explain the stoichiometry, the effects of O_2 on the rate of CO_2 production, the ^{18}O isotope results, and the overreduction beyond the level of half-reduction and requires that an oxide, bridging or otherwise, be present at the active site. Even with a concomitant monoxygenase activity of the P-450 type, it must still be explained how CO can react with ferric iron and/or cupric copper [but see Bickar et al. (1984)].

In conclusion then, although the exact details of the reaction mechanism have yet to be unraveled, the catalytic oxygenation of CO by activated bovine left ventricular cytochrome *c* oxidase cannot be disputed.

Registry No. CcO, 9001-16-5; CO_2, 124-38-9; O_2, 7782-44-7; CO, 630-08-0; mannitol, 69-65-8; superoxide dismutase, 9054-89-1; catalase, 9001-05-2; ferricyanide, 13408-62-3.

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Spectroelectrochemical Study of the Cytochrome *a* Site in Carbon Monoxide Inhibited Cytochrome *c* Oxidase†

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Received June 28, 1985

ABSTRACT: The reduction potential of the cytochrome *a* site in the carbon monoxide derivative of beef heart cytochrome *c* oxidase has been studied under a variety of conditions by thin-layer spectroelectrochemistry. The reduction potential exhibits no ionic strength dependence and only a 9 mV/pH unit dependence between pH 6.5 and 8.5. The weak pH dependence indicates that protonation of the protein is not stoichiometrically linked to oxidoreduction over the pH range examined. The temperature dependence of the reduction potential implies a relatively large standard entropy of reduction of cytochrome *a*. The measured thermodynamic parameters for reduction of cyctochrome *a* are (all relative to the normal hydrogen electrode) $\Delta G°'(25\ °C) = -6.37$ kcal mol^{-1}, $\Delta H°' = -21.5$ kcal mol^{-1}, and $\Delta S°' = -50.8$ eu. When cytochrome *c* is bound to the oxidase, the reduction potential of cytochrome *a* and its temperature dependence are not measurably affected. Under all conditions studied, the cytochrome *a* site did not exhibit simple Nernstian $n = 1$ behavior. The titration behavior of the site is consistent with a moderately strong anticooperative interaction between cytochrome *a* and Cu_A [Wang, H., Blair, D. F., Ellis, W. R., Jr., Gray, H. B., & Chan, S. I. (1985) *Biochemistry* (following paper in this issue)].

Cytochrome *c* oxidase is the terminal enzyme of the electron transport chain in mitochondria. Spanning the mitochondrial inner membrane, this enzyme catalyzes the reduction of dioxygen to water, using protons derived from the matrix side of the membrane and reducing equivalents provided by ferrocytochrome *c* on the cytosol side. During electron transport, cytochrome *c* diffuses from the membrane-bound cytochrome bc_1 complex to a binding site (Michel & Bosshard, 1984; Antalis & Palmer, 1982) or sites (Wilms et al., 1981) on cytochrome *c* oxidase. Electron transfer from bound cytochrome *c* to cytochrome *a* is rapid (the apparent first-order rate constant is greater than 1000 s^{-1} under some conditions;

† This is Contribution No. 7184 from the Arthur Amos Noyes Laboratory of Chemical Physics. This work was supported by NSF Grant CHE82-18502 to H.B.G. and NIH Grant GM-22432 to S.I.C. D.F.B. was the recipient of National Research Service Award 5T32GM-07616 from the National Institute of General Medical Sciences.

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0006-2960/86/0425-0161$01.50/0

Wilms et al., 1981), indicating that these sites are fairly close to each other in the bimolecular complex. The oxidase contains three other prosthetic groups: One of these is a copper ion called Cu_A, which exhibits unusual spectroscopic properties [Stevens et al. (1982) and references cited therein] and which also accepts electrons from cytochrome *c* rapidly; another heme called cytochrome a_3 and another copper ion called Cu_B are close (ca. 5 Å) to each other and together form the site of dioxygen reduction. In recent years, considerable progress has been made toward elucidating the structures of these prosthetic groups and the mechanisms of electron transfer and dioxygen reduction [for recent reviews, see Malmström (1980), Brunori et al. (1981), Wikström et al. (1981), Blair et al. (1983), Wainio (1983), and Freedman & Chan (1984)].

Each of the four metal centers of the oxidase is known to function in a redox capacity, as evidenced by optical and EPR[1] measurements at various stages during enzymatic turnover with solution oxidoreductants and dioxygen (Greenwood & Gibson, 1967; Gibson & Greenwood, 1965; Chance et al., 1975; Clore et al., 1980; Antalis & Palmer, 1982). An electrochemical titration has demonstrated that the enzyme accepts four electrons upon complete reduction (Heineman et al, 1972). In an early spectrophotometrically monitored titration with cytochrome *c*, Minnaert (1965) measured the midpoint potential of cytochrome *a* to be 278 mV vs. NHE. However, the titration behavior of cytochrome *a* was not that expected for a Nernstian $n = 1$ (one-electron acceptor) site; the observed Nernst plot slope was close to $n = 0.5$. It was subsequently realized that the metal sites of the enzyme possess similar (within 100 mV) reduction potentials and interact anticooperatively (Malmström, 1974; Wikström et al., 1976; Kojima & Palmer, 1983; Goodman, 1984). In such a situation, a departure from simple Nernstian behavior is predicted (Malmström, 1974; Cornish-Bowden & Koshland, 1975).

