Cyanide Stimulated Dissociation of Chloride from the Catalytic Center of Oxidized Cytochrome *c* Oxidase†

Marian Fabian,* Ludovit Skultety,‡ Christian Brunel, and Graham Palmer

Department of Biochemistry and Cell Biology-MS 140, Rice University, 6100 Main St., Houston, Texas 77005-1892

Received January 9, 2001; Revised Manuscript Received March 13, 2001

ABSTRACT: A comparison of bovine cytochrome *c* oxidase isolated in the presence and the absence of chloride salts reveals that only enzyme isolated in the presence of chloride salts is a mixture of a complex of oxidized enzyme with chloride (CcO·Cl) and chloride-free enzyme (CcO). Using a spectrophotometric method for chloride determination, it was shown that CcO·Cl contains one chloride ion that is released into the medium by a single turnover or by cyanide binding. Chloride is bound slowly within the heme a_3-Cu_B binuclear center of oxidized enzyme in a manner similar to the binding of azide. The pH dependence of the dissociation constant for the formation of the CcO·Cl complex reveals that chloride binding proceeds with the uptake of one proton. With both forms of the enzyme the dependence of the rate of reaction for cyanide binding upon cyanide concentration asymptotes a limiting value indicating the existence of an intermediate. With CcO·Cl this limiting rate is 10^3 higher than the rate of the spontaneous dissociation of chloride from the binuclear center and we propose that the initial step is the coordination of cyanide to Cu_B and in this intermediate state the rate of dissociation of chloride is substantially enhanced.

The respiratory heme-copper oxidases constitute a superfamily of terminal oxidases in both prokaryotic organisms and the mitochondria of eukaryotic cells (*1*−*3*). The most widely studied enzyme is the cytochrome *c* oxidase of mammalian mitochondria. This multisubunit enzyme (*4*, *5*) catalyzes the reduction of molecular oxygen to water using the reducing equivalents supplied by ferrocytochrome *c*. The free energy made available by this reaction is used to generate a protonmotive force across the mitochondrial inner membrane.

The oxidase contains four redox centers: Cu_A, heme *a*, and the binuclear heme a_3-Cu_B center. Cu_A and cytochrome *a* serve to store electrons delivered by cytochrome *c*; these electrons are then transferred to the binuclear center where dioxygen is reduced to water and most external ligands are bound.

In oxidized enzyme heme a_3 and Cu_B are 4.8 Å apart (*6*−*8*) and are antiferromagnetically coupled (*9*). On the basis of EXAFS and ENDOR studies (*10*) and the continuous electron density between heme a_3 and Cu_B it has been suggested that water plus hydroxide (*11*) are bridging ligands between the two metal ions in oxidized enzyme. A competing explanation invokes peroxide as the bridging ligand (*12*).

Previous EXAFS measurements on bovine oxidase have indicated the presence of a heavy atom (S/Cl) bridging heme a_3 and Cu_B (*13*−*19*). It was proposed that this ligand should be chloride rather than S since there are not any cysteine or methionine residues that could conceivably provide a sulfur atom (*6*−*8*) and since no acid-labile sulfur has been detected (*14*, *18*). That chloride can be bound to the oxidized enzyme can be inferred from spectral changes and from the reactivity of the chloride complex with other external ligands (*20*−*27*). It was suggested that chloride is released from the binuclear center when the enzyme is subjected to a cycle of reduction and reoxidation (*14*, *27*).

A recent EXAFS study on the related bacterial heme-copper bo_3 oxidase showed that chloride could be bound at the Cu_B site in the complex of fully reduced oxidase with carbon monoxide (*28*). Moreover, a second chloride ion, not located in the catalytic center of the bo_3 enzyme, and not tightly bound, seemed essential for intramolecular electron transfer (*29*). It appears that chloride can be bound to both the oxidized and the reduced binuclear center and this raises the possibility that it might participate as a cofactor in the catalytic mechanism.

In view of the possible catalytic relevance of chloride, we have undertaken a more thorough characterization of the interaction of chloride with the oxidized bovine enzyme. Here we provide data that extend the previous observations by specifying the stoichiometry, reactivity, and the kinetic characteristics of the process. The surprisingly rapid kinetics of cyanide binding to both chloride-ligated oxidase and to unligated enzyme at basic pH prompted us to compare the ligand exchange reactions of these two forms. We present new kinetic evidence that suggests that heme a_3 is accessible to the external ligand after transient coordination to Cu_B possibly with both chloride and cyanide bound simultaneously at the binuclear center. We suggest that in this intermediate the dissociation of chloride is substantially enhanced relative to the rate of spontaneous dissociation. We also conclude that chloride is not directly involved in catalysis because its rate of binding to oxidized chloride-free enzyme is a very slow process.

† Supported by the National Institutes of Health (GM 55807) and the Robert A. Welch Foundation (C-636).

* To whom correspondence should be addressed. E-mail: fabian@bioc.rice.edu. Phone: (713) 348-2373. Fax: (713) 348-5365.

‡ Permanent address: Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic.

10.1021/bi010059y CCC: $20.00
Published on Web 04/26/2001

MATERIALS AND METHODS

Cytochrome *c*, catalase, ascorbic acid sodium salt, Tris,[1] Hepes, EPPS, reduced 3-acetylpyridine adenine dinucleotide (APADH), and phenazine methosulfate (PMS) were purchased from Sigma. Hydrogen peroxide, MES, and AMPSO were purchased from Aldrich, MOPS was from Fluka, iron perchlorate hydrate, mercury (II) perchlorate hydrate, and perchloric acid were obtained from Aldrich, NaCN was from Mallinckrodt, Bio-Gel P6 was from Bio-Rad, and Triton X-100 (peroxide-free) was from Boehringer. Water was purified by passage through a Milli-Q system.

