A NEW HIGH POTENTIAL REDOX TRANSITION FOR CYTOCHROME aa_3

RICHARD W. HENDLER AND GURMEL S. SIDHU
Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT Using cyano-complexes of iron, tungsten, and molybdenum and a platinum working electrode, we have been able to attain and hold voltages in the range of 400 to 900 mV (vs. standard hydrogen electrode) in an aqueous medium. With this system we have obtained additional information in support of an earlier conclusion that cytochrome a_3 has a high E_m transition (i.e. >460 mV) in addition to its E_m in the 180–200 mV range (Hendler, R. W., K. V. S. Reddy, R. I. Shrager, and W. S. Caughey. 1986. *Biophys. J.* 49:717–729; Reddy, K. V. S., and R. W. Hendler. 1986. *Biophys. J.* 49:693–703). The proposed new transition has an E_m near 770 mV and an n value >1. The reduced form of the high-potential species of cytochrome a_3 does not bind CO, in contrast to the reduced form of the low-potential species which does. A possible reaction scheme for cytochrome aa_3 which incorporates the new information is presented.

INTRODUCTION

Mammalian cytochrome *c* oxidase has been known for the past 60 years and intensively studied during the last 30 years (see Wikström et al., 1981, for a complete synopsis and references). In spite of this, newer findings continue to remind us that there is much about the enzyme that is not understood. For example, Zn and Mg were recently found to be integral parts of the enzyme (Einarsdóttir and Caughey, 1984; Einarsdóttir and Caughey, 1985*a*). On the basis of careful metal analyses of six bovine heart cytochrome *c* oxidase preparations, Einarsdóttir and Caughey (1985*b*) proposed that an additional Cu center is present in the dimer such that there are 5 Cu, 4 Fe, 2 Zn, and 2 Mg per unit dimer. Subsequently, Steffens et al. (1987) and Bombelka et al. (1986) proposed that there is an extra Cu center present per monomer (i.e., 3 Cu, 2 Fe, 1 Zn, and 1 Mg). That energy of respiration is conserved at the cytochrome oxidase locus through active proton pumping as well as through the formation of a $\Delta\Psi$ became a consensus in 1986 when West et al. (1986) accepted Wikström's view (see Wikström and Saraste, 1984, for references). Neither the mechanism of proton pumping nor the kinetic sequence of the four electrons used for O_2 reduction and which involve the active redox centers of the molecule has been established. The current view of the thermodynamic reduction potentials at pH 7 for the two hemes is either that two distinct $n = 1$ centers exist with $E_m \sim 220$ mV for cytochrome *a* and $E_m \sim 380$ mV for cytochrome a_3 (Wilson et al., 1972) or that a form of redox cooperativity linking the two heme centers exists whereby the first electron is accepted with an E_m near 360 mV and is shared by both centers and that a negative cooperative interaction exists so that the second electron is added with an apparent E_m ~230 mV (Nicholls and Peterson, 1974; Malmström, 1974). We have recently described results of the application of new, more powerful techniques for performing and analyzing potentiometric titrations of mammalian cytochrome *c* oxidase (Reddy and Hendler, 1986; Hendler et al., 1986). These procedures involve the use of controlled electrical potentials on a platinum electrode to fix the solution potential, the collection of full visible optical spectra at each voltage, and the use of all of the spectral data in the analysis. The analytical techniques involve singular value decomposition of data matrices, the use of second derivatives of absorbance peaks, and nonlinear regression fitting procedures. These studies produced some unexpected results, and it is the purpose of this and other papers in preparation to provide further experimental characterization of the new findings as well as to reconcile them with existing views on the properties of mammalian cytochrome *c* oxidase.

The current paper focusses on the indication, published previously (Reddy and Hendler, 1986; Hendler et al., 1986), that cytochrome a_3 has (at least) two redox potentials, one below 200 mV (~180 mV) and one above 460 mV. In one of the earlier studies (Hendler et al., 1986), it was found that at the highest potential that could be maintained with $K_3Fe(CN)_6$ as a redox mediator (~460 mV), cytochrome a_3 was reduced while cytochrome *a* was oxidized. As the solution potential was lowered to ~200 mV, cytochrome *a* became reduced while cytochrome a_3 became oxidized. The continued lowering of the solution potential resulted in the re-reduction of cytochrome a_3. These results suggest a high level of redox cooperativity

such that when a particular redox center (different from cytochrome *a*) is oxidized, the midpoint potential of cytochrome a_3 is high (>460 mV) and that when that controlling center is reduced, the midpoint potential of cytochrome a_3 is low (~180 mV). In the current paper we describe potentiometric titrations in the range of 450 to 900 mV. The key findings are that when the completely oxidized resting enzyme is introduced into a solution having a potential between 400 and 750 mV (vs. standard hydrogen electrode [SHE]),[1] cytochrome a_3 become reduced, and that in the voltage range of 750 to 790 mV the reduced cytochrome a_3 becomes oxidized. Additional findings of interest are that the reduced form of high-potential cytochrome a_3 does not bind CO and that exposure of the enzyme to ambient voltages above 750 mV selectively destroys the ability of the enzyme to re-form the reduced species of high potential cytochrome a_3.

EXPERIMENTAL PROCEDURES

The chemical sources, titration procedures, apparatus, and general procedures have been previously described (Reddy and Hendler, 1986; Reddy et al., 1986; Hendler et al., 1986). Two new mediators were used in the current studies: octacyanotungstate ($K_4W[CN]_8\ 2H_2O$, E_m = 510 mV [vs. SHE], n = 1) and octacyanomolybdate ($K_4Mo[CN]_8$, E_m = 778 mV [vs. SHE], n = 1). An initial sample of octacyanotungstate was a gift from James A. Fee and subsequent quantities of both mediators were provided by Jay M. Johnson of Yellow Springs Instrument Co., Yellow Springs, OH. The enzyme used for most of the studies was prepared from bovine heart muscle by the method of Yoshikawa et al. (1977). The preparations were 0.9–1.6 mM in 0.01 M sodium phosphate at pH 7.4 with respect to heme A, and 10–11 nmol heme A/mg protein (ϵ = 20 $mM^{-1}\ cm^{-1}$). The preparation has nine polypeptide chains, a metal stoichiometry of 5 Cu/4 Fe/2 Zn/2 Mg per dimer and is optically clear under the experimental conditions. Recently this preparation of the enzyme has been crystallized and studied by x-ray diffraction (Yoshikawa et al., 1988). Additional information about the preparation is contained in the reference just cited. The concentrated enzyme was distributed into 10- or 20-μl aliquots and stored aerobically at −80°C. Confirmatory studies were performed with cytochrome oxidase prepared according to the procedure of Yonetani (1961) (gift of David Bickar).

Procedure for Generating Reduced High-Potential Cytochrome a_3 from the Resting Enzyme

50 μl each of 12 mM $K_3Fe(CN)_6$ and $K_4W(CN)_8\ 2H_2O$, and 10 μl of 12 mM $K_4Mo(CN)_8$ were added to 2.87 ml of 0.125 KCl/0.0625 M potassium phosphate buffer (pH 7.1) followed by 18 nmol cytochrome aa_3 in 10 or 20 μl. The difference spectrum before and after addition of the enzyme is representative of its resting state. The solution, in an open cuvette, was stirred for 2 h under constant illumination from the light source of the spectrometer, and spectra were recorded every 15 min. During this time the voltage of the medium decreased from ~460 mV (vs. SHE) to <300 mV and the position of the Soret peak shifted from ~420 nm (oxidized) to 429 nm with a pronounced shoulder at 448 nm, and an α peak at 607 nm became evident. The difference spectrum (end of the incubation minus the beginning) showed a trough at 416 nm and peaks at 448 and 607 nm. These spectral shifts indicate the reduction of cytochrome a_3 with its Soret peak at 429 nm and the higher potential form of cytochrome *a* (Hendler et al., 1986) with its Soret peak at 448 nm and its α peak by 607 nm. The reducing power during this incubation comes from the mediators and is markedly stimulated by illumination from the light source of the spectrometer. The cuvette was then closed, the voltage of the medium brought to 468 mV (using a potentiostat), and the flow of argon gas initiated. This second phase was continued for 1 h with spectra recorded every 15 min. During this time, the shoulder at 448 nm disappeared and the α peak diminished and shifted to ~602 nm. The difference spectrum obtained from the spectra at the end and the beginning of the anaerobic incubation at fixed voltage showed the disappearance of the Soret (at 448 nm) and α (at 607 nm) peaks of cytochrome *a*. The absolute spectrum at the end showed a clean Soret peak at 429 nm and a low broad absorbance feature near 602 nm. The difference spectrum obtained from the end of the entire incubation minus the first spectrum (resting state) showed a sharp trough at 416 nm and a sharp peak at 435 nm.

