High voltage redox properties of cytochrome *c* oxidase

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ABSTRACT In earlier studies evidence was obtained for the existence of both high and low redox potential forms of cytochrome a_3 (Hendler et al. 1986. *Biophys. J.* 49:717–729; Hendler and Sidhu. 1988. *Biophys. J.* 54:121–133). The current paper describes additional experiments that support this conclusion and then reviews a large number of experimental observations that appear to be consistent with the view that cytochrome a_3 displays at least (see Sidhu and Hendler. 1990. *Biophys. J.* 57:1125–1140) two different forms, which are distinguishable by their redox potentials, spectra, and reactivity with CO.

INTRODUCTION

Cytochrome *c* oxidase is an efficient energy transducing machine powered by the transfer of four electrons from a donor, cytochrome *c*, with a midpoint potential of ~265 mV to an acceptor, O_2, with a midpoint potential of ~810 mV. This represents ~50 kcal or 209 KJ of energy. If O_2 is reduced in two steps, the first pair of electrons would be accepted with an E_m of ~0.3 V to form peroxide, and the second pair with an E_m of ~1.3 V to form H_2O (Wood, 1988). A monomer of cytochrome *c* oxidase contains two hemes, three coppers, one magnesium, and one zinc (Einarsdottir and Caughey, 1985; Bombelka et al., 1986; Steffens and Buse, 1988). Many potentiometric titrations using potassium ferricyanide as the oxidant and the highest potential redox mediator have revealed two Nernstian redox transitions with E_m values of ~230 and ~360 mV (Brunori et al., 1988). We have shown that the lower E_m transition can be resolved into three independent Nernstian transitions (Hendler et al., 1986; Sidhu and Hendler, 1990). A redox titration system using potassium ferricyanide as the highest potential component cannot explore redox behavior above 400 mV. O_2 is probably not reduced in a single step involving four electrons. Therefore, it is reasonable to expect that the enzyme contains at least an additional redox center with an E_m value between 360 and 1,300 mV.

To explore the redox behavior in the range 400 to 850 mV, we used an oxidizing Pt electrode and two mediators with E_m values of 510 and 778 mV (Hendler and Sidhu, 1988). With this system, direct evidence was obtained for a redox titration with an E_m of ~770 mV. This finding was consistent with earlier suggestive evidence that a high E_m transition for cytochrome a_3 existed; namely, that at 450 mV cytochrome a_3 appeared to be reduced (Hendler et al., 1986). In addition to this high voltage redox transition, direct evidence for a Nernstian redox transition for cytochrome a_3 below 200 mV was also obtained (Hendler et al., 1986). One other pertinent observation concerning the redox behavior of cytochrome a_3 is that upon lowering the voltage from 450 to 200 mV, the simultaneous reduction of cytochrome *a* and oxidation of cytochrome a_3 is seen (Hendler et al., 1986; Hendler and Sidhu, 1988). Our explanation for these observations considered a strong cooperative redox interaction involving a center, whose redox state controls the redox potential of cytochrome a_3.

In this work we have taken another look at the overall evidence for the existence of both high and low redox potential states for cytochrome a_3, and for strong cooperative interactions that regulate these potentials. One aspect of this appraisal concerns the evidence that cytochrome a_3 is actually in a reduced state at 450 mV. This evidence was based on the shift of the Soret band from its position near 420 nm, characteristic of the oxidized species, to ~429 nm, characteristic of a low spin form of reduced cytochrome a_3 (Hendler and Sidhu, 1988). Experiments were designed to see whether the metallocyanide redox mediators used in these titrations could have released CN in sufficient concentration to complex with oxidized cytochrome a_3, forming a spectral complex that has been mistakenly identified as the reduced form of the enzyme.[1]

[1]Although cyanide is photolytically released from the metallocyanide compounds, at the neutral pH of the experiments reported in this paper it would tend to be almost completely in the protonated form. The term cyanide, used in this paper, should be taken to mean both the free protonated forms.