The nature and magnitude of the intersite interactions have been the subject of many recent investigations. In all but one of these studies, the dominant interaction was postulated to be between cytochrome *a* and cytochrome a_3 (Wikström et al., 1976; Babcock et al., 1978; Carithers & Palmer, 1981; Kojima & Palmer, 1983). However, published optically monitored titrations (Minnaert, 1965; Artzatbanov et al., 1978), as well as recent EPR studies (Goodman, 1984), suggest that interaction between cytochrome *a* and Cu_B is also important. The possibility of interactions involving Cu_A has not previously been explored. A consideration of all of the possible intersite interactions requires a complex scheme in which the enzyme may take on any of 16 different states (Carithers & Palmer, 1981). This complexity, together with the difficulty of deconvolving the spectral contributions of each of the chromophores [Wikström et al., 1976; Blair et al. (1982) and references cited therein], has made it very difficult to elucidate the redox properties of cytochrome oxidase in detail.

The redox behavior of the oxidase is simpler in the presence of ligands that bind to cytochrome a_3 and stabilize this site in one redox state. For example, cyanide stabilizes cytochrome a_3 in the oxidized state (Artzatbanov et al., 1978), while evidently allowing Cu_B to undergo oxidoreduction (Jensen et al., 1984; Goodman, 1984). Carbon monoxide evidently stabilizes both cytochrome a_3 and Cu_B in their reduced states (Wilson & Nelson, 1982). Ligand binding thus decreases the number of overall redox states accessible to the enzyme and simplifies the interaction problem accordingly.

[1] Abbreviations: NHE, normal hydrogen electrode; SCE, saturated calomel electrode; OTTLE, optically transparent thin-layer electrode; CO, carbon monoxide; EPR, electron paramagnetic resonance.

Table I: Redox Mediators Employed in This Study

mediator	$E^{\circ\prime}$ (mV vs. NHE)	no. of equiv used
$[(NH_3)_6Ru]Cl_3$	51	1.0
$[(NH_3)_5pyRu](ClO_4)_3$	260	1.0
(hydroxymethyl)ferrocene	405	0.5
1,1′-bis(hydroxymethyl)ferrocene	465	0.5

In order to more completely characterize the thermodynamic properties of the metal centers of cytochrome *c* oxidase, we have undertaken a series of spectroelectrochemical experiments under a variety of conditions of temperature, ionic strength, and pH. The initial studies have employed the carbon monoxide derivative of the enzyme, in which only cytochrome *a* and Cu_A undergo oxidoreduction. By treating this relatively simple problem first, we have been able to characterize the thermodynamic properties intrinsic to cytochrome *a* in considerable detail. The measurements show that the reduction potential of cytochrome *a* is only weakly (−9 mV/pH unit) dependent upon pH in the physiological range, indicating that reduction of the site is not stoichiometrically linked to protonation. A negligible dependence upon ionic strength was observed. The temperature dependence of the reduction potential, and thus the standard entropy of reduction, is larger than that of other cytochromes, suggesting that a substantial conformational change accompanies cytochrome *a* reduction. Complexation by cytochrome *c* (2 equiv) has no measurable effect upon either the reduction potential of cytochrome *a* or its temperature dependence. Even in the carbon monoxide inhibited enzyme, markedly non-Nernstian behavior was observed. This result is most readily interpreted in terms of an anticooperative interaction between cytochrome *a* and Cu_A. Such an interaction, which has not previously been suggested, is likely to have an effect upon the steady-state electron-transfer properties of the enzyme. These results are discussed with respect to the mechanisms of electron transfer and proton pumping by the oxidase.

Materials and Methods

Materials. Cytochrome *c* oxidase was purified from beef heart mitochondria by the method of Hartzell & Beinert (1974) and was stored frozen at −80 °C. Protein concentration was determined by the method of Lowry et al. (1951) and heme concentration by the reduced minus oxidized extinction of the uninhibited enzyme at 604 nm with an extinction coefficient of 24 mM^{-1} cm^{-1} (van Gelder, 1966). The purified enzyme contained 8 nmol of heme *a*/mg of protein. Just prior to use, the enzyme was thawed and dialyzed into potassium phosphate buffer containing 0.5% Tween-20. Phosphate buffer was used because the pH of phosphate, unlike that of Tris, is relatively insensitive to temperature. Following dialysis, samples were centrifuged at 35000*g* for 30 min to remove insoluble material. Enzyme concentrations were typically 0.10–0.15 mM. Horse heart cytochrome *c* (Sigma type VI) was chromatographically purified on Whatman CM-52 cellulose prior to use (Brautigan et al., 1978).