RESULTS

The highest voltage that can be maintained in a medium is determined by the midpoint potential of the mediator that is the most difficult to oxidize. In potentiometric titrations of respiratory components, this maximum voltage has usually been set using ferricyanide. Although with 0.2 mM ferricyanide, it is possible to maintain voltages as high as 550 mV (Fig. 1 *a*), this voltage can not be used in a titration. This is because (*a*) the $K_3Fe(CN)_6$ oxidizes the other mediators that are present as well as any reduced substance in the sample which has a lower E_m, (*b*) some of the other mediators display light-activated reducing power, and (*c*) the presence of protein in the medium slows down the rate of reaction of the mediators with the platinum electrode. In our experience, the highest starting voltage attained with the full titration medium has been under 400 mV. With additions of small aliquots of 1 M $K_3Fe(CN)_6$ it has been possible to raise the voltage to ~460 mV. There are, however, other inorganic compounds whose structure and solution chemistry are similar to ferricyanide and which have higher E_m values. Two such substances that have been characterized electrochemically are octacyanotungstate (E_m = 510 mV) and octacyanomolybdate (E_m = 778 mV). The reversible interaction of octacyanomolybdate with a tin oxide electrode has been demonstrated by cyclic voltometry (Szentrimay et al., 1977). Similarly, both octacyanotungstate and octacyanomolybdate react reversibly with a gold electrode as demonstrated by cyclic voltometry (J. M. Johnson, personal communication). These compounds have been used in the reversible titration of laccase and ceruloplasmin (Reinhammer, 1972; Fee and Malmström, 1968; Deinum and Vänngård, 1973). No chemical oxidant of sufficiently high E_m has been found to attain and maintain voltages in the 450–800 mV range; instead, solutions containing dissolved O_2 have been used as oxidants. In Fig. 1, we show how the three high-voltage mediators are able to stabilize voltages in the 400–900 mV range under ideal circumstances (i.e., in the dark and in the absence of protein or electron-donating substances). In each case, the voltage of the medium was first raised to

[1] *Abbreviatons*: SHE, standard hydrogen electrode; n value refers to the Nernst equation, $E = E_m + (RT/nF)\ \ln K$, where n is the number of electrons involved in the redox transition.

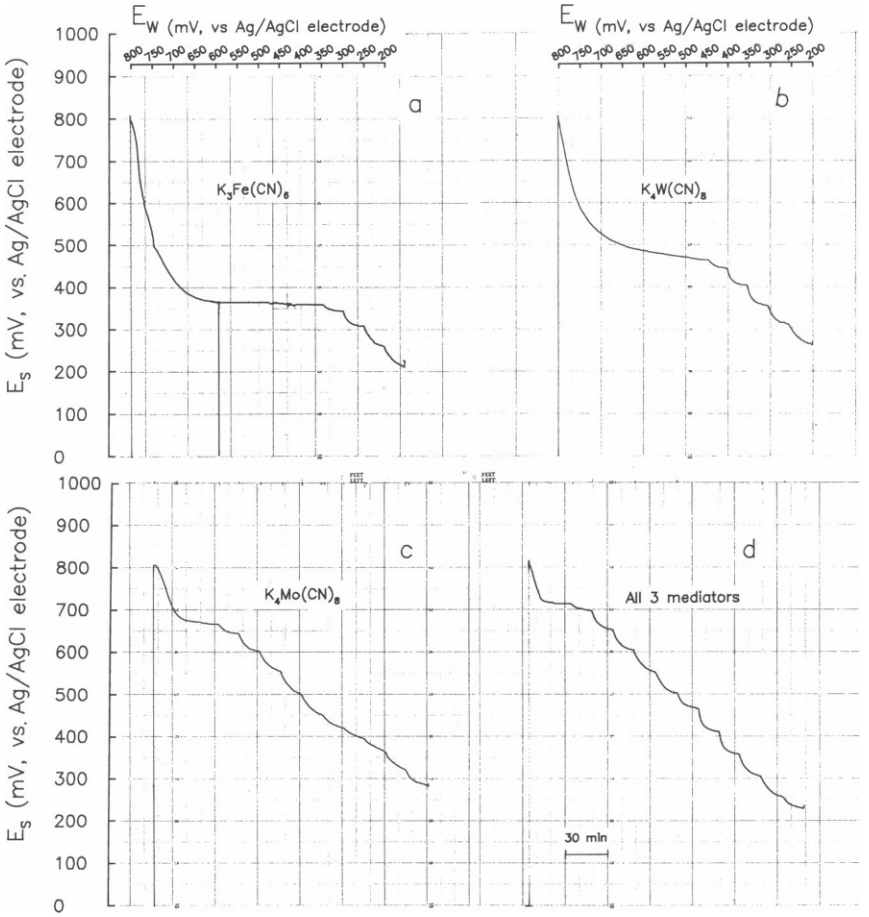


FIGURE 1 The three mediators, $K_3Fe(CN)_6$, $K_4W(CN)_8$, and $K_4Mo(CN)_8$, were tested alone and together for their ability to hold solution potentials in the voltage range of 200 to 800 mV (vs. Ag/AgCl electrode; Ag/AgCl electrode had a voltage of 200 mV vs. SHE). The mediators were present at 0.2 mM each in 3 ml of 125 mM KCl and 62.5 mM potassium phosphate in an unilluminated cuvette at pH 7.0. The voltage of the medium was first raised to ~800 mV (vs. Ag/AgCl electrode) using a constant current generator and then the working Pt electrode was switched to a potentiostat for voltage control. The voltage on the Pt electrode (E_w) was first set to 800 mV, held for 15 min, and then stepped down in 50-mV increments with holding period of 15 min at each voltage as indicated at the top of the figure. The chart was moving at a rate indicated in panel *d* by the bar showing the displacement in 30 min. The voltage of the medium (E_s) was continuously monitored with a measuring Pt electrode vs. a reference Ag/AgCl electrode. The voltage range showing "steps" indicates where the mediator or mixture is competent as a redox buffer by virtue of its ability to exchange electrons with the working and measuring Pt electrodes.

~1,000 mV (vs. SHE) electrically and the Pt electrode was set to 1,000 mV and then reduced in 50-mV steps to 400 mV (vs. SHE), waiting 15 min at each step. In the figure, actual readings vs. Ag/AgCl electrode are shown. These are 200 mV lower than readings referenced to the SHE. Although the thermodynamic midpoint potential of H_2O at pH 7 is ~0.8 V, little or no electrolysis occurs because of the overpotential of H_2O at a smooth Pt electrode which amounts to ~0.45 V (Glasstone, 1948). In Fig. 1 *b* it is seen that with octacyanotungstate, a voltage of ~650 mV (vs. SHE) was maintained; with octacyanomolybdate, this limit was raised to ~850 mV (vs. SHE) (Fig. 1 *c*). However, in the latter case, buffering was minimal below 700 mV as evidenced by the lack of steps. With all three mediators together (Fig. 1 *d*), there was good voltage control throughout the 400–900 mV (vs. SHE) region. Using these mediators and cytochrome oxidase in an illuminated cuvette still provided voltage control in the higher voltage region.

The resting enzyme, which is in the fully oxidized state (Wikström et al., 1981) shows a prominent Soret peak near 420 nm. When this enzyme was added to a medium containing the redox mediators and preadjusted to 468 mV, and held at this voltage, a slow progressive process ensued which resulted in the shift of the position of the major Soret absorbance to 429 nm (Fig. 2). The source of

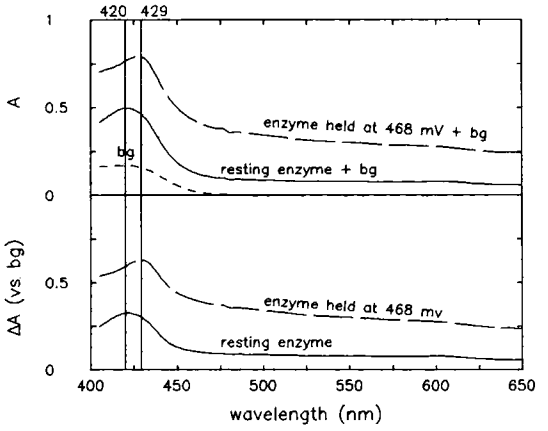


FIGURE 2 An aqueous medium (3 ml) containing 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0), and the following redox mediators: 0.2 mM $K_3Fe(CN)_6$, 0.2 mM $K_4W(CN)_8$, and 0.04 mM $K_4Mo(CN)_8$ was brought to 468 mV (vs. SHE) using a Pt working electrode and a constant current generator as described in the Methods. Resting cytochrome aa_3 (6 μM [heme A], 0.47 mg protein) was added and the solution potential was held at 468 mV using a potentiostat to set the voltage on the Pt working electrode. Spectra were taken just before the addition of the enzyme (*bg*) and at 2 min after its addition (*resting enzyme*). Spectra were then taken at 5-min intervals from 5 to 260 min. The top panel shows the absolute spectra for bg, the resting enzyme, and the enzyme held for 260 min at 468 mV. The bottom panel shows the difference spectra ($-bg$) for the two enzyme spectra shown in the upper panel.