0006-3495/90/10/957/11 $2.00

The metallocyanides of Fe, W, and Mo are notoriously stable with respect to hydrolysis, and exhibit virtually no exchange with radioactive cyanide for periods up to several days in the dark (Adamson et al., 1950; Taube, 1950; MacDiarmid and Hall, 1954; Zielinski, 1978). For these reasons, they have been called "covalent" complex salts (Clark et al., 1954). The dissociation constant for the reversible release of cyanide from $Fe(CN)_6^{-3}$ is estimated to be $<10^{-12}$ M (Adamson et al., 1950). Light, however, can effectively increase this constant and the photolysis of all of these compounds has been actively studied (Sieklucka and Samotus, 1980; Nya and Mohan, 1984). In the current work, we have assayed the concentration of cyanide released by each of these metallocyanide complexes during the course of spectral potentiometric titrations, determined the amount of spectral change caused by the same or higher concentration of cyanide added directly to the enzyme, and performed similar studies at high voltage where cyanide release has been prevented. In addition, we have evaluated a further possible complication in the use of these redox mediators. It is thermodynamically possible for cyanide to be oxidized to cyanogen (C_2N_2) under the conditions of potentiometric titration. Cyanogen may possibly have a strong binding tendency to cytochrome a_3 heme. We are not aware of any studies testing this possibility. Therefore, we determined whether C_2N_2 is present during normal potentiometric studies above 400 mV and whether it can bind to cytochrome oxidase and cause spectral changes. Finally, we review all of the evidence obtained so far bearing on the existence of high and low redox potential forms of cytochrome a_3.

EXPERIMENTAL METHODS

Spectrometer, sample illumination, and experimental details

The spectrometer, experimental conditions, enzyme preparation, and metallocyanides have been previously described (Hendler and Sidhu, 1988). The most pertinent aspects of the experimental set-up, in terms of the release of cyanide from the metallocyanide complexes, however, will be reviewed here. Because the spectrometer has sensitive photodiode array detector, low levels of illumination of the sample are required. Light from a 100-W quartz halogen filament lamp operated at ~85% of the recommended voltage (i.e., ~10 V) was passed through an infrared and a 2.0-A (units) neutral density filter before impinging on the sample cuvette. Light at 365 nm is commonly used in photolysis studies of metal cyanides. We have used a calibrated photodetector with a model 550 radiometer (EG & G, Salem, MA) to measure light flux falling on the sample cuvette with appropriate filters to quantify the energy of irradiation in different wavelength ranges.

Assay for cyanide and cyanogen concentrations

Cyanide concentration was determined by the method of Epstein (1947). In this method, cyanide is oxidized to cyanogen chloride with chloramine T. Color is developed by reaction of the CNCl with pyridine in the presence of bis-pyrazolone (cat. #6969; Kodak) and 1-phenyl,-3-methyl,5-pyrazolone (cat. #17564; Alfa products, Danvers, MA). The latter is made by mixing five parts of an aqueous suspension of the phenyl methyl pyrazolone (made by stirring 1.5 mg per ml intermittently for ~1 h before use) with one part of a solution of 0.1% (wt/vol) of bis-pyrazolone in pyridine. The color was quite stable and read 50 min after addition of the pyridine/pyrazolone reagent.

The assay for cyanogen (C_2N_2) was based on the disproportionation of C_2N_2 occurring in 0.1 M KOH (Corain et al., 1981), which produces 1 mol each of cyanide and CNO^- from 1 mol of C_2N_2. The CNO^- does not produce any color in the cyanide assay, nor does it interfere with color development from cyanide. The disproportionation in 0.1 M KOH proceeds rapidly during the first 5 min, is ~90% complete in 15 min, and is ~100% complete in ~60 min. The assay for C_2N_2 is based on the difference in color for paired samples in which one has been exposed to the alkali and the other has not. Routinely the 0.5-ml disproportionation sample, in duplicate, was treated with 0.1 ml 0.6 N KOH for 15 min followed by 0.1 ml 0.6 N HCl and 0.1 ml 1 M potassium phosphate buffer (pH 7). Water was added to 1 ml total volume, followed by 0.2 ml 1% aqueous chloramine T (cat. #13665; Alfa Products) and then after 2.5 min by 6 ml of the pyridine/pyrazoline reagent. The paired samples were treated as above except that no KOH or HCl was used and no 15-min waiting period was involved. Typical assay curves are shown in Fig. 1.

Fig. 1 *A* shows results obtained for assays using a stock KCN test solution of 100 μM KCN with (solid points) and without (open symbols) KOH treatment. There is no significant difference in the two assays. For the experiments shown in Fig. 1 *B*, one of the test solutions contained both C_2N_2 and KCN. A saturated aqueous solution of C_2N_2 was diluted 1:2,000 in the 100-μM test KCN solution. The KOH-treated samples (solid line and symbols) produced significantly more color than did the non–KOH-treated samples (short dashed line with open symbols). The difference between these two curves is a measure of the amount of C_2N_2 that was present and these data are plotted in Fig. 1 *C*. The long dashed line