Bovine cytochrome *c* oxidase was isolated using the detergent Triton X 100 by the method of Soulimane and Buse (*30*) slightly modified such that in the solubilization of mitochondria we have used protein concentration of 20 and 10 mg/mL to extract bc_1 and CcO, respectively. The isolated enzyme was stored in liquid nitrogen at a concentration of 250 μM (in 10 mM Tris-HCl buffer, pH 7.6 containing 0.1% TX 100) and used without detergent exchange. Dodecyl maltoside was used for the bulk of the experiments; however, because of the large volumes of buffer needed the DM was replaced with TX 100 for the quantitative Cl determinations.

The isolation procedure (*30*) was utilized to prepare enzyme in two ways. In the first, the enzyme was prepared in the presence of chloride salts as was originally published. However, during the chromatography on the Sepharose Q column, we used a discrete NaCl concentrations first washing the bound oxidase by buffer (10 mM Tris-HCl, pH 7.6, 0.1% TX 100) then with three column volumes of buffer plus 110 mM NaCl and finally eluting oxidase with 300 mM NaCl in the same buffer. Enzyme isolated in this way is a mixture of the complex of oxidized oxidase with chloride (CcO·Cl) and chloride-free enzyme (CcO).

To obtain chloride-free enzyme, all chloride salts used during the preparation were replaced by K_2SO_4 and Sepharose-bound enzyme was washed with this modified buffer, as before, then with this buffer containing 70 mM K_2SO_4 and finally eluted with 240 mM K_2SO_4 in the same buffer.

Before analysis for chloride content, the enzyme from each preparation was desalted either by gel filtration on a Bio-Gel P 6 (Bio-Rad) column at 4 °C equilibrated with 10 mM Hepes, pH 7.4, 0.1% TX 100 or by multiple filtrations of the enzyme solution with chloride free buffer using Centriflo ultrafiltration membrane cones (Amicon). When using gel filtration, a catalytic amount of catalase was first passed through the P6 column.

Enzyme concentration was determined at pH 8.0 from the Soret absorbance of oxidized oxidase at 424 nm using $A = 158\ mM^{-1}\ cm^{-1}$. For the oxidase isolated at the presence of chloride salts the fractions of CcO and CcO·Cl in the purified oxidase were routinely determined from the reaction kinetics of oxidized oxidase with 2 mM hydrogen peroxide at pH 8.0 by optical spectroscopy. The concentration of CcO was calculated from the absolute spectrum at the end of the fast phase *minus* that of oxidized enzyme using $\Delta A_{438-414} = 65\ mM^{-1}\ cm^{-1}$ (*31*). The fraction of CcO·Cl in the same sample was then deduced from the known concentration of total enzyme. Up to 70% of the oxidase isolated in the presence of chloride salts can be in the CcO·Cl form.

Displacement of chloride from CcO·Cl was achieved either by a cycle of reduction and reoxidation or by cyanide binding to oxidized enzyme. The reoxidation procedure was performed in two ways. For the single turnover procedure, the enzyme was fully reduced under anaerobic conditions by a slight excess of APADH in the presence of 10 μM PMS and then reoxidized by air. For multiple turnovers, the reduction was performed aerobically with 10 mM ascorbate in the presence of 1 μM cytochrome *c*. For the displacement of Cl^- from CcO·Cl by cyanide 20 mM cyanide was used. In each of these experiments, the sample size was 2.5 mL of approximately 40 μM cytochrome oxidase (in 10 mM Hepes, pH 7.4 containing 0.1% Triton X-100).

To determine whether-or-not the binding of carbon monoxide to fully reduced enzyme could influence chloride displacement, we have also prepared reoxidized enzyme from the carbon monoxide complex of fully reduced oxidase. The progress of both redox turnover and of cyanide binding was followed optically at appropriate wavelengths.

The measurement of chloride concentration in solution was based on the colorimetric estimation of SCN^- following its displacement from $Hg(SCN)_2$ by chloride anion (*32*) using the absorption difference of ΔA (464−550 nm). A calibration curve up to 900 μM sodium chloride was nonlinear but highly reproducible. Typically, 0.5 mL portions of a chloride containing sample and the colorimetric reagent were mixed together and incubated at room temperature for 10 min. Then the absorbance difference at 464−550 nm of the sample measured against a reference prepared with deionized water as a chloride free blank (*32*). All samples analyzed had a chloride concentration in the range 70−300 μM.

Halide ions are known to interfere with this method (*32*, *33*) and we have also observed that dithionite, cyanide, thiocyanide, ferrocyanide, azide, and denatured enzyme also interfered with this analysis. It was therefore necessary to remove the denatured protein from the reaction medium by filtration using Centricon 10 centrifugal filters (Amicon). The excess of cyanide present following the ligand exchange reaction was removed by acidification of sample to about pH 1.0 by HNO_3, heating to 70 °C and evaporating under a slow stream of nitrogen.