electrons for this reductive process is the mediators and the reducing power of the mediators is augmented by illumination. A sequence of difference spectra during this time showed a continual growth of a peak at 435 nm and a trough at 416 nm (Fig. 3). A plot of ΔA (435–416) shows the time-course of this spectral change at the constant voltage (Fig. 4). SVD analysis of the series of absolute spectra showed only two major fundamental spectral components which were coupled in their changes with voltage so that as one grew (at 427 nm) the other simultaneously diminished (at 419 nm) (not shown). SVD analysis of the difference spectra showed essentially a single spectral change with a peak at 435 nm and a trough at 416 nm (not shown). There were no spectral changes at 448 or 607 nm, which mark the positions of the reduced Soret and α absorption bands of cytochrome *a*. The superposition of a positive peak at 427 nm and a negative peak at 419 nm results in the displacement of the peak and trough positions accounting for the 416- and 435-nm features seen in the difference spectrum. As has been previously shown (Hendler et al., 1986), a reductive titration to 237 mV of the enzyme after this period of holding at 468 mV resulted in the reduction of cytochrome *a* as evidenced by the appearance of its characteristic Soret and α peaks and the oxidation of cytochrome a_3 as evidenced by a trough near 429 nm (Fig. 5). Having established that oxidized cytochrome a_3 becomes reduced at 468 mV, it was of interest to determine the highest voltage at which the reduction of cytochrome a_3 would occur. For this experiment, the resting enzyme was introduced into a medium at 750 mV, held at this voltage for ~60 min, with spectra taken every 10 min, then the voltage of the medium was reduced to 700 mV and the process of holding and spectra-taking repeated. The whole procedure was continued with voltage steps of ~50 mV until a voltage of 467 mV was reached. It was found that the reduction of cytochrome a_3 ensued from the

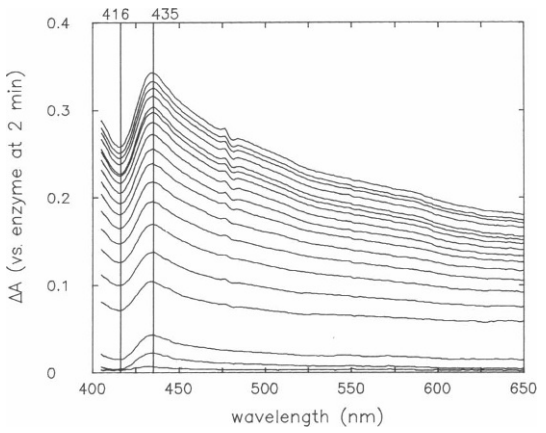


FIGURE 3 For the experiment described in Fig. 2, the spectrum of the resting enzyme was taken as a reference spectrum and subtracted from the spectra of the enzyme held at 468 mV for different amounts of time. The difference spectra from 5 to 260 min for each 15-min interval are shown in this figure with increasing time from the bottom to the top of the figure. The two vertical lines are drawn at 416 and 435 nm.

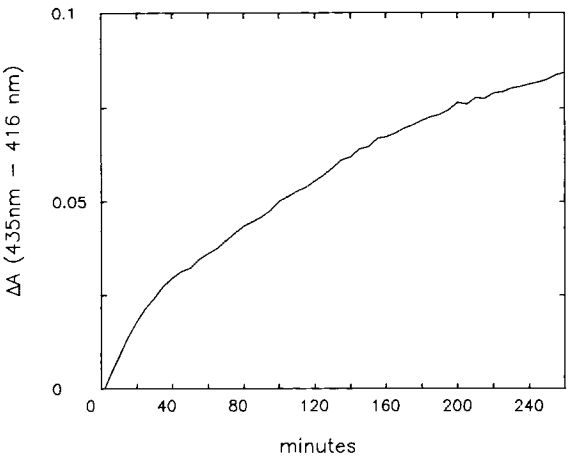


FIGURE 4 The ΔA (435–416 nm) for the spectra taken during the time course described in Fig. 2 are plotted as a function of time of incubation at 468 mV.

beginning at 750 mV (Fig. 6). The difference spectra taken during this experiment were essentially the same as seen in the experiments just described (not shown, but cf. Fig. 3). A more complete test of the reducibility of cytochrome a_3 at 750 mV and of the possible influence of cytochrome *c* in this process is shown in Fig. 7. Oxidized cytochrome *c* was added at 74 min into the time of holding the enzyme at 750 mV and a spectrum was taken at 76 min. After correcting for the initial offsets in the magnitudes of the second derivatives of wavelength vs. *A* at 435 and 416 nm, due to the addition of oxidized cytochrome *c*, it is seen that the rate of reduction of cytochrome a_3 was not markedly changed by the presence of cytochrome *c*. Difference spectra for the changes which occurred before and after the addition of cytochrome *c* were essentially the same (not shown).

The experiments just described show that the midpoint potential of high potential cytochrome a_3 is above 750 mV.

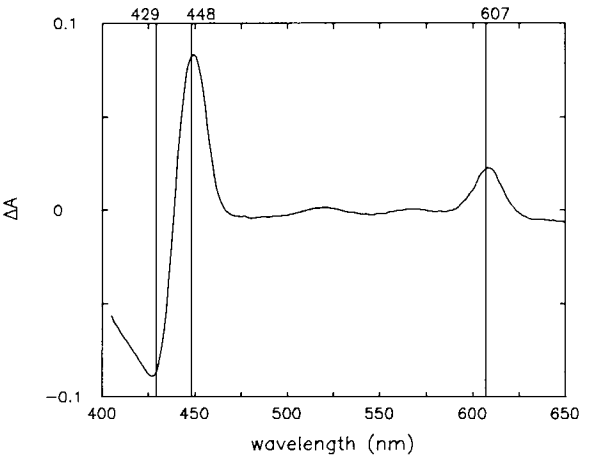


FIGURE 5 After holding the enzyme at 468 mV for 260 min as described in the legend to Fig. 2, an electrical reduction titration to 237 mV was performed. The reduced minus oxidized difference (237 to 468 mV) spectrum is shown in the figure.

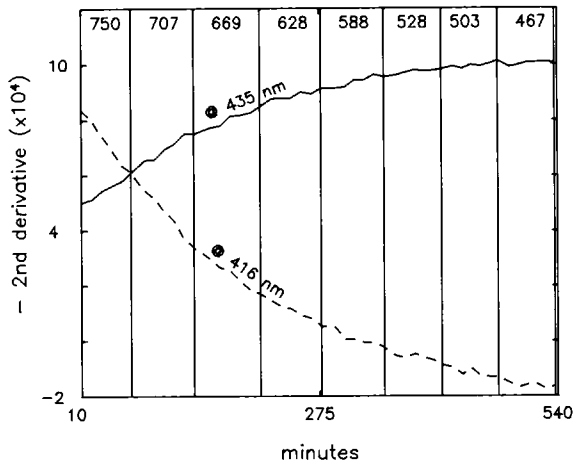


FIGURE 6 The voltage of the medium was pre-set to 750 mV as described in the legend to Fig. 2 and then cytochrome aa_3 (6 μM heme A) was added. Voltage control was shifted from the constant current generator to the potentiostat, and spectra were taken every 10 min for 1 h. The voltage of the medium was decreased in steps of approximately 50 mV and the enzyme held at each new voltage for 1 h with spectra taken every 10 min. The second derivatives of absorbance vs. wavelength centered at 435 nm (reduced Soret absorbance) and at 416 nm (oxidized Soret absorbance) are plotted here as functions of time of incubation. The vertical lines set off the voltages of each 60-min holding period. The numbers at the top of the figure denote the voltages in mV (vs. SHE).

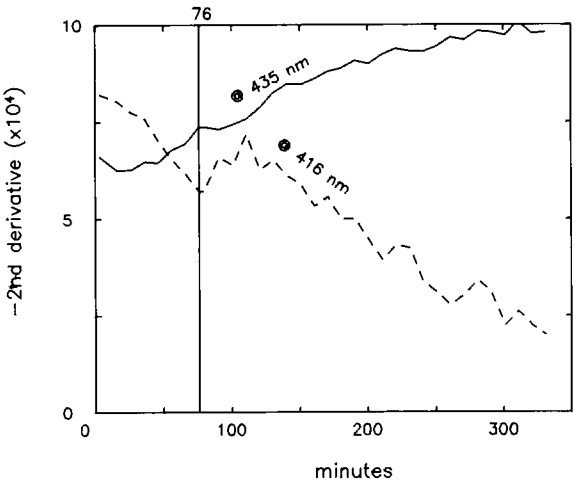


FIGURE 7 The voltage of the medium was set to 750 mV, the resting enzyme was then added, and the voltage of the medium held as described in the legends to Fig. 2 and Fig. 6. Spectra were taken every 10 min. At 74 min, 10 μM cytochrome *c* was added and a spectrum taken 2 min later. The second derivatives of absorbance vs. wavelength, centered at 435 and 416 nm, were computed. The magnitudes of these derivatives before the addition of cytochrome *c* are shown during the first 76 min of incubation. The sudden change in the second derivatives which occurred after the addition of cytochrome *c* (i.e., 76 min vs. 74 min) were computed and used as offsets to correct the second derivatives obtained during the time course following the addition of cytochrome *c*. The rationale for this procedure was that the oxidized cytochrome *c* should be contributing a fixed increment to these derivatives, which were changing because of the change in oxidation state of cytochrome a_3.