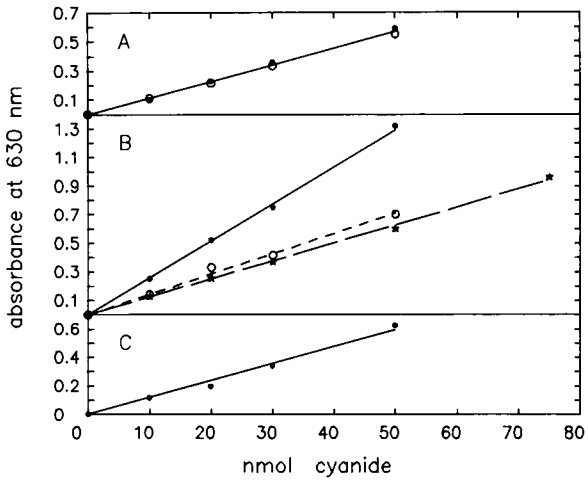

FIGURE 1 Cyanide was assayed by the procedure of Epstein (1947) as described in Methods. *A* shows that exposing the KCN samples to 0.1 M KOH for 15 min before neutralization and assay (*solid points*) produced the same amount of color as for samples not exposed to KOH (*open symbols*). *B* shows that for a test solution containing both C_2N_2 and KCN, the KOH treatment (*solid curve with solid points*) produced more color than for the same samples not exposed to KOH (*short dashed line with open symbols*). The curve obtained with KCN alone is shown by the long dashed line with star symbols. The difference between the assays from the KOH-treated and nontreated samples is a measure of the C_2N_2 present and these data are plotted in *C*.

with star symbols in Fig. 1 *B* was obtained from the C_2N_2 and KCN test solution without KOH treatment. It appears that ~10% free cyanide compared with the amount of C_2N_2 was present in the original C_2N_2, saturated aqueous sample. This either came from the original tank of C_2N_2 or was generated from the C_2N_2 in the aqueous solution. This small amount of cyanide could explain the observation described below and depicted in Fig. 5, that at 10- and 100-μM amounts of C_2N_2 added to cytochrome oxidase there was a slight reduction of cytochrome *a*. Expressing the difference curve in Fig. 1 *C* in terms of cyanide concentration using the standard KCN assay curve, and allowing for ~90% of disproportionation in 0.1 M KOH in the 15-min exposure time of the assay leads to a calculated concentration of 0.2 M for C_2N_2 in the initial saturated aqueous solution. This is the solubility for C_2N_2 in water in the literature (Perry et al., 1963).

C_2N_2 was obtained in a cylinder from Matheson Gas Products, Inc. (Secaucus, NJ). Working solutions were obtained by saturating 1 ml of H_2O with the gas during a 10-min bubbling period in a specially constructed scintered glass scrub bottle with electrically operated valves on both the input and exit tubes and a rubber port through which small quantities of the saturated solution could be removed with a Hamilton syringe.

RESULTS

1. Release of cyanide from metallocyanide complexes under the light intensity used for spectroscopic studies.

The level of light flux impinging on the cuvette was 120 μJ/s below 550 nm and 18 μJ/s using a UV filter centered at 340 nm with a band width of 100 nm at half height. The concentrations of cyanide achieved over the first 2 h of illumination for the three cyanide complexes studied in an open stirred cuvette are shown in Fig. 2.