All buffer solutions were prepared with glass-distilled water and research-grade reagents. Hexaammineruthenium trichloride was obtained from Alfa and purified by the method of Pladziewicz et al. (1973). (Hydroxymethyl)ferrocene (Strem Chemicals) and 1,1′-bis(hydroxymethyl)ferrocene (Research Organic/Inorganic Chemical Corp.) were used as received. Pentaammine(pyridine)ruthenium perchlorate was prepared as previously described (Cummins & Gray, 1977). The above redox mediators (see Table I) were selected because they have a negligible absorbance in the 400–700-nm region

at the concentrations used and have reduction potentials that allow the solution potential to be poised from −50 to 550 mV vs. NHE. Equilibrium dialysis experiments indicate that these mediators do not bind strongly to the enzyme.

Apparatus. Reduction potential measurements were made in an optically transparent thin-layer electrode (OTTLE) cell with an electrode configuration similar to that previously described (Taniguchi et al., 1982). The OTTLE cell was machined from lucite and employed quartz windows (path length ca. 0.47 mm). The working electrode material consisted of two pieces of 500 lines/in. electroformed gold mesh (Buckbee-Mears Co., Minneapolis, MN) soldered to 18-gauge copper wire. A Sargent-Welch S-30080-17 miniature calomel reference electrode was used along with a platinum wire counterelectrode. The OTTLE cell/electrode assembly was contained in a gas-tight stainless steel shroud in order to remain strict anaerobicity. The shroud and spectroelectrochemical cell designs will be described in a future publication. Cell temperatures were varied with a Forma Model 2095 constant temperature bath and measured directly (±0.2 °C) with an Omega Engineering, Inc. miniature copper–constantan thermocouple placed in the protein solution in close proximity to the thin-layer cavity of the OTTLE cell. A Princeton Applied Research Model 174A potentiostat was used to control the potentials applied across the protein solutions, which were measured (±0.1 mV) with a Keithley Model 177 microvolt digital multimeter. UV–visible spectra were obtained with a Cary 219 spectrophotometer (0.55-nm spectral bandwidth) interfaced to a Spex Industries SCAMP SC-31 data processor.

Methods. After the addition of redox mediators, enzyme and buffer solutions were thoroughly degassed on a high-vacuum apparatus and subsequently allowed to stir under 1 atm of CO (99.99%, Matheson) for at least 30 min. The CO-saturated solutions, OTTLE cell, electrodes, and shroud were then transferred into an inert atmosphere box where the spectroelectrochemical cells were loaded (ca. 0.7-mL dead volume), fitted with the reference and auxiliary electrodes, and placed into the shroud. Once brought out into the laboratory, the sealed shroud maintains an inert atmosphere for at least 1 week. A specially constructed aluminum mount was used to support the shroud in the spectrophotometer sample compartment.

Enzyme/mediator solutions were poised at −50 mV vs. NHE for at least 2 h in order to reduce the enzyme fully and form the CO-associated derivative (verified by a change in the Soret λ_{max} from 443 to 430 nm). Reduction potentials were determined by sequentially applying a series of potentials across the OTTLE cell. Each potential was maintained, typically for ca. 1 h, until electrolysis ceased (verified by cessation of spectral changes at 443 nm) so that the equilibrium value of the ratio of concentrations of oxidized to reduced forms, oxid/red, of all redox couples in solution was established as defined by the Nernst equation:

$$E(\text{applied}) = E^{\circ\prime} + [2.303RT/(nF)] \log (\text{oxid/red}) \quad (1)$$

where $E^{\circ\prime}$ is the reduction potential of the redox couple (the prime signifies that the present measurements were made near neutral pH). Redox couples were converted in increments from one oxidation state to the other by the series of applied potentials, for which each value of oxid/red was determined from the corresponding overlay spectra. Care was taken to ensure that oxidations were reversible: Rereduction led to data closely superimposable with oxidative data in all cases (cf. Figure 2). Absorption spectra were stored on magnetic disk; difference spectra were obtained by computer subtraction. At least 10 points were included in each Nernst plot.

Data Analysis. The Nernst plots obtained from titrations of the cytochrome *a* site did not exhibit the slope expected for a one-electron acceptor. This kind of behavior suggests that the reduction potential of the cytochrome *a* site is modulated by an interaction with another site in the enzyme (Malmström, 1974; Cornish-Bowden & Koshland, 1975). In such an interactive situation, the conventionally defined midpoint potential is no longer useful as a measure of the intrinsic properties of the site, since it is sensitive not only to the properties of the site but to the potential of its interaction partner and the strength of the interaction. The equilibrium equations appropriate to such an interacting system were used in a nonlinear curve fitting program to obtain estimates of the potential of the cytochrome *a* site both when its interactive partner is oxidized and when its interactive partner is reduced (and hence an estimate of the magnitude of the interaction). A more detailed description of this interaction model is given in the accompanying paper (Wang et al., 1985). The potentials reported here are those that pertain when the interacting partner is *oxidized*; in the present situation, where the interaction is anticooperative, this is the higher of the two potentials which may be taken on by cytochrome *a*. The potential defined in this way is a meaningful measure of the intrinsic thermodynamic properties of the cytochrome *a* site, because it pertains under a single, specific set of conditions with regard to the interaction(s) that influence(s) the potential.