The following experiment shows the oxidation of the reduced high-potential species at voltages above 750 mV. It was found that the reduced form of the high potential cytochrome a_3 species could be generated by a 2-h incubation in an open cuvette in an illuminated medium containing a mixture of redox mediators (see Methods). The cuvette was then closed, placed under an argon atmosphere and stirred for 60 min at ~430 mV. An oxidative titration was started at 430 mV using steps of 50 mV and equilibration times of 15 min, up to a voltage of 730 mV. From 730 to 855 mV, the voltage steps were decreased to 10 mV. Plots of the second derivatives at 416 nm (oxidized species) and at 435 nm (reduced species) (Fig. 8) show that oxidation commenced rather abruptly at ~760 mV and continued with a pronounced slope until 792 mV and that another abrupt change was instituted at this voltage. The absolute spectrum at 738 mV shows a Soret peak at 429 nm, characteristic of the reduced cytochrome a_3 species (Fig. 9 *a*). Using a more sensitive scale for absorbance reveals a minor broad absorbance near 604 nm (not shown, but see Fig. 15). At 792 mV, the position of the Soret had shifted to 422 nm, close to the position of the Soret peak in the resting state of the enzyme (Fig. 9 *b*). The absolute spectrum at 855 mV showed none of the characteristic absorption features of the enzyme, indicating its destruction (Fig. 9 *c*). The peak near 435 nm and the low broad absorbance feature near 604 nm in the difference spectrum (738–792 mV) confirm the reduced to oxidized transition (Fig. 10 *a*). The trough expected at 416 nm is not evident, and this could be due in part to the destructive changes

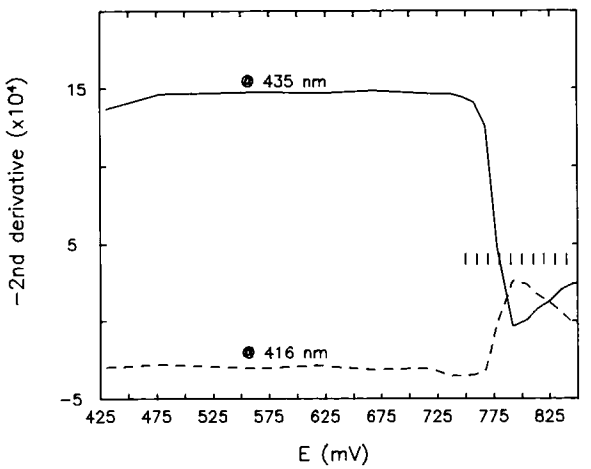


FIGURE 8 Cytochrome aa_3 (6 μM [heme A]) was added to the medium described in the legend to Fig. 2 and held, open to air, for 156 min. The cuvette was then closed, placed under argon, and stirred for 1 h. The voltage was then brought to 430 mV (vs. SHE) and increased in steps of ~50 to 730 mV. From 730 to 855 mV, the voltage steps were close to 10 mV. At each new voltage, 15 min were allowed for equilibration after which a spectrum was taken. The second derivatives of absorbance vs. wavelength were computed at 435 and 416 nm and are plotted as functions of voltage in the figure. The tick marks shown in the lower right-hand part of the figure mark the voltages from 750 to 850 mV in steps of 10 mV.

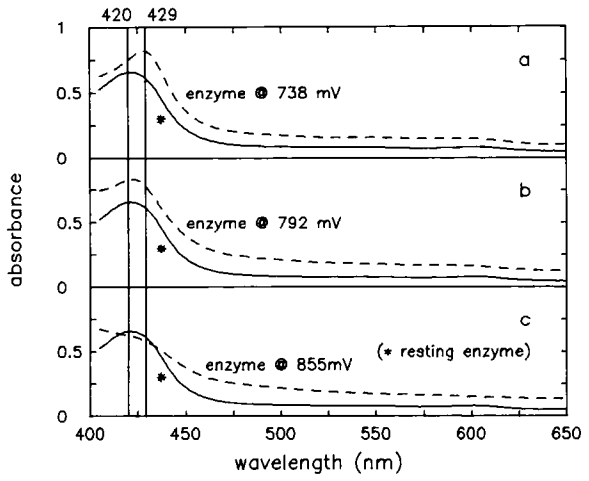


FIGURE 9 The spectrum taken 3 min after adding cytochrome aa_3 to the medium open to air, as described in the legend to Fig. 8, is taken as the spectrum for the resting enzyme and is shown (*solid line*) in all three panels. The spectrum of the enzyme, taken at 738 mV (reduced) is shown in panel *a* (*dashed line*). The spectrum of the enzyme taken at 792 mV (oxidized) is shown in panel *b* (*dashed line*). The spectrum taken at 855 mV (altered) is shown in panel *c* (*dashed line*).

which become more evident at higher voltages. The difference spectrum (792–855 mv) shows that the characteristic Soret (i.e., near 420 nm) and α absorbance of the oxidized enzyme were lost in this high voltage range (Fig. 10 *b*).

We have interpreted the spectral changes shown in Figs. 8–10, which occurred in the voltage range of 750 to 792 mV as the result of the anaerobic oxidation of cytochrome a_3 by the Pt electrode via the redox mediators. In view of the closeness of this voltage range to the thermodynamic potential for the oxidation of H_2O, one may wonder whether the spectral changes seen may reflect the liganding of O_2, released by electrolysis, to the cytochrome a_3

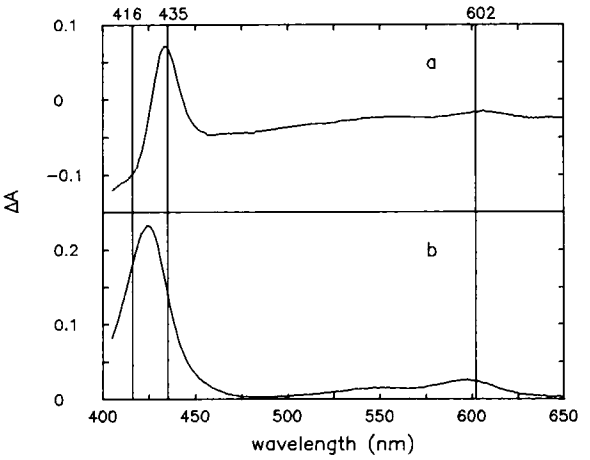


FIGURE 10 Panel *a* shows the difference spectrum for the spectrum in panel *a* of Fig. 9 (at 738 mV) minus the spectrum in panel *b* of Fig. 9 (at 792 mV). Panel *b* shows the difference spectrum for the spectrum in panel *b* of Fig. 9 (at 792 mV) minus the spectrum in panel *c* of Fig. 9 (at 855 mV).

heme center. If the conversion of the 429-nm peak to a 422-nm peak is due to the presence of O_2, then performing the whole titration in an open (aerobic) cuvette should eliminate the spectral changes we have described both in the 470–750 mV range and in the 750–790 mV region. We performed this experiment and found that the 429-nm species was fully formed and maintained up to a voltage of 740 mV and that its conversion to the ~422-nm species occurred in the voltage range of 740 to 789 mV, but the magnitude of the oxidative change was only ~10% of that seen in the anaerobic titration (not shown). The combination of O_2 and high voltage may be destructive to the enzyme, but the spectral changes we have seen in the 750–790 mV range are not due to the liberation of O_2 and its binding to the enzyme.

To see if the oxidative titration which occurs in the voltage range above 750 mV could be reversed, we performed a titration only up to 789 mV to try to prevent or limit the damage to the enzyme which was evident above 790 mV. Fig. 11 shows the magnitudes of the second derivatives at 416 nm and at 435 nm vs. *E*. The reduced species of cytochrome a_3 is present up to a voltage of 752 mV and then the conversion of the reduced Soret at 435 nm to the oxidized spectrum at 416 nm proceeds actively during the titration up to 789 mV. The change in the ΔA (435 to 416 nm) which occurred in this 37-mV range of oxidation accounted for 89% of the change in the ΔA (435 to 416 nm) which arose during the pretreatment when the enzyme was held at 468 mV and thereby reduced. A reductive titration from 789 to 752 mV was then initiated to see if the electrical oxidation could be reversed. How-

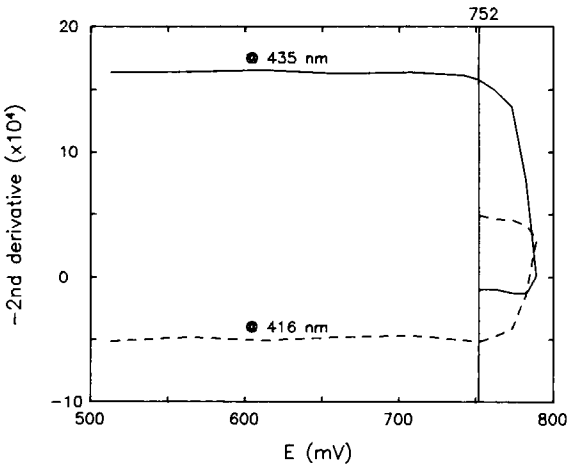


FIGURE 11 The initial stages were as described in the legend to Fig. 8 except that the voltage was held at 468 mV during the 1-h period after changing the gas phase to argon. The voltage was then raised in ~50 mV steps to 740 mV. The voltage steps were decreased to ~10 mV and the titration continued to 789 mV. Equilibration times of 10 min were allowed at each voltage before taking spectra. After reaching 789 mV, the voltage was reduced in steps ~10 mV back to 752 mV, taking spectra after each 10-min waiting time. The second derivatives for absorbance vs. wavelength centered at 435 and 416 nm were computed and are shown in the figure.