Because there are several tightly bound chloride anions per oxidase molecule (*28*), it is only possible to determine the total amount of chloride bound in the binuclear center as the relative change in concentration following elimination of chloride from the center. We have thus performed two measurements on each sample: (i) the total chloride present in the sample (Cl_T), that includes both chloride in the buffer medium (Cl_M) and chloride bound to the enzyme (Cl_E), and (ii) a chloride measurement of the buffer medium (Cl_M) obtained by filtration of the sample using Centricon 10 centrifugal filters. To determine the total chloride present approximately 40 μM enzyme was denatured by incubation

[1] Abbreviations: CcO, cytochrome oxidase isolated in the presence of K_2SO_4; CcO·Cl, complex of oxidized cytochrome *c* oxidase with chloride; CcO/Cl, cytochrome *c* oxidase isolated in the presence of chloride salts (NaCl and KCl) that is a mixture of CcO and CcO·Cl; MV·CO, mixed-valence carbon monoxide complex (a^{3+} Cu_A^{2+} a_3^{2+} CO Cu_B^{+}); FR·CO, complex of fully reduced oxidase with carbon monoxide; Hepes, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; Tris, Tris [hydroxymethyl] aminomethane; APADH, 3-acetylpyridine adenine dinucleotide, reduced form; PMS, phenazine methosulfate; DM, *n*-dodecyl-β-D-maltoside; MES, 4-morpholineethanesulfonic acid; MOPS, 3-morpholino-propanesulfonic acid; EPPS, 9*N*-[2-hydroxyethyl]-piperazine-*N*′-[3-propanesulfonic acid]; AMPSO, 3-[1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid.

Table 1: Stoichiometry of Chloride Strongly Bound to Enzyme

	moles of chloride bound to oxidase		
sample	untreated	reoxidized	CN bound
CcO	3.3 ± 0.2	3.2 ± 0.2	3.3 ± 0.3
CcO·Cl	4.4 ± 0.3	3.3 ± 0.3	3.5 ± 0.3

Table 2: Relative Change in Chloride Content Induced by Redox Cycling or by Cyanide Ligation

	relative change[a] in chloride content induced by	
sample	reoxidation	CN ligation
CcO	−0.1	0.0
CcO medium	+0.08	+0.03
CcO·Cl	−1.1	−0.9
CcO·Cl medium	+1.0	+0.9

[a] Positive values represent an increase, negative values a decrease.

at 100 °C for 15 min in the presence of 30 mM KOH (*28*). The medium was isolated using the Centricon 10 filters and quantified, and the result was equated with Cl_T. The chloride tightly bound to the enzyme was then calculated as the difference $Cl_T - Cl_M$. These two determinations were made for enzyme samples before and after the reoxidation or cyanide binding.

The results of the chloride determinations are presented as number of moles of chloride bound per mole of enzyme. The calculation of this ratio for CcO is straightforward because the whole population is homogeneous with respect to chloride content. In the case of oxidase isolated at the presence of chloride salts, the product is a mixture of about 30% CcO and 70% CcO·Cl. To calculate the amount of chloride per CcO·Cl complex, we had to first subtract the contribution of the chloride contained within the 30% of the CcO subpopulation from the total chloride determined for the mixture of CcO and CcO·Cl. Thus, all number of chloride content presented are calculated per CcO or per CcO·Cl complex (Table 1 and 2).

The concentration of hydrogen peroxide was determined from the absorbance at 240 nm using a molar absorbance of 40 M^{-1} cm^{-1} (*34*). Optical spectra were recorded on IBM 9430 spectrophotometer interfaced to a microcomputer. The slow kinetics of ligand binding were measured on a Hewlett-Packard 8452A diode array spectrophotometer. The stopped-flow method (Kinetic Instrument apparatus with 2 cm optical path) was used to determine the rate constants of cyanide binding to CcO and CcO·Cl and for the determination of the reaction rate of CcO and a rate in the fast phase of reaction of oxidase isolated in the presence of chloride salts (CcO/Cl) with 2 mM H_2O_2. All measurements were performed at 23 °C. EPR spectra were obtained using Varian E-line spectrometer operating at 12 K. Data were analyzed and graphs were prepared using Igor Pro (Wavemetrics, Lake Oswego, OR).

To determine the kinetics of chloride binding to CcO at different pH values the enzyme was incubated at 23 °C with 0.5 M KCl at pH 6.5, 7.0, and 8.0. The amount of CcO ligated by chloride was determined in the reaction with hydrogen peroxide. The stock enzyme (CcO), sufficient for about 10 samples, was incubated at the desired pH with KCl. Subsequently, 2 mL portions of incubation mixture were taken at selected times and the kinetics of reaction with 2 mM H_2O_2 measured. The extent of the fast phase in this reaction, corresponding to unligated CcO, was plotted versus the incubation time (see Figure 2A).

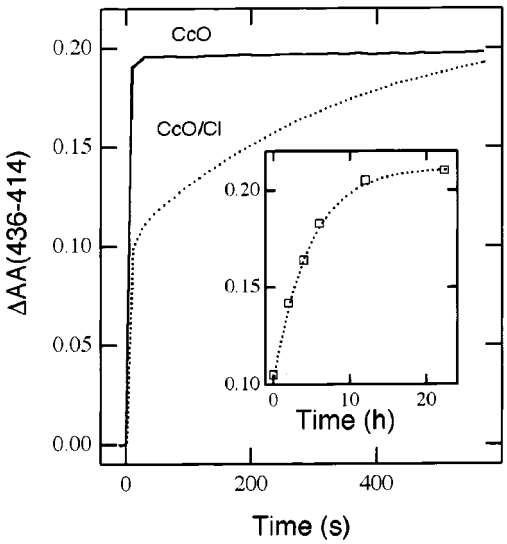


FIGURE 1: Kinetics of hydrogen peroxide interaction with oxidase prepared at the absence (CcO) and the presence of chloride salts (CcO/Cl). The reaction was initiated by the addition of 2 mM hydrogen peroxide to 3.6 μM CcO (−) or to CcO/Cl (···) and the kinetics followed using the absorption change in the Soret band at 436 *minus* 414 nm. The buffer was 100 mM EPPS buffer, pH 8.0, containing 0.1% DM. (Inset) Dependence of the amplitude of the fast phase of reaction of CcO/Cl on the time elapsed after desalting on a P6 column. (□) data; (···) monoexponential fit.