To avoid oxidation of the Fe_{a_3}/Cu_B–CO site, potentials above 420 mV (vs. NHE) were not applied to the sample solutions. Oxidation of the Fe_a site was thus not complete at the highest potentials used. A correction for this incomplete oxidation was applied in the following manner: The values for $E^{\circ\prime}$ (Fe_a) that were estimated from the initial computer fits were used to calculate the fraction of Fe_a that remained reduced at the highest potentials used. The corresponding absorbance difference was then added to the observed absorbance changes, to obtain a corrected fully oxidized end point. By use of the corrected absorbance differences, new values of log (oxid/red) were calculated, and new estimates of $E^{\circ\prime}$ were obtained from computer fits.

Standard entropies of reduction were obtained from the slopes of plots of reduction potential vs. temperature, essentially as described by Taniguchi et al. (1980). In previous metalloprotein titrations using the OTTLE cell (Taniguchi et al., 1980, 1982), the reference electrode was maintained at a constant (room) temperature while the sample temperature was varied. This so-called nonisothermal electrode arrangement substantially simplifies the measurement of standard entropies of reduction if, as has been demonstrated previously (Taniguchi et al., 1980, 1982), the various thermal junction potentials may be neglected. In a strictly nonisothermal electrode arrangement, the observed temperature dependence of the reduction potential is due entirely to the entropy change associated with the redox half-reaction of interest, called ΔS°_{rc}. In order to obtain standard entropies of reduction on a meaningful absolute scale, the reaction entropy of the normal hydrogen electrode half-cell (15.6 eu, by convention; Latimer, 1952) has been subtracted from the reaction entropies estimated from the temperature dependences measured here. The reported values of $\Delta S^{\circ\prime}$ and $\Delta H^{\circ\prime}$ thus pertain to the overall redox reaction involving both the NHE and the redox half-reaction of interest (Taniguchi et al., 1982). Because of the need for strict anaerobicity, the sample cell and reference electrode were placed together inside a stainless steel chamber in the present experiments, which resulted in partial thermostating of the reference electrode along with the sample.

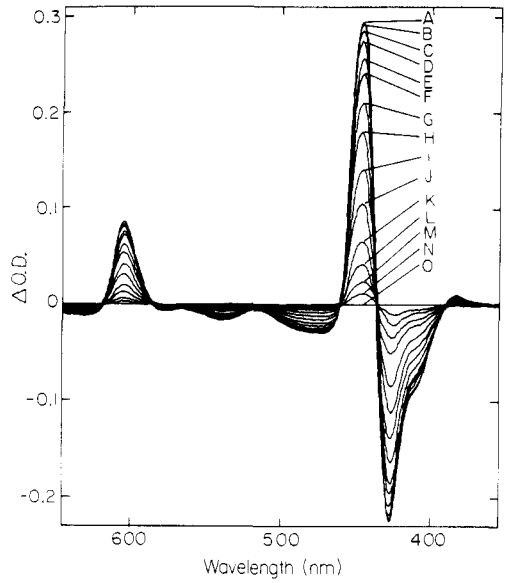

FIGURE 1: Representative set of absorbance difference spectra of cytochrome *a* at different applied potentials in a thin-layer spectroelectrochemical titration of CO-inhibited cytochrome oxidase. Conditions: sample temperature 9.8 °C, SCE temperature 16 °C (because of partial thermostating, cf. Materials and Methods), pH 7.0, ionic strength 0.245 M. Applied potentials (mV vs. SCE): (A) −220, (B) −100, (C) −70, (D) −40, (E) −25, (F) −10, (G) 5, (H) 20, (I) 35, (J) 50, (K) 65, (L) 80, (M) 95, (N) 110, and (O) 140. The most oxidized spectrum (measured at 140 mV vs. SCE) has been subtracted from the spectra recorded at each potential to yield the difference spectra shown.

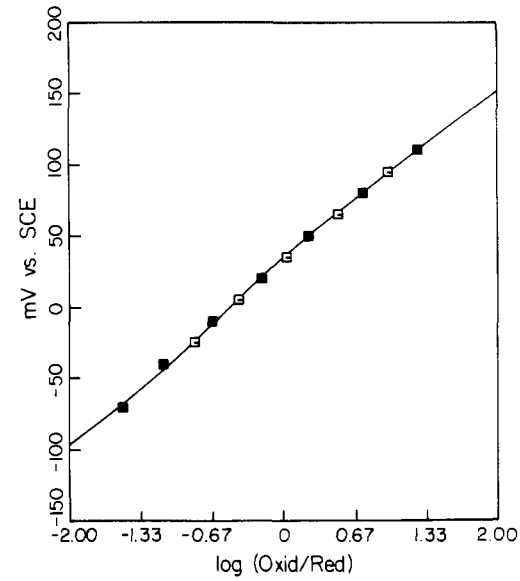

FIGURE 2: Nernst plot calculated from the spectra shown in Figure 1 by using ΔA_{443}. The solid line is the computer-generated best fit to a model in which the cytochrome *a* site participates in an anticooperative interaction of magnitude 24 mV with another site whose potential is 6 mV vs. SCE. (■ and □) Spectra obtained on oxidation and rereduction, respectively.