ever, instead of re-reducing the cytochrome a_3, the oxidation phase continued, during which time the remaining 11% change of the ΔA (435 to 416 nm) was accounted for. The fact that the oxidative process slowly continued while the voltage of the medium was being lowered (second derivative at 416 nm) indicates that the oxidized cytochrome a_3 heme had become less accessible to the aqueous medium where the voltage was set than was the case for the reduced cytochrome a_3 heme that was oxidatively titrated in this same voltage range. This could be the result of a redox-dependent conformational change. It is worth inquiring whether the exposure to the high-voltage medium prevented all further redox interactions of the enzyme with oxidants and reductants in the medium. The following experiment involving a recycling of the enzyme was designed to test this possibility. After the preincubation with mediators in air, followed by holding the enzyme in argon at 468 nm during which time the high voltage species of cytochrome a_3 became reduced, the enzyme was brought to 750 mV in 50-mV steps with 10-min equilibration periods at each step. The voltage of the medium was then brought to 780 mV and held for 20 min, with spectra taken every 5 min. Fig. 12 shows the oxidation which took place at 780 mV. The change in the ΔA (435 to 416 nm) which occurred during this 20 min at 780 mV accounted for 57% of the change in the ΔA (435 to 416 nm) which occurred during the reduction of cytochrome a_3 in the preincubations. The voltage of the medium was then quickly brought to 456 mV with a small aliquot of 1 M $Na_2S_2O_4$. A spectrum was taken after 10 min at this voltage and then the voltage of the medium was lowered to 97 mV with $Na_2S_2O_4$ and another spectrum taken after 10 min. The cuvette was then opened to the air for 1 h with stirring and spectra taken every 10 min. Finally, the cuvette was closed, made anaerobic with argon, taken to 470 mV electrically and held at this voltage for 1 h with spectra taken every 10 min. As had been seen in the previous experiment, reducing the ambient voltage from 789 to 456 mV did not result in the re-reduction of cytochrome a_3 (not shown). In fact, as was indicated before, there was a small continued rise in the second derivative at 416 nm and a further decrease in the second derivative at 435 nm (not shown). Lowering the voltage from 456 to 97 mV resulted in the definite reduction of cytochrome a as seen by the peak near 448 nm and the trough near 420 nm and the α peak near 604 nm (Fig. 13 *a*). The fact that the position of maximum absorbance of this peak was at 603 nm indicates that the low voltage species of cytochrome a_3 was also reduced. When the cuvette was opened to air for 15 min and the voltage of the medium had risen to 233 mV, the oxidation of both cytochrome a and cytochrome a_3 was indicated by the Soret maximum and minimum at ~420 and ~445 nm, respectively, and the trough for a broad α peak at ~602 nm (Fig. 13 *b*). Further oxidation of the cytochrome occurred when the cuvette was made anaerobic and the voltage was

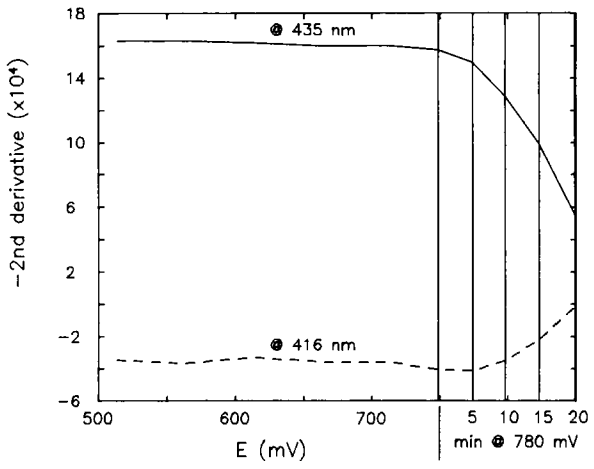


FIGURE 12 The same preincubations in air and argon at 468 mV described in the legend to Fig. 11 were performed to reduce the high-potential species of cytochrome a_3. The voltage was raised in 50-mV steps to 750 mV with 10-min equilibration times before taking spectra. The voltage was then set to 780 mV and held for 20 min with spectra taken every 5 min. The figure shows the second derivatives of absorbance vs. wavelength centered at 435 and 416 nm obtained in the voltage range of 513 to 780 mV.

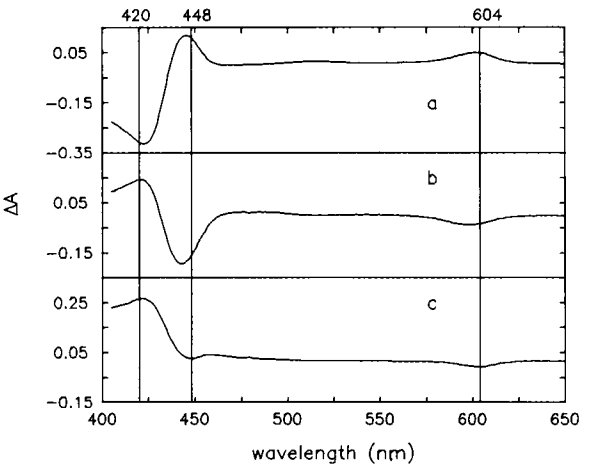


FIGURE 13 After the 20-min holding period of cytochrome aa_3 at 780 mV described in the legend to Fig. 12, a small amount (~1 μl) of 1 M $Na_2S_2O_4$ was used to quickly drop the voltage to 456 mV and after 10 min at this voltage a spectrum was taken. Small aliquots of 1 M $Na_2S_2O_4$ were then used to drop the voltage to 97 mV and then another spectrum was taken after 10 min. In panel *a*, the difference spectrum (97–456 mV) shows the reduction of the cytochromes. The cuvette was then opened to the air and stirring was continued during the next hour while the voltage rose to 233 mV during the first 15 min and to 251 mV at the end of the hour. Most of the spectral change occurred during the first 15 min and the difference spectrum shown in panel *b* shows the oxidation of the enzyme which took place during the first 15 min of exposure to air (i.e., spectrum 15 min in air minus spectrum at 97 mV under argon). The cuvette was then closed, placed under an argon atmosphere, the voltage brought to 470 mV (vs. SHE) electrically and held at that voltage for 1 h. Panel *c* shows the difference spectrum for the spectrum of the enzyme held under argon for 1 h at 470 mV minus the last spectrum obtained under air for 1 h when the voltage of the medium was 251 mV.

brought to 468 mV (Fig. 13 *c*). This experiment shows that the enzyme was not destroyed at 780 mV, although the reformation of reduced high-potential cytochrome a_3 at 750 mV was prevented. The specific loss of the ability of the enzyme to form the reduced species of high-potential cytochrome a_3 is more clearly shown in Fig. 14. In this experiment, the enzyme was held at ~460 mV in three different conditions. During the preincubation of the resting enzyme the reduced form of high-potential cytochrome a_3 was formed (solid line [curve 1], panel *a*). The second time, the enzyme was brought to 468 mV was after having been held at 780 mV for 20 min. The short dashed difference spectrum (curve 2) in panel *a* shows that the reduced Soret at 435 was not present; instead a peak at 448 nm is seen. After the enzyme was reduced and then reoxidized and returned to 468 mV, the reduced form of the high-potential species of cytochrome a_3 was still not present (long dashed line [curve 3], panel *a*). The specific loss of the species with a Soret peak at 435 nm is clearly shown in panel *b* where the difference spectra for the enzyme are shown for curves 2 and 3 in panel *a* referenced to curve 1. The overall results of this experiment show that the principal damage to the enzyme which was electrically oxidized to 780 mV, was its loss of ability to form the reduced form of the high-potential species of cytochrome a_3. The ability to reversibly reduce and oxidize cytochrome *a* and to form the low-potential species of cytochrome a_3 seems to have remained intact.