RESULTS

Heterogeneity of the Binuclear Center. It was recently shown that the reaction of nitric oxide (NO) with bovine oxidized oxidase isolated in the presence of chloride salts (CcO/Cl) is not homogeneous and contains at least two populations of enzyme. One population is resistant to reaction with NO while the second population reacts rapidly with NO (*27*).

The existence of two populations of oxidized enzyme, isolated by the same procedure (*27*) as employed in the study with NO, can also be demonstrated in the reactions of the enzyme with hydrogen peroxide or carbon monoxide (CO). Thus, oxidase isolated in the presence of chloride salts reacts with hydrogen peroxide in a biphasic manner with a fast phase that is complete within 10 s at pH 8.0 and which accounts for 30−50% of the total absorbance change. The remainder of the reaction proceeds approximately 1000 times slower; the rate constant for this slow phase is 2.4×10^{-3} s^{-1} (Figure 1). However, oxidized CcO isolated by the same procedure but using reagents in which chloride has been replaced by potassium sulfate reacts with 2 mM hydrogen peroxide in a single fast phase (Figure 1).

A similar difference in reactivity between chloride-containing and chloride-free CcO can be demonstrated using carbon monoxide. Under anaerobic conditions carbon monoxide converts chloride-free CcO to the mixed-valence CO complex (MV·CO) in a single phase with a yield of about 95% in 5 min at pH 8.0. However, the interaction of CO with CcO/Cl is biphasic. In the first 5 min, the yield of MV·CO is about 40%. The rest of the enzyme reacts slowly with CO and over the next 90 min of anaerobic incubation in the dark an additional 30% of MV·CO is formed.

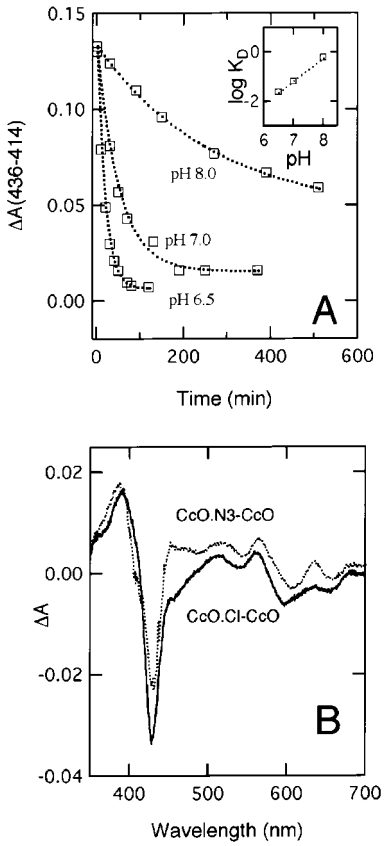


FIGURE 2: pH dependence of chloride binding kinetics to CcO and a comparison of the changes in optical spectrum of CcO induced by chloride and azide binding. (A) The kinetics of chloride binding to CcO at pH 6.5, 7.0, and 8.0. The reaction was initiated by the addition of 0.5 M KCl to 2.2 μM of CcO in 100 mM MOPS (pH 6.5 and pH 7.0) or 100 mM EPPS (pH 8.0) each with 0.1% DM. The decrease of CcO during the reaction was monitored by using 2 mM hydrogen peroxide to quantify the fast phase of reaction using the wavelength pair $\Delta A(436-414)$ (see Materials and Methods). The extent of the fast phase, corresponding to the amount of CcO remaining, is plotted vs time of incubation in the chloride medium. (□), experimental values; (•••) single-exponential fits. (Inset) A plot of log K_d vs pH for CcO•Cl. (□) Experimental values; (•••) linear fit. (B) The difference spectra of CcO•Cl and CcO•N_3 *minus* CcO at pH 7.0. Azide: 8.5 μM CcO was reacted with 40 μM azide and the spectrum referenced against untreated CcO. Chloride: 3.9 μM CcO was incubated with 0.5 M KCl for 90 min. and the spectrum referenced against that recorded immediately after addition of chloride. The buffer was 100 mM MOPS, pH 7.0 containing 0.1% DM; 23 °C.

In support of our contention that the fast and slow phases observed in the reaction of CcO and CcO/Cl with hydrogen peroxide or CO reflect the presence of enzyme either lacking or containing chloride within the binuclear cavity, we have the following observations. (i) The rate constant for the fast phase of the reaction of 2 mM hydrogen peroxide with CcO/Cl is essentially the same as that observed with CcO, namely 0.88 and 0.84 s^{-1}, respectively. (ii) The biphasic interaction of peroxide with CcO/Cl can be eliminated by desalting the enzyme and incubating it in chloride free buffer. Immediately after desalting CcO/Cl on a P6 column the reaction with hydrogen peroxide is still biphasic and identical to the reaction before gel filtration. However, with time, the amplitude of the fast phase increases with a corresponding decrease in the amplitude of the slow phase (Figure 1, inset). This growth of the fast phase is very slow and has a half-time of about 4 h (pH 8.0, 23 °C) corresponding to a first-order rate constant of 6×10^{-5} s^{-1}; we suppose that this change in reactivity reflects the slow dissociation of chloride from the enzyme into the chloride-free buffer. The conversion of CcO/Cl to the homogeneous, rapidly reacting enzyme can also be observed after a cycle of reduction and reoxidation in chloride-free buffer. The kinetics of reaction of this "pulsed" enzyme with hydrogen peroxide is identical to that shown for CcO in Figure 1. A similar activation was previously demonstrated in the reaction with NO (*27*). (iii) Preincubation of CcO that was isolated in sulfate buffers with chloride converts the fast monophasic reaction with peroxide to the biphasic reaction that is observed with CcO/Cl. For example, incubation of CcO with either 0.5 M NaCl or KCl for 12 h at 4 °C and pH 8.0 results in a reaction with peroxide essentially indistinguishable from that observed with CcO/Cl (Figure 1). The fast phase can be reduced even further, and the complementary slow phase increased, by preincubating the enzyme with 0.5 M KCl at pH 6.5 for 90 min. After incubation the extent of the hydrogen peroxide induced fast phase is reduced to 7−10% when measured at either pH 6.1 or 8.0.