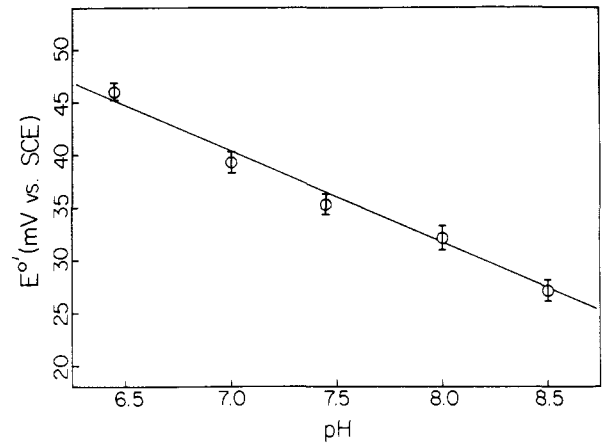

FIGURE 3: pH dependence of the cytochrome *a* reduction potential in CO-inhibited cytochrome oxidase (9.8 °C, ionic strength 0.245 M). The solid line is a linear least-squares fit to the data, with slope of −9 mV/pH unit.

Control experiments using two thermocouples were carried out to measure the extent of this partial thermostating of the reference electrode. The deviation of the reference electrode temperature from room temperature was found to be linearly related to the difference between the sample temperature and room temperature, so that the fractional isothermal character of the electrode arrangement could be simply defined:

$$\mathrm{FIC} = \Delta T_r / \Delta T_s$$

where ΔT_s is the difference between the sample temperature and room temperature and ΔT_r is the difference between the reference electrode temperature and room temperature. The correction to the standard entropy of reduction, $\delta(\Delta S°)$, is therefore given by

$$\delta(\Delta S°) = \mathrm{FIC} \times \Delta S°_{rc}(\mathrm{SCE})$$

where $\Delta S°_{rc}(\mathrm{SCE})$ is the entropy of reduction of the SCE (−15.3 eu; W. R. Ellis, unpublished experiments) obtained directly from a plot of the temperature dependence of the potential of the SCE. The correctness of this treatment was verified by control experiments using myoglobin. The standard entropy of reduction of myoglobin was found to be the same, within experimental uncertainty, in the partially nonisothermal situation as in the strictly nonisothermal situation when the above correction was applied.

RESULTS

Overlay difference spectra obtained in a typical experiment are displayed in Figure 1. A linear relation between ΔA_{605} and ΔA_{443}, with a slope consistent with measurements of the reduced minus oxidized spectrum of cytochrome *a* (Vanneste & Vanneste, 1965; Blair et al., 1982), was observed (plot not shown), indicating that only cytochrome *a* is titrated at the potentials used. Potentials above 420 mV were not applied, because the ferrocytochrome a_3–CO complex is partially oxidized at these high potentials.

A Nernst plot of the spectral data is shown in Figure 2. Equilibration at each applied potential was determined from the absorbance change at 443 nm. The plot is not like that expected for a simple $n = 1$ Nernstian process (which, from eq 1, is a straight line with a slope of 56 mV/decade at 9.8 °C). The slope of the observed Nernst plots is a strong indication of an anticooperative interaction with one or more of the other metal sites in the enzyme (Malmström, 1974; Cornish-Bowden & Koshland, 1975). The line through the data points is the computer-generated best fit to a model in which cytochrome *a* participates in an anticooperative interaction of magnitude 24 mV with another site whose potential is 256 mV vs. NHE. Similar experiments on the Cu_A chromophore at 830 nm (Wang et al., 1985) provide evidence that the interacting site is Cu_A.

The effect of pH on the reduction potential of cytochrome *a* is shown in Figure 3. A linear least-squares fit to the data yields a slope of −9 mV/pH unit. Significant degradation of the enzyme took place at pH values outide the extremes shown.