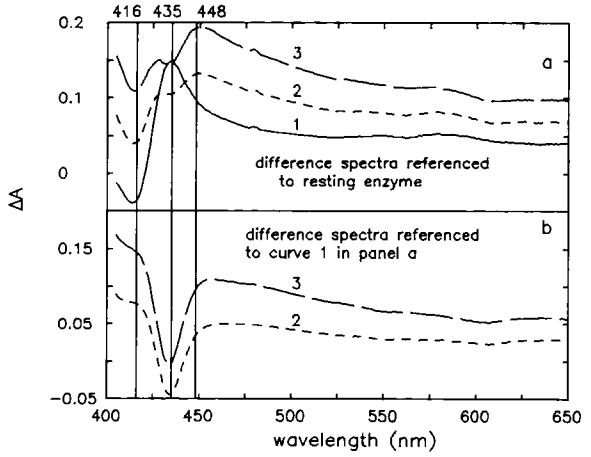


FIGURE 14 For the experiment described in the legend to Figs. 12 and 13, a spectrum of the resting enzyme was taken 3 min after adding it to the medium open to the air. As described in the legends to Figs. 12 and 13 (and the text), the enzyme was exposed to a voltage near 460 mV at three different times during the experiment. The three curves in the upper panel are difference spectra for each of these times minus the resting enzyme. Curve 1 (*solid*) is for the first exposure during the initial preincubations. Curve 2 (*short dashed*) was after bringing the enzyme to 780 mV and then quickly down to 456 mV with $Na_2S_2O_4$. Curve 3 (*long dashed*) was after lowering the voltage to 97 mV with $Na_2S_2O_4$, air oxidation, and anaerobic electrical titration back to 470 mV. The lower panel (*b*) shows curves 2 and 3 in the upper panel after curve 1 was subtracted from each.

If the enzyme can be damaged in the voltage range above 750 mV, and if the resting enzyme, which is fully oxidized, must be exposed to an effective potential >750 mV, the question arises that why isn't the resting enzyme damaged? The essential difference between voltages above 750 mV in a titration protocol and in storage of the enzyme is the presence of redox mediators. These mediators transmit the ambient voltage to all of the amino acid residues in contact with the medium. In this way, oxidizable groups can be oxidized. The high voltage component in contact with the resting enzyme is O_2. Its oxidizing potential is transmitted specifically through the cytochrome a_3-Cu_B center to the other redox-active centers in the molecule. Oxidation of amino acid residues by O_2 would be expected to be a very slow process, especially at −80°C. A long period of exposure of the enzyme to high concentrations of O_2 at elevated temperature may be expected to cause the kind of damage to the enzyme we have seen in the high voltage transition.

It is known that when cytochrome a_3 is reduced with dithionite, it binds CO strongly, shifting the position of its Soret from 429 to ~435 nm. The following experiment was designed to see if the reduced high-potential species of cytochrome a_3 was also capable of binding CO. After the usual preincubation which resulted in the formation of the reduced species of cytochrome a_3 at 468 mV, the voltage of the medium was raised to 660 mV, where the reduced species is stable, and the argon atmosphere was replaced by CO. Spectra were taken every 10 min for 70 min. During this time the 2nd derivatives at 416 nm and 435 nm did not change (not shown). The absolute spectra of the enzyme just before the addition of CO and after 70 min exposure to this ligand show that the peak of the unliganded Soret at 429 nm did not shift to 435 nm (Fig. 15). The difference spectrum for these two spectra in the same figure also shows no evidence for CO liganding. These data show that the reduced form of the high-potential cytochrome a_3 cannot bind CO (at least directly to its heme), in contrast to the reduced form of the low-potential species which binds CO strongly. The enzyme in the CO atmosphere was then taken to 700 mV and subsequently to 850 mV in 50-mV steps. At each new voltage the enzyme was held for 40 min with spectra taken every 10 min. The oxidation commenced at 747 mV (as seen in the absence of CO) and continued for the first 20 min at 799 mV after which the destruction of the enzyme set-in as just described and indicated by the reversal of the second derivatives at 416 and 435 nm and the difference spectra (not shown).

DISCUSSION

The current studies are based on findings described in two earlier papers on cytochrome oxidase, one using intact mitochondria and one using the pure enzyme (Reddy et al., 1986; Hendler et al., 1986). In both cases, three Nernstian transitions were seen in the voltage range of 100 to 450

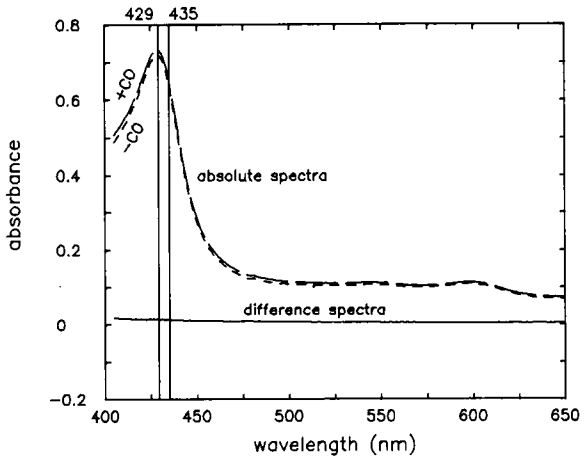


FIGURE 15 After the usual preincubations as described in the legend to Fig. 11, the voltage was raised to 660 mV in steps of ~50 mV. The argon atmosphere was replaced by CO and spectra were taken every 10 min for 70 min. The dashed line curve shows the absolute spectrum just before switching to CO, and the solid line curve shows the absolute spectrum after 70 min in CO. The difference spectrum for the +CO spectrum minus the −CO spectrum is shown in the same figure.

mV. The E_m values were, respectively, ~200 mV with n = 2, ~260 mV with n = 2, and ~340 mV with n = 1. Each transition was characterized by a unique reduced minus oxidized difference spectrum. The low-voltage component displayed a Soret peak at 429 nm and an α peak at 602 nm, the middle component showed a Soret peak at 446 nm and an α peak at 606 nm, and the high-voltage component had a Soret peak at 448 nm and an α peak at 607 nm. In the presence of CO, the spectra, E_m, and n values of the middle and high E_m components were hardly different but the low E_m component, with its Soret peak at 429 nm and α peak at 602 nm, was no longer seen. In its place was the characteristic difference spectrum for the CO-liganded enzyme with a Soret peak near 435 nm and α peak near 595 nm. On the basis of these results the low E_m transition seen in the absence of CO, with a Soret peak at 429 nm and an α peak at 602 nm is attributed to cytochrome a_3, and the two other transitions to different forms of cytochrome a. The different forms of cytochrome a could be monomer/dimer or coupled/uncoupled to Cu_A. The E_m of the CO-liganded species was close to 200 mV, which is an unexpected result, because of the view that CO binds much more tightly to the reduced form of cytochrome a_3 than to the oxidized form and therefore its E_m should have been greatly elevated. The finding is also in apparent conflict with the fact that reduced CO-liganded cytochrome a_3 cannot be oxidized by additions of concentrated $K_3Fe(CN)_6$ (Wikström et al., 1981). As a possible explanation to the two apparently divergent experimental results we suggested that cytochrome a_3 may have two different E_ms. This would be the result of redox cooperativity whereby the presence or absence of electrons or another (controlling) redox center could influence the reduction potential of cytochrome a_3. When the controlling center was reduced, cytochrome a_3 would have a low redox potential (ie., ~180–200 mV) and when the controlling center was oxidized, cytochrome a_3 would have a high redox potential (i.e., >460 mV). A thermodynamic analysis of this kind of redox cooperativity involving all of the redox centers of cytochrome oxidase has been completed and is being published separately (Hendler, R. W., and H. V. Westerhoff, manuscript in preparation). According to this explanation, the inability of $K_3Fe(CN)_6$ to oxidize the reduced CO-liganded species would be due to its oxidation of the controlling center and the consequent rise in the E_m of cytochrome a_3. Support for this view was present in the actual titration data and spectra obtained in the earlier study. At voltages around 400 to 460 mV, attainable with $K_3Fe(CN)_6$, the controlling center should be oxidized, the E_m of cytochrome a_3 should be greater than 460 mV and therefore cytochrome a_3 should be reduced. As the voltage was lowered and the controlling center reduced, the E_m of cytochrome a_3 should be lowered to ~180–200 mV, and in the voltage range 460 to 200 mV, cytochrome oxidase should have become oxidized. At the same time cytochrome a should have become reduced. These changes were seen (Hendler et al., 1986). Difference spectra in the range 460 to 200 mV showed the rise of the characteristic Soret peak for cytochrome a at 448 nm and for its α peak at ~606 nm, and the simultaneous disappearance of the characteristic Soret peak for cytochrome a_3 at 429 nm. A plot of the second derivatives at 448 nm (cytochrome a) and at 429 nm (cytochrome a_3) in this voltage range showed the former rising while the latter decreased. Upon continued lowering of the voltage below 200 mV, the second derivative at 429 nm and the Soret peak it measured both turned around from decreasing and showed increases due to the reduction of the low E_m species of cytochrome a_3. A possible alternative explanation to the one just offered is that the 429-nm peak in the higher voltage region is due to oxidized cytochrome a and that in the lower voltage region it is due to reduced cytochrome a_3. This requires that the electron distribution in the oxidized heme A of cytochrome a is so similar to that in the reduced heme A of cytochrome a_3 that the optical absorption spectra are essentially the same.