The solid line in Fig. 2 *A* was obtained during an

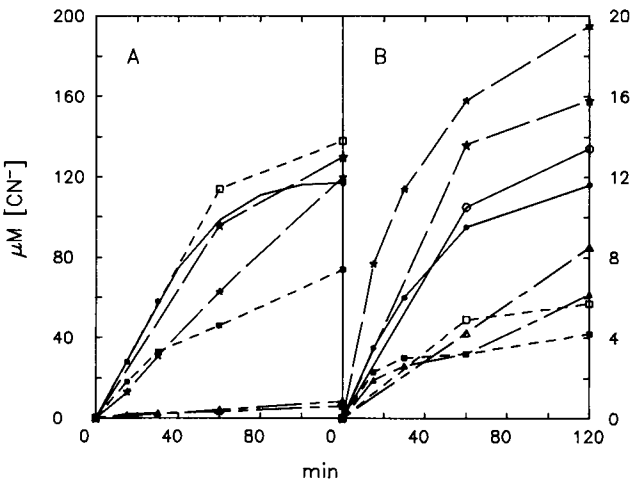

FIGURE 2 Solutions of metallocyanide redox mediators were incubated with stirring in 125 m KCl, 62.5 mM potassium phosphate (pH 7.0) in the spectrometer in an open cuvette or left sitting on the bench top in ambient light. Cyanide concentration was assayed as described in Methods. (*A*) The concentration of metallocyanides was 0.2 mM in all cases. Solid curve, $K_3Fe(CN)_6$ and $K_4W(CN)_8$ in the spectrometer; short dashed curves, $K_4W(CN)_8$, upper curve with open squares in ambient light, lower curve with solid squares in the spectrometer; long dashed curves, $K_4Mo(CN)_8$, upper curve with large open stars in ambient light, lower curve with small closed stars in the spectrometer; alternating short, long dashed curves, $K_3Fe(CN)_6$, upper curve in ambient light, lower curve in the spectrometer. (*B*) Note 10-fold expansion of ordinate. The four upper curves were obtained with 3 mM of the iron cyanide mediators and the four lower curves with 0.2 mM concentrations. The alternate short, long dashed curves with triangles in the lower part of the panel are the same two as shown in the lowest part of Fig. 1 *A* and represent 0.2 mM $K_3Fe(CN)_6$. The short dashed curves in the lower part of *B* were obtained with 0.2 mM $K_4Fe(CN)_6$. The open squares represent the incubation in ambient light and the closed squares represent the incubation in the spectrometer. In the upper part of the panel the solid curves were obtained from an incubation with 3 mM $K_3Fe(CN)_6$, open circles in ambient light, closed circles in the spectrometer. The long dashed curves represent incubations with 3 mM $K_4Fe(CN)_6$, the upper curve was from the incubation in ambient light, and the lower curve was from the incubation in the spectrometer.

incubation of 0.2 mM each of $K_3Fe(CN)_6$ and $K_4W(CN)_8$ in the spectrometer with no voltage control. An incubation of the same solution outside of the spectrometer on the bench top in ambient light produced an amount of cyanide in excess of 200 μM in 1 h (not shown). The short dashed line with solid square symbols shows cyanide release from 0.2 mM $K_4W(CN)_8$ in the spectrometer. A comparable sample held in ambient light released the higher levels of cyanide shown in the upper short dashed line curve with open square symbols. The two long dashed lines with star symbols show cyanide release from 0.2 mM $K_4Mo(CN)_8$ with the lower curve representing the sample held in the spectrometer. It appears that in all of these cases the level of light intensity was significantly lower in the spectrometer than in normal room illumination. $K_3Fe(CN)_6$ was significantly more resistant to photolysis as shown by the two lowest curves in the figure obtained with 0.2-mM concentration in the spectrometer (lower curve) and on the bench top (upper curve).

A further study of the stability of the iron complexes is shown in Fig. 2 *B*, where the *y* axis is expanded 10-fold. The alternating short and long dashed curves with the triangle symbols (shown in the bottom part of the figure) are the same as shown at the bottom of Fig. 2 *A* for 0.2 mM $K_3Fe(CN)_6$. The short dashed lines with closed (spectrometer) and open (ambient) square symbols were obtained with 0.2 mM $K_4Fe(CN)_6$. It is not uncommon for 3-mM concentrations of the iron cyanide complexes to be used with cytochrome oxidase (e.g., Wikström, 1981). The upper curves in Fig. 2 *B* show cyanide release from these higher concentration incubations. The solid line curves were obtained with $K_3Fe(CN)_6$ and the long dashed line curves with $K_4Fe(CN)_6$. In both cases the points taken at 60 and 120 min for the samples held in ambient light were higher than those from the samples in the spectrometer.

2. Stability of the complexes in the dark and reversibility of the light-stimulated cyanide release by a dark period

In Fig. 3, four incubations in the spectrometer are shown in which 0.2 mM $K_3Fe(CN)_6$ and 0.2 mM $K_4W(CN)_8$ were present. Incubations with the sample illuminated are shown by solid lines and incubations in the dark are shown by dashed lines. In the dark, the level of cyanide was between 0 and 2 μM. In the light, ~40 μM cyanide was attained in 20 min. The curves shown with triangle and star symbols were for incubations in the presence of cytochrome oxidase. The rate of rebinding of photolyzed cyanide is indicated in the curve with open circle symbols where about half of the released cyanide disappeared in ~20 min in the dark. Levels of free cyanide released in