The incubation of CcO at pH 6.5 even in the presence of Cl salts could convert oxidase to the "slow" form of enzyme (*20*, *35*−*37*). However, the EPR spectra of Cl derivative (66 μM CcO•Cl) recorded at pH 6.1 and pH 8.0 with 120 mM KCl present in the medium (not shown) were very similar to that of control enzyme. The $g = 12$ signal, characteristic for the "slow" form (*20*, *35*−*37*), is absent and the only differences were a small high-spin heme signal at $g = 6$ suggesting the reduction of a small amount of Cu_B, the presence of a small $g = 4$ feature due to non-heme iron and some slight changes in the line shape and position of the $g = 3$ signal of cytochrome *a*; these changes were previously documented (*38*). The signal at $g = 4$ originates from non-heme iron and if this was the result of the demetalation of heme then less than 3% of enzyme would have been denatured otherwise there would be obvious changes in the visible spectrum. This upper limit of 3% is based on the exaggerated assumption that Cl binding does not change the optical spectrum of oxidase, which is of course not true (*23*, *25*), and that the observed decrease in the Soret band during the incubation with Cl salts is exclusively the result of enzyme denaturation.

Kinetics and Stoichiometry of Chloride Binding. The chloride analysis for both CcO and CcO•Cl populations are summarized in Table 1 and shows that the enzyme contains from 3 to 4 tightly bound chloride ions as determined colorimetrically. No significant difference was found even after extensive dialysis of CcO against chloride-free buffer (10 mM Hepes, pH 7.4, plus 0.05% Triton X 100). However after a two day dialysis of CcO•Cl at pH 7.4 and 4 °C a slight decrease in chloride content was observed; this we explain as being due to the slow dissociation of chloride from the catalytic binuclear center. Subjecting CcO to reduction-reoxidation or to reaction with cyanide does not change the chloride content of CcO nor is any chloride found in the medium (Table 2). However, both of these two treatments cause the loss of approximately one chloride from CcO•Cl (Table 1) and the chloride that is released could be detected in the reaction medium (Table 2).

Both the dissociation constant and the rate of binding of chloride to CcO is pH dependent (Figure 2A). To determine

the rate of association of chloride with bovine CcO, we followed the time dependence of the decrease in the extent of the fast phase of reaction with hydrogen peroxide following addition of 0.5 M KCl to CcO. The time dependence of the decrease in amplitude was exponential and had values of 9.2×10^{-4}, 3.3×10^{-4}, and 6×10^{-5} s^{-1} at pH values of 6.5, 7.0, and 8.0. From the relative extent of the fast and slow phases, we can calculate the K_d for chloride binding; the values are 24, 67, and about 600 mM at pH 6.5, pH 7.0, and pH 8.0, respectively. A plot of log K_d versus pH is linear with a slope of 0.93 (Figure 2A, inset) indicating that binding of one chloride to the catalytic center is linked to the uptake of one proton. The pH dependence of chloride binding to the pulsed *bo* oxidase from *Escherichia coli* is consistent with this conclusion (*25*).

The presence of chloride in the binuclear center can also be demonstrated by optical spectroscopy (Figure 2B). Thus the spectrum of CcO recorded 90 min after addition of chloride *minus* that of CcO recorded immediately after addition of chloride shows a slight blue shift of 2 nm from 424 to 422 nm and a small decrease in the intensity of the Soret band (Figure 2B). The difference spectrum is very similar to that induced by low concentration of azide (Figure 2B). The chloride induced difference spectrum has a minimum at 430 nm and a maximum at 392 nm in the Soret band with visible peaks at 513, 562, 635, and 680 nm and trough at 598 and 655 nm. The spectral changes caused by 0.5 M KCl at pH 7.0 (Figure 2B) are also observed at pH 6.5 and pH 8.0. Moreover the kinetics of the spectral changes at pH 7.0 coincide with the kinetics of Cl binding determined by hydrogen peroxide (Figure 2A).

Ligand Exchange Reactions. Quite different behavior is found in the reaction of CcO and CcO·Cl with cyanide. The binding of 10 mM cyanide to either CcO or CcO·Cl at pH 8.0 can be fit by single exponential with rate constants of 0.018 s^{-1} and 0.027 s^{-1}, respectively (Figure 3A). This small difference in rate constants is undoubtedly the reason kinetic heterogeneity is not routinely detected in the reaction of CcO/Cl with cyanide even though two forms of the enzyme are present.

Because the absolute spectrum of CcO·Cl is slightly blue-shifted with respect to that of control enzyme, the addition of cyanide actually leads to absorbance changes in the Soret some 8% larger than that observed with CcO (Figure 3A). This is undoubtedly a consequence of the combination of chloride displacement and the spin-state change of cytochrome a_3 induced by cyanide binding. Both processes lead to red-shifts of the Soret band and are summed in the overall spectral shift observed with chloride-treated enzyme.

It is known (*39*−*41*) that the rate constant for the reaction of cyanide with oxidized enzyme asymptotes a limiting value indicating a two step mechanism consisting of a rapid preequilibrium followed by a first-order isomerization. To establish which of the kinetic parameters of this process (k_{max} or K_d) are affected by chloride binding we have determined the saturation behavior for both enzyme forms at pH 8.0 (Figure 3B). For CcO we obtained $k_{max} = 0.18$ s^{-1} and $K_d = 140$ mM; the values for CcO·Cl are $k_{max} = 0.083$ s^{-1} and $K_d = 21$ mM.