In any case, the results of the present paper rule out the alternative explanation. Both hemes are oxidized in the resting enzyme. Therefore the growth of the Soret peak at 429 nm seen when the resting enzyme is introduced into a medium in the voltage range of 430 to 750 mV is not due to the oxidation of reduced cytochrome a. Furthermore, the shift of the position of the peak from 429 to near 420 nm, the position characteristic of the oxidized hemes in the resting enzyme which occurs when the voltage is raised through the 750–790 mV region, is not due to the reduction of cytochrome a. We can also rule out that the peak at 429 nm which arises at 468 mV is due to reduced cytochrome a. The recognized spectrum for reduced cytochrome a has a

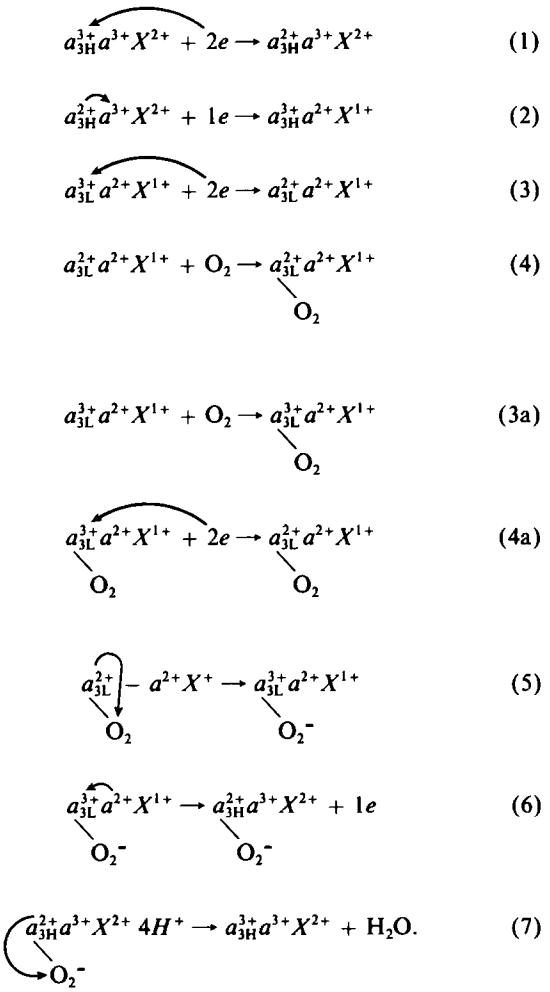
Soret peak at 448 nm and an α peak near 605 nm. We can generate this spectral change by lowering the voltage from 468 mV. On this basis, we attribute the 429-nm absorbance to reduced cytochrome a_3. The reduction of cytochrome a_3 at voltages up to 750 mV means that it must have an E_m above 750 mV. From the fact that O_2 is the natural electron acceptor of the enzyme, it is reasonable to expect the high voltage E_m to be 800 mV or less. The spectral change that occurs in the voltage range of 750 to 790 mV resulting in the shift of the 429-nm peak back towards the 420-nm position of the oxidized enzyme is consistent with the oxidation of the reduced cytochrome a_3. However, because of the selective alteration of the enzyme which occurs during the high-voltage titrations it has not been possible to reverse the high-voltage oxidation. Therefore, we have not established the actual values of the E_m and n. Nevertheless, because of the high percentage of the absorbance which undergoes the apparent reduced-to-oxidized transition, in a rather narrow voltage region we conclude that the E_m in near 770 mV and the n value is >1. Although most of the studies on the high voltage behavior of cytochrome oxidase were performed with the enzyme prepared according to Yoshikawa et al. (1977) we have observed the same results with a Yonetani-type preparation (Yonetani, 1961) made by David Bickar.

It must be stressed before any further consideration of the possible implications of these findings that the interpretations rest entirely on optical absorbance measurements. To be specific, we assign the Soret position near 420 nm as due to the oxidized hemes of cytochromes a and a_3. The spectrum representing reduced cytochrome a is taken to have a Soret maximum at 448 nm, and an α peak near 605 nm (our location is 606–607 nm). Both of these assignments are in agreement with the work of others (Wikström et al., 1981). Based on our earlier observations where potentiometric titrations were performed both in the presence and absence of CO, we recognize a spectrum with a Soret maximum near 429 nm and an α peak at 602 nm as due to a reduced form of cytochrome a_3 (Hendler et al., 1986). The concomitant disappearance of the (oxidized) peak at 420 nm and appearance of a peak at 429 nm is interpreted to be the result of a reduction of a heme center. All of our subsequent interpretations rest on the validity of this conclusion. If instead of signaling the reduction of a heme, the shift in absorbance can be due to a conformational change of the protein, resulting in a dramatic modification of the environment around a heme center, then our interpretations will be wrong. In the absence of an added ligand this kind of phenomenon has not been reported for cytochromes. If such a dramatic shift in the position of the Soret (or any other characteristic) absorbance can occur without a change in the redox state of a heme center, then not only this work but most of the kinetic and equilibrium studies performed with this enzyme will have to be reevaluated. The rise in the magnitude of the 429-nm absorbance that we observe in the 200–460 mV range is completely reversible, and the changes in absorbance are fit by the Nernst equation. If the formation of the Soret peak at 429 is due to a conformational change in the protein, then this change is controlled by the titration of some redox center. Although this is not the interpretation we propose it would not be without interest, especially because the same 429-nm absorbing peak is formed when the resting enzyme is exposed to any voltage between 460 and 750 mV. In any event, it is important to try to establish by some other independent technique that the rise in the 429 nm absorbance is due to a change in redox state of cytochrome a_3. We are planning to explore the use of resonance Raman spectroscopy for this purpose.

There is one other of our observations which requires some special consideration. Cytochrome a_3 is, by definition, the cytochrome that binds exogenous ligands such as CO. Every laboratory which has examined this property, including our own, has confirmed this fact. Therefore, our failure to observed any change in the optical spectrum, which we attribute to a reduced form of cytochrome a_3, in the presence of CO needs some explanation. To start, it is worth noting that all of the previous information and expectations concerning the binding of exogenous ligands to cytochrome a_3 are based on the low potential form of this cytochrome. Obviously, something must be quite different for the environment of the heme group in the case of the high-potential form. One possibility, which we are not actually proposing, is that an additional internal ligand is involved. Because the generation of the high-potential form is controlled by the oxidation of another center (X), it could be that the strong ligand is originally associated with X and that when X is oxidized, the ligand moves to the heme, resulting in its elevated E_m and loss of ability to bind CO. An alternative consideration of potential relevance is that the electronic configuration around the high-potential heme group must be quite different from that around the low-potential form. The stability of a ligand bond to CO could be seriously affected by such an alteration in the electronic configuration.

The newer findings reported in this paper must be accommodated in some way into a possible reaction scheme consistent with the role of the enzyme in cycling electrons from cytochrome c to O_2. The obligation to present at least one such possibility prompts us to show the following tentative and admittedly speculative reaction sequence. In this scheme, H and L refer to high- and low-potential forms of cytochrome a_3. A separate controlling center, X, is present because it is thermodynamically required to account for the turnaround in titration of cytochrome a_3 that has been observed (Hendler and Westerhoff, 1988). The controlling center may be a free radical or the "extra" Cu site proposed by Einarsdóttir and Caughey (1985*b*) and by Steffens et al. (1987) and Bombelka et al. (1986). Although separate copper centers are not specifically shown, based on our titration analysis, we assume that each of the traditionally known coppers

(i.e., Cu_A and Cu_B) can be coupled to a heme so that a pair of electrons is required to reduce each heme center.



$$a_{3H}^{3+}a^{3+}X^{2+} + 2e \rightarrow a_{3H}^{2+}a^{3+}X^{2+} \quad (1)$$

$$a_{3H}^{2+}a^{3+}X^{2+} + 1e \rightarrow a_{3H}^{3+}a^{2+}X^{1+} \quad (2)$$

$$a_{3L}^{3+}a^{2+}X^{1+} + 2e \rightarrow a_{3L}^{2+}a^{2+}X^{1+} \quad (3)$$

$$a_{3L}^{2+}a^{2+}X^{1+} + O_2 \rightarrow a_{3L}^{2+}a^{2+}X^{1+} \quad (4)$$

$$O_2$$

or

$$a_{3L}^{3+}a^{2+}X^{1+} + O_2 \rightarrow a_{3L}^{3+}a^{2+}X^{1+} \quad (3a)$$

$$O_2$$

$$a_{3L}^{3+}a^{2+}X^{1+} + 2e \rightarrow a_{3L}^{2+}a^{2+}X^{1+} \quad (4a)$$

$$O_2 \qquad O_2$$

$$a_{3L}^{2+} - a^{2+}X^{+} \rightarrow a_{3L}^{3+}a^{2+}X^{1+} \quad (5)$$

$$O_2 \qquad O_2^-$$

$$a_{3L}^{3+}a^{2+}X^{1+} \rightarrow a_{3H}^{2+}a^{3+}X^{2+} + 1e \quad (6)$$

$$O_2^- \qquad O_2^-$$

$$a_{3H}^{2+}a^{3+}X^{2+}\ 4H^{+} \rightarrow a_{3H}^{3+}a^{3+}X^{2+} + H_2O. \quad (7)$$