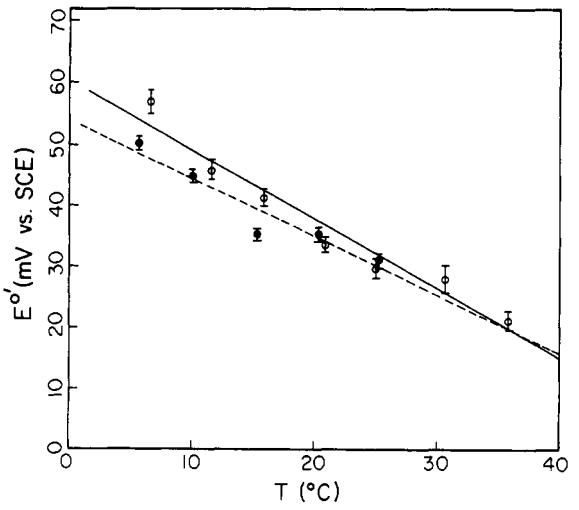

FIGURE 4: Temperature dependence of the cytochrome *a* reduction potential in CO-inhibited cytochrome oxidase (pH 7.0, ionic strength 0.245 M), in the uncomplexed oxidase (○) and in the presence of 2 equiv of cytochrome *c* (●). The lines (solid = uncomplexed; dashed = complex with cytochrome *c*) are linear least-squares fits to the data, corresponding to the following thermodynamic parameters (after correction for partial thermostating of the reference electrode): for uncomplexed enzyme, $E^{\circ\prime}$(25 °C) = 276 ± 3 mV vs. NHE, $\Delta G^{\circ\prime}$(25 °C) = −6.37 ± 0.08 kcal mol^{-1}, $\Delta H^{\circ\prime}$ = −21.5 ± 0.8 kcal mol^{-1}, and $\Delta S^{\circ\prime}$ = −50.8 ● 2.8 eu; for complex with cytochrome *c*, $E^{\circ\prime}$(25 °C) = 274 ± 3 mV vs. NHE, $\Delta G^{\circ\prime}$(25 °C) = −6.32 ± 0.08 kcal mol^{-1}, $\Delta H^{\circ\prime}$ = −20.3 ± 1.2 kcal mol^{-1}, and $\Delta S^{\circ\prime}$ = −46.9 eu.

The cytochrome *a* reduction potential was measured at different ionic strength between 0.05 and 0.42 M (phosphate buffer, supplemented with KCl at the high ionic strengths). Within experimental uncertainty, no ionic strength dependence was observed (data not shown).

The temperature dependence of the cytochrome *a* reduction potential at pH 7.0 is shown in Figure 4. The thermodynamic parameters deduced from a linear least-squares fit to the data are $\Delta G^{\circ\prime}$(25 °C) = −6.37 ± 0.05 kcal mol^{-1}, $\Delta H^{\circ\prime}$ = −21.5 ± 0.8 kcal mol^{-1}, and $\Delta S^{\circ\prime}$ = −50.8 ± 2.8 eu. The temperature dependence of the cytochrome *a* reduction potential in the cytochrome oxidase–cytochrome *c* complex (2 equiv of cytochrome *c*) is also displayed in Figure 4. At 25 °C, $E^{\circ\prime}$ is not significantly different in the complex with cytochrome *c* than in the uncomplexed enzyme (274 mV vs. NHE in the complex; 276 mV vs. NHE in the uncomplexed enzyme). The temperature dependence is also the same within experimental uncertainty, leading to the following estimates for the thermodynamic parameters: $\Delta G^{\circ\prime}$(25 °C) = −6.32 ± 0.05 kcal mol^{-1}, $\Delta H^{\circ\prime}$ = −20.3 ± 1.2 kcal mol^{-1}, and $\Delta S^{\circ\prime}$ = −46.9 ± 3.9 eu.

The reduction potential of cytochrome *c* and its temperature dependence were also measured in the complex with cytochrome oxidase. The thermodynamic parameters for reduction of oxidase-complexed cytochrome *c* deduced from the data are $\Delta G^{\circ\prime}$(25 °C) = −5.77 ± 0.05 kcal mol^{-1}, $\Delta H^{\circ\prime}$ = −17.0 ± 0.35 kcal mol^{-1}, and $\Delta S^{\circ\prime}$ = −37.8 ± 1.1 eu. For comparison, the values measured for uncomplexed cytochrome *c* are $\Delta G^{\circ\prime}$(25 °C) = −6.00 ± 0.05 kcal mol^{-1}, $\Delta H^{\circ\prime}$ = −14.5 ± 0.4 kcal mol^{-1}, and $\Delta S^{\circ\prime}$ = −28.5 ± 1.2 eu (Taniguchi et al., 1982). Thus, $\Delta G^{\circ\prime}$(25 °C) is slightly different from that measured in uncomplexed cytochrome *c*; this small net change in $\Delta G^{\circ\prime}$ is caused by a larger change in $\Delta H^{\circ\prime}$, which is mostly compensated by a change in $\Delta S^{\circ\prime}$.

Discussion

Although several measurements of the cytochrome *a* reduction potential have been reported (Schroedl & Hartzell, 1977a,b; Anderson et al., 1976; Wikström et al., 1976) for the CO derivative of cytochrome oxidase, none has carefully evaluated the effects of pH, ionic strength, and temperature. The available measurements ($E^{\circ\prime}$ = 250–255 mV vs. NHE at 24–25 °C) are in close agreement with the values determined here.