It has been pointed out that the saturation behavior of the cyanide reaction might be caused by the presence of a small amount of one-electron reduced enzyme (*42*). For example

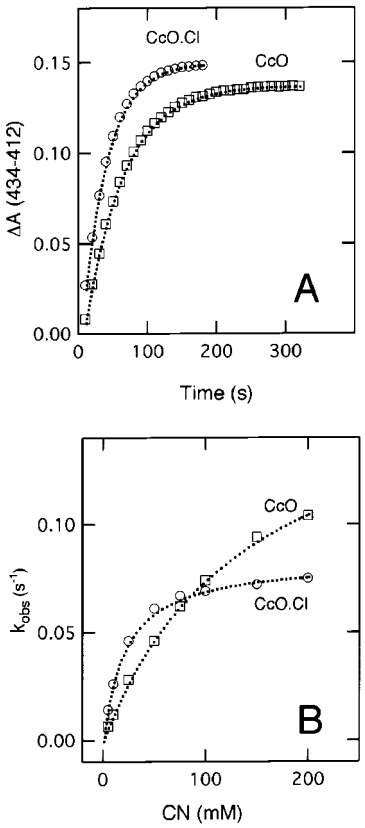


FIGURE 3: Comparison of cyanide binding kinetics to CcO and CcO·Cl at pH 8.0. (A) Kinetics of reaction of 10 mM cyanide with 2.3 μM CcO (□) or CcO·Cl (○) measured as the change in the Soret band absorption. Points, experimental data. (···) single-exponential fits. (B) The dependence of k_{obs} derived from the single-exponential fits of reaction kinetics on cyanide concentration. Conditions: 3.6 μM CcO or CcO·Cl. The buffer was 100 mM EPPS pH 8.0 containing 0.1% DM. The ionic strength of 0.3 was maintained using K_2SO_4. The dotted lines are fits to the equation: $k_{obs} = k_{max}[HCN]/(K_d + [HCN])$.

in a published experiment, the rate constant for the binding of 0.2 mM cyanide to the enzyme preincubated with ferricyanide was almost one-half that of untreated enzyme (*42*). However, this is not the case in the present experiments because enzyme preincubated with 20 mM ferricyanide at room temperature and pH 8 for 20 min had the same rate for cyanide binding as did untreated enzyme.

DISCUSSION

One test for the quality of preparations of cytochrome oxidase is the kinetic pattern observed upon reaction of the enzyme with cyanide and the enzyme is commonly divided into two classes. The "fast" form of cytochrome oxidase reacts monoexponentially with cyanide in a relatively rapid manner while the "slow" form reacts 1000-fold less readily; preparations that contain significant contributions from both "fast" and "slow" forms exist. The basis for this difference in behavior is thought to be due to variations in the binuclear center but the origin of these variations remains unknown.

The enzyme preparation isolated by the original method of Sulimane and Buse (*30*) has a catalytic activity similar to that observed in mitochondria; the kinetics of reaction with cyanide indicates that the enzyme is in a "fast" form and it would thus appear that the active center is structurally homogeneous. However, when enzyme isolated by this

procedure was reacted with nitric oxide, it was observed that the kinetics contained several phases (*27*). As nitric oxide is known to react with the binuclear center this observation implied that there was an unsuspected additional inhomogeneity at this center. In agreement with this observation, we find that both hydrogen peroxide and carbon monoxide react with the original Soulimane-Buse enzyme heterogeneously (Figure 1). However, enzyme prepared by the same protocol but modified so that potassium sulfate replaced chloride in all reagents reacts homogeneously with hydrogen peroxide and the rate of this reaction is the same as the most rapid phase observed with the original, unmodified preparation. The preparation of homogeneous chloride-free enzyme can also be accomplished by prolonged incubation of desalted enzyme at pH 8.0. At this pH, the dissociation constant for chloride is about 600 mM (Figure 2A, inset) and we assume that chloride slowly dissociates from the binuclear center during this incubation.

It should be pointed out that the complex of oxidized oxidase with Cl is not a "slow" form of the enzyme. The "slow" form of the enzyme is characterized by (i) a Soret maximum at 418 nm, (ii) a $g' = 12$ signal in the EPR spectrum and, as is mentioned above, (iii) slow and often multiphasic cyanide binding kinetics (*20*, *35*−*37*). These are in contrast to CcO•Cl which has a Soret maximum at 422 nm, lacks the $g' = 12$ EPR signal and reacts with cyanide in a monoexponential manner with a rate comparable to "fast" CcO (Figure 3A).

The ability of the oxidized oxidases to bind chloride has been observed previously (*20*−*27*). The total number of chloride ions bound to our bovine enzyme (Table 1) is in agreement with previously published data that show that the bovine enzyme binds 3.5−5 chloride ions tightly (*28*). It has also been suggested that chloride is released from the binuclear center by reoxidation because the reactivity of reoxidized enzyme with NO is enhanced (*27*). We have observed the same enhanced reactivity for hydrogen peroxide (Figure 1) and our data show directly, as was expected, that chloride is released from the binuclear center into the medium by reoxidation and moreover that the stoichiometry is one chloride released per binuclear center (Tables 1 and 2).

However, the total chloride content among oxidases is variable (*28*, *11*). For example, there are one to two chloride ions found in the bo_3 oxidase from *E. coli* (*28*) and one chloride ion in the *Paracoccus* enzyme (*28*), but there is no evidence for tightly and stoichiometrically bound chloride in the cytochrome aa_3-600 isolated from *Bacillus subtilis* (*11*).