$$O_2^-$$

In Eq. 1, the fully oxidized molecule accepts the first pair of electrons on the high E_m cytochrome a_3 center. The reduction of this center by cytochrome *c* will be highly exergonic. The reaction shown in Eq. 2 implies a conformational change whereby the high E_m form of cytochrome a_3 is converted to the low E_m form when the controlling center becomes reduced (by either a single electron or a pair). This implied conformational change could be important in the role of the enzyme as a proton pump. A consequence of the change of redox potential of cytochrome a_3 relative to that of cytochrome *a* would be the internal transfer of electrons from the latter to the former. In Eq. 3, the second pair of electrons is added to the oxidized form of low E_m cytochrome a_3. This transfer is expected to be slightly endergonic. The fully reduced molecule would bind O_2 (Eq. 4). Alternate Eq. 3a and 4a are shown because our finding that the redox potential of CO-liganded low-potential cytochrome a_3 was not appreciably different from that of the free enzyme suggests that the oxidized form of low-potential cytochrome a_3 may also bind O_2 and then be reduced. In Eq. 5, the first pair of electrons is transferred to O_2, generating the peroxidatic stage of reduction. At this point, (Eq. 6) it is assumed that the ambient voltage would be suitable for the oxidation of the controlling center and regeneration of the high-potential form of cytochrome a_3. This would entail the opposite conformational change to that in Eq. 2 and could provide for the repositioning of the proton carrying group(s?) in a pumping cycle. Electrons in cytochrome *a* would then be transferred to cytochrome a_3 and then as shown in Eq. 7, from cytochrome a_3 to bound O_2^- with which four protons from the medium would complete the formation of H_2O. It is worth noting that the first pair of electrons is passed to O_2 from the low-voltage form of cytochrome a_3 and that the second pair of electrons is passed from the high-voltage form. This is consistent with the facts that the midpoint potential for the reduction of O_2 by a pair of electrons is ~0.3 V and that the midpoint potential for the reduction of O_2^- by a pair of electrons to form H_2O is ~1.3 V (see Naqui and Chance, 1986). Therefore, the electron transfer shown in Eq. 7 is expected to be exergonic.

We realize that this scheme differs from the usual view that cytochrome *c* reduces cytochrome *a* which then reduces cytochrome a_3 (see Wikström et al., 1981). The job of distinguishing a spectral change as due specifically to either cytochrome *a* or cytochrome a_3 is not easy, especially when the two cytochromes are probably undergoing a change of redox state at the same time so that the spectra are overlapping. Furthermore, the assignment of a particular spectral feature to either cytochrome a_3 or cytochrome *a* is not unambiguous. These assignments go back more than 25 years to studies using ligands to one or the other cytochrome and are based on the following assumptions. (*a*) No redox cooperativity affecting spectra exists. (*b*) The liganded heme offers zero spectral contribution. (*c*) Liganded hemes are completely protected from oxidations or reductions which change the redox state of the unliganded heme. (*d*) Combinations of difference spectra involving different preparations of CO- and CN-liganded enzymes in different states of reduction strictly follow the rules of algebraic addition (Yonetani, 1960; Vanneste, 1966; Horie and Morrison, 1963; Lemberg, 1969). Therefore, we believe that the changes in redox state attributed to one or the other particular heme center are not without question. Another factor is that the usual view is based on the concept that cytochrome a_3 has a single redox potential which is higher than that of cytochrome *a*. Even in the "neoclassical" view (Nicholls and Petersen, 1974; Malmström, 1974), the kind of redox cooperativity we have incorporated into the scheme presented above is absent. In the "neoclassical" scheme the first electron introduced from cytochrome *c* is shared equally by kinetic exchanges between two isopotential heme centers. The second electron enters at a lower effective E_m to complete the total reduction of the two heme centers. There is no redistribution of electrons held uniquely by each of the heme centers as proposed in the current scheme based on our new

findings. Our proposal that electrons from cytochrome *c* may not have to pass through cytochrome *a* en route to O_2, though based on different reasons, may be compared to the same conclusion reached by Hill and Greenwood (1984) based on rapid kinetic measurements of the oxidation of fully reduced cytochrome aa_3 by O_2 in the presence and absence of cytochrome *c*.

The reaction scheme encompassed in these equations predicts several unique spectral changes that should be seen during a complete four-electron oxidation or reduction of the enzyme. The internal electron transfers between cytochromes a_3 and *a*, due to the change in redox potential of the former in relation to the latter as shown in Eqs. 2 and 6, should lead to the kind of difference spectra we have observed in potentiometric titrations in which the orientation of the features for cytochrome *a* at ~448 nm and at ~605 nm is opposite to the orientation of the characteristic feature for cytochrome a_3 at 429 nm. The direct reduction of cytochrome a_3 when cytochrome *a* is already reduced, as shown in Eq. 3 should lead to a spectrum with a Soret peak at 429 nm and an α peak at 602 nm. In addition, a unique prediction of this scheme is that there should be several reversals in the direction of change of individual spectral peaks as electrons come on and off of a particular center several times during the sequence. For example, the peak at 429 nm should change its direction (from increasing to decreasing) several times as indicated in Eqs. 1–3 and 5–7. In the vicinity of the α absorbances near 605 nm, changes in the direction of growth or decline should occur as indicated in Eqs. 2 and 3 compared with Eqs. 5 and 6. The presence of O_2 will introduce additional spectral modifications to those we have outlined on the basis of our anaerobic titrations.

Evidence for a reciprocal relationship for the Soret peaks at 429 and 448 nm was first presented in 1980 (Scott and Gray). We have conducted a preliminary analysis of three sets of timed sequences of spectral changes obtained at different low temperatures after the photo-release of bound CO from the fully reduced enzyme in the presence of O_2. In these studies, full optical spectra were available and singular value decomposition of the spectra was performed. In addition, direct difference spectra during different time periods of each kinetic run were examined.

In the preliminary studies we have observed (*a*) the reciprocal spectra with the opposite orientation of peaks at ~448 nm and ~605 nm relative to the peak at 429 nm, (*b*) a difference spectrum showing only a Soret peak at 429 nm and an α peak at 602 nm, and (*c*) Several reversals of direction in the growth and decrease of peaks in the Soret and α regions. These studies, although preliminary, show that singular value decomposition is a valuable tool for separating the many overlapping spectral changes which occur during the complete oxidation of the reduced, initially CO-liganded molecule. A more thorough application of these methods to kinetic spectra at low temperature is planned.

Finally, the similarity between the spectral interrelations of the 429-nm peak and the peak near 420 nm discussed throughout this paper and that of the 427- and 420-nm peaks described by Kumar et al. (1984) deserves comment. The 420-nm peak of Kumar et al. (1984) was generated from the resting enzyme by oxidation with either O_2 or ammonium chloroiridate. Its spectrum was close to but subtly different from that of the resting enzyme. The 420-nm form was converted to the 427-nm form by addition of 5 mM H_2O_2 in the presence of a trace of catalase. These results were interpreted as showing that the 427-nm form (analogous to the 428 nm form of Brunori et al., 1981) was a complex of a peroxide and an oxidized species of cytochrome a_3. We have attributed our 429-nm peak to an unliganded form of reduced cytochrome a_3. There are three prominent possibilities, that we see, to reconcile these observations.

(*a*) Reduced nonliganded cytochrome a_3 and oxidized, peroxide-liganded cytochrome a_3 produce very similar absorption spectra.

(*b*) Even though we observe the formation of the 429-nm form from the oxidized enzyme both by a reductive titration below 200 mV and by holding the enzyme anaerobically at 430 mV, and the presence of O_2 does not change the latter transition, and we have somehow generated H_2O_2 from H_2O, to produce the peroxide form of oxidized cytochrome a_3 in both cases. In addition to all of this, condition (*a*) must also hold.

(*c*) The 420-nm form of Kumar et al. represents our oxidized cytochrome oxidase in which the E_m of cytochrome a_3 is near 770 mV. The addition of H_2O_2 results in electron transfer from H_2O_2 to high potential cytochrome a_3 producing reduced cytochrome a_3 plus either O_2 or O_2^-. The E_m for the one-electron oxidation of H_2O_2 at pH 7 is 890 mV and for the two-electron oxidation it is 281 mV.

We strongly discount possibility (*b*) and are in no position to decide between possibilities (*a*) and (*c*).

The kinetic spectra for the oxidation of cytochrome *c* oxidase by O_2 at a variety of low temperatures were performed by Dr. Ali Naqui in the laboratory of Dr. Britton Chance at the University of Pennsylvania and analyzed by Dr. Richard W. Hendler and Mr. Richard I. Shrager at National Institutes of Health. The high-voltage mediators, octacyanotungstate and octacyanomolybdate, were gifts from James A. Fee and Jay M. Johnson. The enzyme used for most of these studies was prepared by Gary Yewey according to the procedure of Yoshikawa et al. (1977). Confirmatory experiments were performed with a Yonetani (1961) preparation isolated by David Bickar. We thank Winslow Caughey for his interest, cooperation, and discussions.

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