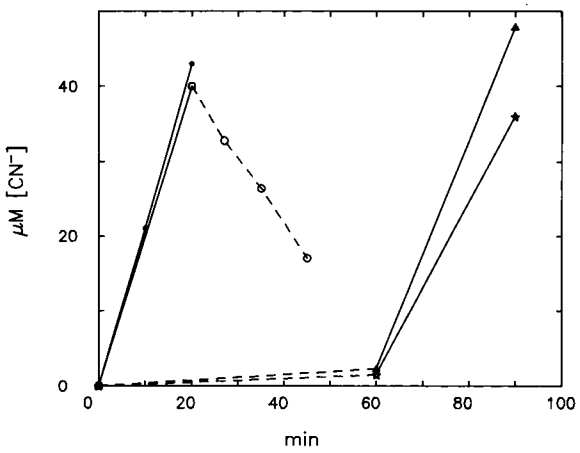

FIGURE 3 Solutions of 0.2 mM each of $K_3Fe(CN)_6$ and $K_4W(CN)_8$ in 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0) were incubated in the spectrometer under voltage-controlled conditions (Hendler and Sidhu, 1988) at ~470 mV. The major difference between these conditions and an actual experiment was that removal of samples for cyanide assays at intermediate times lowered sample volume and thereby changed the level of irradiation per unit volume. Four separate experiments are shown. Cytochrome aa_3 (6 μM heme A) was present for the experiments shown with triangles and stars. The solid line curves represent incubations with the sample exposed to the light source, attenuated as described in Methods. The dashed line portions show the results of incubation with the light source blocked from the sample. Cyanide concentration was assayed as described in Methods.

these experiments generally tended to be higher than in actual experiments (c.f. Figs. 5 and 6) because samples were removed for cyanide assay at intermediate times, which decreased the total volume of solution, leaving a higher proportion in the path of the strongest light intensity.

3. Ability of cyanide to reduce cytochrome *a*

It is well known that many preparations of cytochrome *c* oxidase react very slowly with cyanide (Naqui et al., 1984; Baker et al., 1987; Wrigglesworth et al., 1988). The modified Volpe-Caughey preparation used for most of the studies performed in this laboratory is particularly sluggish in its reactivity with cyanide (ibid.). It is not generally appreciated that in addition to its well-known tendency to form complexes with the ferric heme A of cytochrome a_3, cyanide is also a potential reducing agent, theoretically capable of reducing both hemes in cytochrome oxidase. Corain et al. (1981) list the standard reduction potential of the C_2N_2/HCN couple at 370 mV. At pH 7.0 the E'_m of the couple would be -50 mV. From the fact that cyanide is energetically capable of reducing the hemes in cytochrome oxidase it does not follow that it

is a competent reductant. It is worth noting, however, that C_2N_2 is produced from cyanide by oxidation with $CuSO_4$ (Janz, 1957). Among a number of experiments in which resting cytochrome oxidase was incubated with 100 μM cyanide, in only two cases did the Soret and α peaks for reduced cytochrome *a* (at ~446 and ~606 nm) clearly stand out. These two cases were with one particular enzyme preparation, which is no longer available. In all of the other cases the only evidence for reduction of cytochrome *a* was in the existence of a shoulder at 446 nm on the major Soret peak at ~433 nm due to cyanide complex formation with oxidized heme a_3 and of a small α absorbance at ~606 nm. Fig. 4 shows two such incubations of the enzyme in 100 μM KCN under anaerobic conditions. In the top panel, anaerobiosis was maintained by a flow of argon over the solution at a rate of 300 cm^3/min. In the bottom panel the argon flow rate was reduced at ~20 cm^3/min. In both cases the difference spectrum for 60-min incubation in 100 μM KCN was corrected for the spectral changes occurring in control incubations with the appropriate argon flow but no cyanide present. Indications of cytochrome *a* reduction are the shoulder in absorbance at 446 nm on the peak at 433 nm and the small but definite α peak at ~606 nm. At lower concentrations of cyanide, evidence for some cytochrome *a* reduction is still seen. Incubations for 60 min in 2 and 50 μM cyanide are shown in Fig. 6, *b* and *d* below.

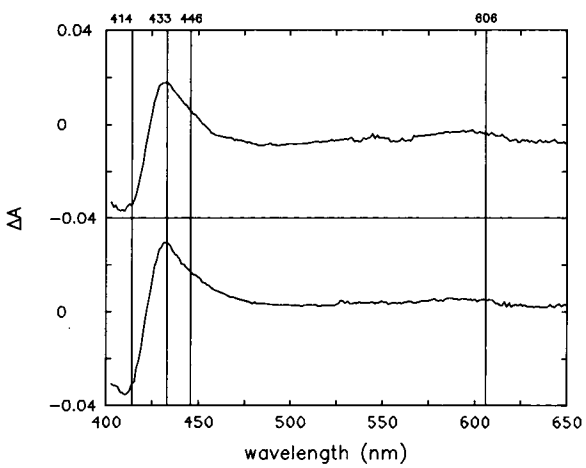

FIGURE 4 Cytochrome aa_3 (6 μM heme A) was incubated in 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0) under flowing argon for 30 min, followed by the addition of 100 μM KCN and a subsequent 60-min incubation. For the experiment depicted in the top panel, the argon flow rate was 300 cm^3/min. For the experiment depicted in the bottom panel, the argon flow rate was reduced to ~20 cm^3/min after the pregassing phase. For both cases, control experiments were conducted in the absence of added KCN. The difference spectra shown in the figure (60 min minus 30 s) were corrected for spectral changes occurring in the absence of KCN.