The analytical method we have employed identified one chloride bound to the catalytic binuclear center with a possible additional three chlorides bound elsewhere. However, the presence of the three additional chlorides may reflect limitation of the analytical procedure. As mentioned in the Materials and Methods, these additional chlorides are only released upon denaturation of the enzyme, and the presence of denatured enzyme in the medium interferes with the chloride determination. Even though the medium is separated from the denatured protein, we cannot unequivocally exclude some possible interference.

Assuming that the additional chloride ions are legitimate constituents, we know that they are not removed or displaced by gel filtration, dialysis, and cyanide binding and are only released upon denaturation of the enzyme. It would therefore appear that the binding sites must be located elsewhere within the protein. Whether-or-not these chlorides have a role in bovine oxidase function is not clear at present but obviously they are not analogous to the chloride of the *E. coli* bo_3 oxidase (*29*) that is easily removable and yet essential for intramolecular electron transfer.

However, the bovine enzyme does have a Cl binding site analogous to that of *E. coli* bo_3 oxidase (*29*) with an easily removable Cl as is indicated by our preliminary EPR data (D. Jancura, M.F., and G.P., unpublished results). It is known that chloride (*38*, *43*), cyanide (*44*, *45*), and azide (*46*) affect the shape and position of the $g = 3$ peak of low spin heme *a*. Our data indicate that Cl shifts peak of the $g = 3$ signal about 24 G to higher magnetic field; the estimated K_D for this transition is about 5 mM in 10 mM Hepes, pH 7.6, and 0.1% TX100. In addition the rate constants of Cl binding and dissociation from this site have to be larger than 200 M^{-1} s^{-1} and 0.1 s^{-1}, respectively. The estimated K_D of 5 mM is very close to the value of $K_D = 3.6$ mM found for Cl binding to the *E. coli* bo_3 oxidase (*29*). It might be that this Cl binding site is the same as that site suggested for azide binding to cytochrome *a* (*46*) and recently identified in the crystal structure of complex of oxidized enzyme with azide (*47*). This site is located on the surface of the protein in the proximity of heme *a* and could be an anion binding site (*47*).

With the bo_3 oxidase from *E. coli*, it was observed that the binding of CO to the reduced binuclear center is associated with a major structural change at Cu_B site in which one histidine ligand is replaced by chloride (*28*). This observation could be taken to indicate, for example, that in the complex of reduced oxidase with oxygen (compound A) chloride might be bound at Cu_B. However, the crystal structures of the complex of the fully reduced bovine enzyme with CO (FR•CO) show that Cu_B is still coordinated to three histidine residues and that CO is the only entity between heme a_3 and Cu_B (*48*). Despite this fact we have tested whether the binding of CO to reduced enzyme might stabilize chloride in the binuclear center and have analyzed the chloride content of the enzyme following reoxidation of the FR•CO complex. No difference was observed between CO-treated enzyme and CO-free enzyme.

Obviously, this measurement does not establish whether it is reduction or reoxidation that is releasing chloride from the binuclear center. However, we assume that chloride is displaced by reduction because the reduction of enzyme substantially decreases its affinity for ligands such as cyanide (*39*, *49*, *50*) and formate (*51*). The reduced binuclear center does have a weak, residual affinity for cyanide but this is a consequence of cyanide's ability to function as a π-acid; there is no documented example of the reduced enzyme binding simple anions with any significant affinity.

That the binuclear center of heme-copper oxidases can bind up to two external ligands was demonstrated by the simultaneous binding of chloride plus NO or two NO to the bo_3 oxidase from *E. coli* (*24*). However, under our conditions, with cyanide in large excess and the chloride concentration in the buffer essentially zero, these two ligands cannot be bound simultaneously because chloride appears in the medium following reaction of cyanide with CcO•Cl (Table 1 and 2).

Homogeneous chloride-free CcO can be quantitatively converted to the chloride adduct (CcO·Cl) by incubation with chloride salts under suitable conditions. Both the rate of this reaction and the dissociation constant for chloride binding are pH dependent and complete reaction requires a low pH. As the plot of log K_d versus pH (Figure 2A, inset) has unit slope it would appear that the binding of chloride is accompanied by the uptake of a single proton. We suppose that during this reaction a single chloride ion is bound by the heme-copper center for then the overall process is electroneutral.

The binding of chloride induces almost the same change in the optical spectrum of heme a_3 as is observed with low concentrations of azide (Figure 2B). On this basis, we propose that the mode of binding for both ligands, azide and chloride, are similar. At low concentrations azide is a bridging ligand between the iron of heme a_3 and Cu_B (*46*, *52*), and according to the crystal structure of the complex of bovine CcO with azide (*47*, *48*), all three histidine residues His 290, His 291 and His 245 remain coordinated to Cu_B.

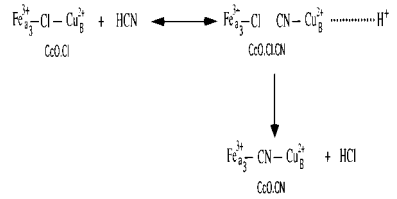
The same conclusion is substantiated by three other observations: (i) the EXAFS analysis of the bovine enzyme; (ii) the dependence of the redox potential of cytochrome a_3 on KCl; and (iii) the EPR spectra of CcO·Cl. If chloride is a bridging ligand then the Fe−Cl bond length is expected to be about 2.3 Å and indeed a S/Cl scatterer is observed at this distance in the iron EXAFS of the bovine enzyme (*53*). As the Fe−Cu distance in oxidized enzyme is 4.8 Å, simple metrics predicts that the Cu_B-chloride distance should be 2.5 Å. In the same EXAFS study, the copper EXAFS provided evidence for a S/Cl scatterer present in significant excess than can be accounted for by the known sulfur content of the Cu_A center; this scatterer was found at 2.3 Å. Thus, as a minimum, chloride should be weakly coordinated to Cu_B. While the EXAFS analysis cannot discriminate between chloride or sulfur, the X-ray structure gives no indication of any sulfur atoms within the vicinity of the binuclear center.