Unlike previous studies, the present results are of sufficiently high precision to detect deviations from simple Nernstian behavior. Since cytochrome a_3 and Cu_B remain reduced during the course of these experiments, the slightly sigmoidal appearance and nonintegral slope of the data in Figure 2, if caused by intersite interaction, indicate that there is a previously unsuspected interaction between cytochrome *a* and Cu_A. In parallel near-infrared spectroelectrochemical experiments monitoring Cu_A, similar behavior was observed; Cu_A also has a potential in the correct range (i.e., sufficiently close to that of Fe_a) to allow an interaction with cytochrome *a* to be expressed (Wang et al., 1985). The evidence for a cytochrome *a*–Cu_A interaction and a discussion of its probable nature are presented in that study. We note here that in the course of the various experiments on cytochrome *a* some batch to batch variation in the interaction behavior was observed: The interaction energy, which measures the decrease in electron affinity of one site accompanying reduction of the other site, varied between ca. 20 and 40 mV in different enzyme batches.

The absence of a measurable ionic strength dependence suggests that charge solvation does not play a large direct role (as opposed to an indirect role via its effect upon the overall conformation of the protein) in determining the reduction potential of cytochrome *a*. Spin-label–spin probe studies employing compounds of dysprosium (Ohnishi et al., 1979) suggest that the heme of cytochrome *a* is less accessible to the solvent than the heme of cytochrome *c*. The absence of any significant effect of complex formation upon the reduction potential of cytochrome *a* is consistent with the suggestion that the solvent plays a relatively small direct role in tuning the cytochrome potential, and may in fact be the physiologically desirable consequence of "designing" the protein so that the cytochrome *a* reduction potential is not very sensitive to the immediate solvent environment. Another beneficial consequence of shielding the metal site from the solvent is that the outer sphere reorganizational barrier to electron transfer will be smaller. The cytochrome *c* reduction potential is only slightly effected ($E^{\circ\prime}$ changes by ca. 6 mV) by complexation with cytochrome oxidase in our experiments; the enthalpy and entropy of reduction undergo larger but compensating changes. Since cytochrome *c* was present in excess, only a fraction of the cytochrome *c* was complexed with the oxidase. In experiments using an excess of the oxidase, Vanderkooi & Erecińska (1974) saw a somewhat larger effect ($E^{\circ\prime}$ changed by ca. 30 mV). The reduction potential of cytochrome *c* is only very weakly dependent upon ionic strength in the 0.01–0.23 M range, but the enthalpy and entropy of reduction again undergo larger but compensating changes (Margalit & Schejter, 1970), consistent with a somewhat greater influence of the solvent in tuning the cytochrome *c* potential.

The observed pH dependence of the cytochrome *a* reduction potential (−9 mV/pH unit) is similar to that observed by Hinkle & Mitchell (1970). Cytochrome oxidase is known to function as a proton pump (Wikström et al., 1981a,b). The site of redox-linked proton translocation is still unclear, although either cytochrome *a* or Cu_A is implicated on energetic grounds. Babcock & Callahan (1983) suggest, on the basis of resonance Raman data, that the heme *a* formyl group of

cytochrome *a* is involved in a redox-coupled hydrogen-bonding interaction with a proton donor supplied by the enzyme. Such interactions are commonplace among hemoproteins containing propionate groups. However, if a strongly redox-linked protonation were occurring at pH 7.4 (the pH where the redox-coupled hydrogen-bonding interaction is inferred from the Raman data), one would expect a ca. 59 mV/pH unit change in $E^{\circ\prime}$. The data shown in Figure 3 show only a very shallow pH dependence. It should be noted, however, that the lack of a pronounced pH dependence in $E^{\circ\prime}$, and hence of a strong redox Bohr effect, does not rule out the possibility that cytochrome *a* is involved in proton pumping (Blair et al., 1986).

The large, negative values of $\Delta S^{\circ\prime}$ obtained from analyses of the $E^{\circ\prime}$ vs. temperature data (Figure 4) suggest that reduction of cytochrome *a* is accompanied by a significant conformational change. It is known that reduction of the enzyme causes ORD spectral changes (Urry et al., 1972) and an increase in the volume of the protein (Cabral & Love, 1972). The $\Delta H^{\circ\prime}$ values are negative, most likely as a result of favorable metal to ligand π back-bonding, which tends to stabilize iron(II). The redox thermodynamic parameters reported here are substantially more negative than those for small low-spin cytochromes (Taniguchi et al., 1980, 1982; Reid et al., 1982; Margalit & Schejter, 1970). An entropy–enthalpy compensation effect has been noted (Huang & Kimura, 1984) for cytochromes *c*. This situation also holds for cytochrome *a*: The $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values deduced from Figure 4 correlate well with a plot of $\Delta S^{\circ\prime}$ vs. $\Delta H^{\circ\prime}$ for *c*-type cytochromes.

