to results obtained in experiments involving flash photolysis of CO-bound reduced CeO in the presence of oxygen (27, 28). It was proposed that the pH dependence observed in the flow-flash experiments is due to a group with a pKₐ of 9, which is involved in proton delivery to the binuclear center (28). However, the current work shows that the pH dependence is completely absent in preparations involving conversion to monomeric CeO. Note that the monomeric oxidase preparations used in the current work have been shown previously to be active in proton pumping (13), and have the same steady state and Cu₆ to heme a electron transfer activity as dimeric cytochrome oxidase. It seems unlikely that these treatments of the enzyme would affect a critical group directly involved in proton transfer without resulting in impairment of function. It is more likely that the pH dependence of the rate of reduction of compound F that is observed with the untreated dimeric CeO is due to the deprotonation of one or more residues which indirectly affect the rate of proton transfer. This indirect conformational pH effect is apparently imposed by structural considerations that are eliminated by treatments that convert the enzyme to the monomeric form. Whether it is the monomeric species per se that is responsible or an unrelated structural perturbation caused by the treatment of the enzyme cannot be ascertained. However, the fact that two very different protocols that have traditionally been used to yield monomeric enzyme each have the same effect on the kinetics of reduction of compound F suggest that monomerization is important.

It is perhaps significant that the rate constant for the reduction of compound F is faster in monomeric cytochrome oxidase than in the dimeric enzyme. Possibly, the quaternary structure may regulate the rate of reduction of oxyferryl heme, and this regulation is removed in the monomeric enzyme. The fast phase of compound F reduction has a large deuterium isotope effect of 8.0 in the Triton X-100 preparation, indicating that electron transfer remains strongly coupled to proton transfer during the reduction of compound F. The rate of delivery of protons to the binuclear center during this step in the mechanism might be influenced by conformational changes determined by the subunit arrangement. This raises the possibility that the enzyme might be allosterically regulated in vivo by alterations in protein conformation induced by post-translational modifications (e.g., phosphorylation) or by interactions with other proteins. The state of aggregation of cytochrome oxidase in the mitochondrial membrane is not known definitively (8), nor is it known whether the state of aggregation is biologically regulated. In any event, it is clear that the rate of electron transfer within the enzyme is strictly coupled to proton transfer and, therefore, is subject to any factor that might control the pK values of critical proton donors or acceptors or to the connectivity of the proton-conducting pathway within the enzyme.

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