Further evidence for a direct interaction between chloride and heme a_3 is provided by the observation that the reduction potential of cytochrome a_3 is decreased upon addition of KCl, as would be expected by the stabilization of the ferric cation by the chloride anion (*54*). The third indication is that EPR spectra of both forms of enzyme, CcO and CcO·Cl, are essentially identical and only exhibit EPR signals from the magnetically isolated low-spin heme *a* and Cu_A. The absence of the signals from the heme a_3−Cu_B center imply that the magnetic coupling remains intact in the presence of chloride (*55*).

There is electron density between heme a_3 and Cu_B present in the X-ray data, suggesting a direct mechanism for this coupling. However, its origin is controversial: one interpretation has water bound to a_3 and hydroxide to Cu_B (*11*) while a competing interpretation rationalizes the electron density as due to a bridging peroxide (*12*). In CcO·Cl, we believe that the native ligand(s) is replaced by a single chloride ion that interacts with both metal centers as has previously been deduced by earlier EXAFS data on both bovine cytochrome *c* oxidase and bacterial quinol oxidases (*13*−*19*).

The reaction of both CcO and CcO·Cl with cyanide results in the formation of the complex of CcO·CN as is apparent from the identical optical spectrum of the product in each case. The displacement of chloride is further rationalized by the larger absorbance changes observed when CcO·Cl is converted to CcO·CN (Figure 3A) reflecting the combined spectral consequences of loss of chloride plus reaction with cyanide.

Scheme 1



The absorbance changes following reaction of cyanide with both CcO and CcO·Cl are monoexponential with the rate constant exhibiting a hyperbolic dependence upon cyanide concentration (Figure 3B). This hyperbolic dependence can be explained by at least two mechanisms: (i) there are two conformations in slow equilibrium and only one of these conformers can react with external ligands, or (ii) the reaction proceeds through an intermediate that slowly converts to final product. In the first case, the limiting rate constant should be independent of the nature of the ligand and equal to the rate of conversion of the inactive to the active conformation. This mechanism can be ruled out, at least for CcO·Cl, because we observe that the limiting rate when hydrogen peroxide is used as the ligand is ∼20-fold smaller at pH 8.0 than that observed when cyanide is the ligand. Thus, by supposing that both ligands interact with the binuclear center using the same general mechanism our data favor the explanation that binding proceeds via some intermediate.

As chloride and CN^- compete for the same bridging position between heme a_3 and Cu_B the binding of CN^- to CcO·Cl should be limited by the rate of chloride dissociation. However, the limiting rate of cyanide binding to CcO·Cl is a 1000-fold faster then the rate at which chloride spontaneously dissociates. This apparent enhancement of chloride dissociation by cyanide has also been observed with the bo_3 oxidase (*25*). Our explanation requires the existence of an transient intermediate state in which the rate of chloride dissociation is substantially increased, and it is this intermediate that is responsible for the observed saturation kinetics. We suppose that this intermediate is a state in which both chloride and cyanide are transiently bound (Scheme 1).

The binuclear center is located inside the protein and isolated from the solvent and the crystal structure suggests that there may be as many as four channels that connect the binuclear center with the bulk solvent (*6*, *56*); one of these channels is hydrophilic and other three are hydrophobic. Two of the hydrophobic channels terminate at Cu_B but all four channels are closed and some reversible conformational change of CcO is required for ligand access (*56*). We conclude that cyanide can reach the binuclear center of the chloride-ligated oxidase as the neutral hydrocyanic acid by using one or both of the hydrophobic channels that approach Cu_B. The interaction of HCN with Cu_B liberates a proton and triggers a structural change that either weakens or breaks the Cl−Cu_B bond. The proton that is released from HCN can be captured by the chloride anion thus preserving electroneutrality, and the electrically neutral HCl can now diffuse through the hydrophobic barrier connecting the

binuclear center and bulk solvent where it dissociates to H^+ and chloride.

The existence of transient intermediate with two simultaneously bound ligands in the binuclear center is supported by several studies on the bo_3 oxidase from *E. coli* and the bovine aa_3 oxidase. It has been observed that oxidized bo_3 oxidase can bind either two NO or NO plus chloride (*24*), oxidized bovine enzyme can bind cyanide plus NO (*57*) and the reduced bovine enzyme can bind cyanide plus CO (*58*). The participation of Cu_B in the transient binding of CO or O_2 (*59*−*63*) as these gaseous ligands proceeds from solvent to the iron of heme a_3 has also been demonstrated, while transient binding to Cu_B is also indicated in the reaction of cyanide with a partially reduced oxidase (*64*).

A comparison of the concentration dependence of cyanide binding to CcO and CcO·Cl (Figure 3B) reveals that the main effect of the presence of chloride is to lower the K_d about 7-fold. If we suppose that the maximal rate is slower than the rate of ligand dissociation from the intermediate the proposed mechanism for ligand binding to oxidized enzyme implies that the changes in K_d reflect changes in the affinity of Cu_B for cyanide. On the basis of the similar saturation kinetics observed for CcO·Cl and CcO (Figure 3B), we assume that cyanide is bound to the binuclear center by the same mechanism. In both cases, we expect a transient coordination of cyanide at the Cu_B site that stimulates the rate of dissociation of the ligand previously located in the heme a_3−Cu_B center.

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BI010059Y