4. Absence of correlation between the spectral shift occurring at ~470 mV with cytochrome oxidase and the presence or concentration of cyanide

Fig. 5 shows time courses for the spectral shift of the Soret peak from its position near 420 nm in the resting oxidized enzyme to near 428 nm while sitting in a medium held at ~470 mV. The difference spectrum resulting from this shift shows a peak near 435 nm and a trough near 416 nm (Hendler and Sidhu, 1988). This shift has been attributed to the reduction of a high voltage form of cytochrome a_3. The main purpose of the current studies was to see if this spectral shift can be attributed to the formation of a cyanide complex of oxidized cytochrome a_3 with cyanide released from the metallocyanide complexes present as redox mediators.

The solid line curves represent experiments performed in the usual way under continuous illumination of the sample cuvette in the optical spectrometer with a continuous flow of 300 cm^3/min of argon over the solution. The medium contained 0.2 mM $K_3Fe(CN)_6$ and 0.2 mM

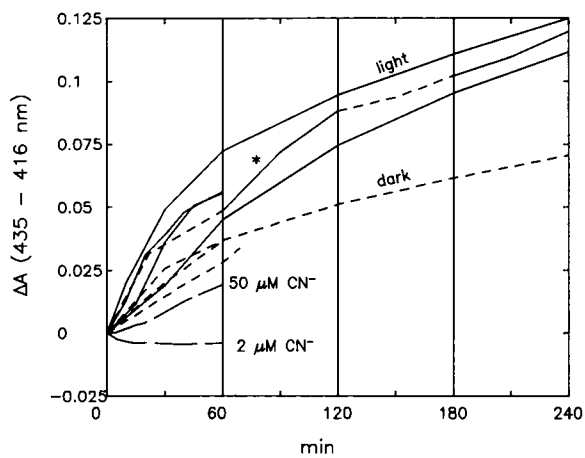

FIGURE 5 Cytochrome aa_3 (6 μM heme A) in 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0) and 0.2 mM each of $K_3Fe(CN)_6$ and $K_4W(CN)_8$ were incubated at ~470 mV in the spectrometer as previously described (Hendler and Sidhu, 1988). The solid line curves represent incubations in the normal attenuated light from the spectrometer. For these illuminated samples a 20-min pregassing period under illumination conditions was performed just before the zero time point shown in the figure. The short dashed curves were obtained with the light source blocked. In these cases, the pregassing phase was also performed in the dark. The curve marked with an asterisk was pregassed in the dark and then subjected to alternating 60-min periods of light and dark. The ordinate represents the shift of the Soret peak from ~420 nm for the resting enzyme to ~428 nm during the incubations at 470 mV. The long dashed curves represent incubations with no metallocyanide mediators present, but with the addition of either 2 or 50 μM KCN. Cyanide concentration was assayed as described in Methods.

$K_4W(CN)_8$, voltage was held at ~470 mV, and before the start of the incubation the cuvette was pregassed with argon for 20 min in the spectrometer under continuous illumination, but no applied voltage. At the end of the pregassing period, which is the zero time for the incubation, ~20 μM cyanide was usually present. This level increased to ~30 μM at 60 min. Curves with short dashed lines represent incubations in which the spectrometer light was blocked from impinging on the sample cuvette. For these incubations, the light was also blocked during the 20-min pregassing stage. The only direct illumination on the samples occurred during ~10 s of each 30 min for the recording of spectra. The level of cyanide present under these conditions was <2 μM. An average of four experiments held in the dark for pregassing followed by 60-min incubation at 470 mV in the dark showed 65% of the shift of the Soret peak compared with an average of four incubations under complete illumination. A subsequent period of full illumination from 60 to 120 min after an initial 60-min dark incubation caused a possibly slight enhancement in the rate of the spectral shift (curve marked with an asterisk), but two subsequent alternations of dark and light periods showed minimal effects. When incubations were carried out in the absence of metallocyanide mediators, but in the presence of 2 μM cyanide, no shift in position of the Soret peak occurred (lower long dashed curve and Fig. 6 *b*.) This is consistent with the K_D of 10^{-4} M for the cytochrome a_3–cyanide complex, and it shows that *the shift that occurred during the dark incubations could not be due to the free cyanide in the medium* (cf. Fig. 6, *a* and *b*). The higher of the two long dashed curves in Fig. 5 shows the extent of the ΔA (435–416 nm) occurring in the presence of 50 μM KCN (absence of mediators) which is considerably above the cyanide concentrations actually found in the illuminated incubations. Fig. 6 *c* shows the spectral change that occurred during 60-min incubation in the light where the average cyanide concentration was <26 μM. Fig. 6 *d* shows the spectral change occurring during 60-min incubation in the presence of 50 μM KCN. The shoulder at ~446 nm and small elevation at ~606 nm are consistent with some small degree of reduction of cytochrome *a* during the incubation as discussed above. The shape of the difference spectrum resulting from the 60-min incubation in the presence of mediators at 470 mV (*c*) is quite different from the one incubated in the presence of a higher amount of KCN (*d*).