The large entropy changes (reduced *minus* oxidized) reported here for the cytochrome *a* center indicate that a relatively large electron-transfer reorganizational barrier might be expected for this site. Electron-transfer reorganizational barriers ($\Delta G^{\ddagger}_{\lambda}$) have been shown to correlate with observed entropy changes for small metal complexes (Sutin et al., 1980). For comparison, $\Delta S^{\circ\prime}$ and the reorganizational enthalpy $\Delta H^{\ddagger}_{\lambda}$ are −28.5 eu and 7.8 kcal mol^{-1}, respectively, for horse heart cytochrome *c* (Nocera et al., 1984). The corresponding values for sperm whale myoglobin (Crutchley et al., 1985) are −39.1 eu and 20 kcal mol^{-1}. As noted above, the outer sphere contribution to the reorganizational barrier is likely to be small. This circumstance, coupled with the facilitating effect of the π-electron system of the heme, could readily account for the rapidity of electron transfer between cytochrome *c* and cytochrome *a*.

Since cytochrome a_3 is believed to participate in interactions with one or more of the other metal sites in the enzyme and might also undergo spin-state transitions during oxidoreduction, spectroelectrochemical results for this site will be of interest. A study of the redox thermodynamics of both cytochromes in the native enzyme has just been completed and will be the subject of a forthcoming paper.

Registry No. Cu, 7440-50-8; cytochrome *a*, 9035-34-1.

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Temperature Dependence of the Reduction Potential of Cu_A in Carbon Monoxide Inhibited Cytochrome *c* Oxidase†

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Received June 28, 1985

ABSTRACT: The temperature dependence of the reduction potential of the Cu_A site in carbon monoxide inhibited cytochrome *c* oxidase has been measured with a spectroelectrochemical method adapted to the relatively weak near-infrared absorption of this copper ion. These measurements, together with parallel measurements on the 604-nm absorption due to Fe_a, indicate that an interaction between Cu_A and Fe_a causes the reduction potential for one of these sites to be decreased by approximately 40 mV upon reduction of the other. The temperature dependence of the Cu_A reduction potential indicates a relatively large and negative standard entropy of reduction of Cu_A ($\Delta S^{\circ\prime} = -48.7 \pm 2.3$ eu). Possible implications of the intersite redox interaction and the large standard entropy of reduction of the Cu_A site are discussed.

The Cu_A site in cytochrome *c* oxidase exhibits unique spectroscopic properties (Beinert et al., 1962; Peisach & Blumberg, 1974; Stevens et al., 1982), which have made it the subject of many chemical [Gelles & Chan (1985) and references cited therein] and spectroscopic (Stevens et al., 1982) investigations. Since the flow–flash kinetic studies of Gibson & Greenwood (1965), which demonstrated that this copper ion can undergo oxidoreduction on a time scale comparable to or faster than the overall oxidase turnover, it is generally accepted that Cu_A functions as a catalyst of electron transfer between cytochrome *c* and the dioxygen reduction site (Fe_{a_3} and Cu_B) of the enzyme. The unusual spectroscopic parameters of the site, which must reflect a structure different from those of other metalloprotein copper sites, have led Chan et al. (1979) to suggest that Cu_A is also important in the energy-transducing functions of cytochrome oxidase, specifically as the site of redox-coupled proton pumping.

The reduction potential of the Cu_A site has been measured by van Gelder et al. (1977) at a range of pH values and in different detergents. These potentiometric titrations were carried out by the addition of oxidizing and reducing agents to achieve the desired potentials while the level of oxidation of Cu_A was monitored by an absorption band at 830 nm that is associated with cupric Cu_A (Beinert et al., 1980). The extinction coefficient of this absorption is approximately 2000 M^{-1} cm^{-1}, which makes monitoring the Cu_A site by this method more difficult and less precise than monitoring the heme sites, which have much stronger absorptions. This circumstance probably accounts for the fact that few room-temperature measurements of the Cu_A reduction potential have been undertaken (Tiesjema et al., 1973; van Gelder et al., 1977; Schroedl & Hartzell, 1977a; Babcock et al. 1978), while measurements of the heme potentials have been made repeatedly (Wilson et al., 1972; Tiesjema et al., 1973, Schroedl & Hartzell, 1977a,b; Babcock et al., 1978; Kojima & Palmer, 1983).

A thorough understanding of the thermodynamics of reduction of Cu_A is essential because of the importance of Cu_A in the electron-transfer functions of cytochrome *c* oxidase and because this copper site may be unique among cuproproteins

† Contribution No. 7206 from the Arthur Amos Noyes Laboratory of Chemical Physics. Supported by Grant GM-22432 from the National Institute of General Medical Sciences, U.S. Public Health Service (S.I.C.), BRSG Grant RR07003 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health (S.I.C.), and Grant AM-19038 from the National Institutes of Health, U.S. Public Health Service (H.B.G.). D.F.B. was the recipient of National Research Service Award 5T32GM-07616 from the National Institute of General Medical Sciences.
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0006-2960/86/0425-0167$01.50/0