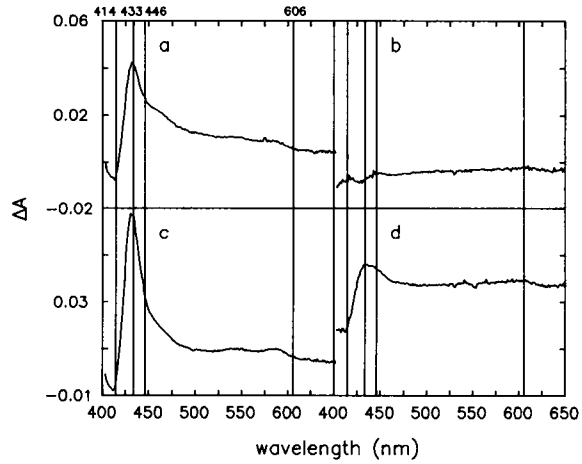

FIGURE 6 (*a*) Difference spectrum resulting from a 60-min incubation in the dark, under argon, at 470 mV (vs. S.H.E.) of cytochrome aa_3 (6 μM heme A) in the presence of 0.2 mM each of $K_3Fe(CN)_6$ and $K_4W(CN)_8$. The 60-min incubation was preceded by a 20-min pregassing with argon, of the mediator buffer solution in the dark before setting of the voltage to 470 mV and addition of the enzyme. The concentration of cyanide in the medium was <2 μM during the whole incubation. (*b*) Difference spectrum resulting from addition of 2 μM KCN to the anaerobic enzyme, under argon, and incubation with full illumination for 60 min in the absence of $K_3Fe(CN)_6$ and $K_4W(CN)_8$. The spectrum has been corrected to account for small background spectral changes occurring during 60 min in the absence of added KCN in a control incubation. (*c*) Difference spectrum resulting from the same kind of incubation described for *a*, but conducted under illumination in the spectrometer. The level of cyanide achieved at the end of the 60-min incubation was 26 μM. (*d*) Difference spectrum resulting from the addition of 50 μM KCN to the anaerobic enzyme, under argon, and incubation for 60 min in the absence of $K_3Fe(CN)_6$ and $K_4W(CN)_8$. The spectrum has been corrected for small background spectral changes occurring during 60 min in the absence of added KCN in a parallel incubation.

5. Experiments with C_2N_2

Cytochrome aa_3 (6 μM heme A) was incubated in the presence of 10 μM C_2N_2 for 1 h, followed by 100 μM C_2N_2 for 1 h, and finally by 1,000 μM C_2N_2 for 1 h. The difference spectra resulting from these three sequential incubations are shown in Fig. 7. During the first 1-h exposure to 10 μM C_2N_2 a typical spectral change is seen for the partial reduction of cytochrome *a* with a Soret peak at ~446 nm and an α peak near 606 nm (top). This is accompanied by the disappearance of a Soret at ~428 nm, which we attribute to the oxidation of reduced high potential cytochrome a_3. During the second 60-min incubation in 100 μM C_2N_2 the Soret maximum position shifts to ~437 nm and the minimum to ~411 nm (middle). The last 60-min incubation in the presence of 1,000 μM C_2N_2 shows a Soret maximum of ~431 nm and a minimum at ~408 nm, accompanied by a broad α band from 580 to 600 nm (bottom). The peak is shifted ~3 nm and the trough ~6 nm to the blue compared with the spectrum obtained when the resting enzyme is held at a voltage of ~470 mV. The spectral change under those conditions shows a Soret maximum near 435 nm and a minimum near 415 nm. The reduction of cytochrome *a* seen at the two lower concentrations of C_2N_2 is probably

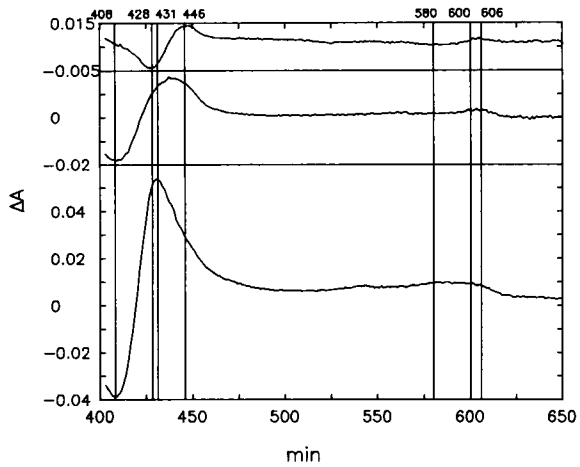

FIGURE 7 Cytochrome aa_3 (6 μM heme A) was incubated in 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0) in the spectrometer without stirring or voltage control in the presence of 10 μM C_2N_2 for 1 h. The upper curve shows the difference spectrum at the end of the incubation minus the resting enzyme. C_2N_2 was increased to 100 μM and the incubation continued for 1 h. The middle panel shows the difference spectrum at the end of the second hour minus that at the end of the first hour. C_2N_2 was increased to 1,000 μM and the incubation continued for an additional hour. The bottom panel shows the difference spectrum at the end of the third hour minus that at the second hour. Vertical lines are drawn at 408, 428, 431, 446, 580, 600, and 606 nm.

due to the presence of some cyanide which we have found to be in the aqueous solutions of C_2N_2 and which is potential reductant for cytochrome *a* at low concentration.

To see if C_2N_2 is formed from cyanide at 470 mV in the presence of 0.2 mM $K_3Fe(CN)_6$, we incubated 100 μM cyanide for 60 min with constant stirring. During this time the cyanide concentration decreased by ~10% but the chemical assay gave no evidence of C_2N_2 formation. The decrease was due to a loss of HCN from the solution. No such loss resulted from an unstirred and stoppered solution. Finally, the incubation at 470 mV was repeated in the presence of cytochrome aa_3 (6 μM heme A) to see if the enzyme could catalyze the oxidation of cyanide to C_2N_2. That no such catalysis occurred is indicated both by the fact that the rate of disappearance of cyanide during the first 15 min was the same in the presence or absence of the enzyme and by the absence of an indication of C_2N_2 formation using the chemical assay.

6. Additional experiments relevant to the existence of a high potential form of cytochrome a_3 and of redox cooperativity

If a form of cytochrome a_3 exists with an $E_m \approx 770$ mV (Hendler and Sidhu, 1988), then some reduced high potential cytochrome a_3 should be present in stored preparations of the enzyme. This is especially so in view of the fact that preparations of pure cytochrome oxidase contain endogenous reducing sources (Young and Caughey, 1987; Fabian and Malmström, 1989). The reduced cytochrome a_3 should be oxidized by O_2. Fig. 8 (top) shows difference spectra resulting from an incubation of the resting enzyme (6 μM heme A) in 125 mM KCl, 62.5 mM potassium phosphate buffer (pH 7.0) in an open cuvette for 1, 2, 3, and 4 h (bottom to top). The oxidation of a reduced form of high potential cytochrome a_3 is indicated by the disappearance of the peak at ~428 nm attributed to the reduced form of the reduced cytochrome (Hendler and Sidhu, 1988).

If the redox potential of cytochrome a_3 is ~770 mV when another controlling redox center, X, is oxidized, but is ~175 mV when X is reduced, then reduction of center X at medium voltages >200 mV and <750 mV should lead to the oxidation of cytochrome a_3 (Hendler et al., 1986; Hendler and Sidhu, 1988; Hendler and Westerhoff,

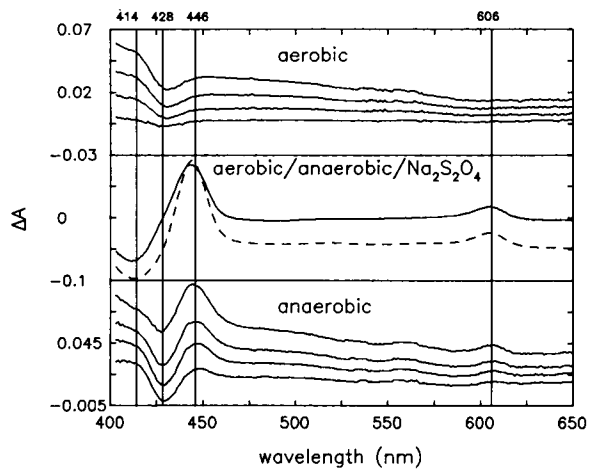

FIGURE 8 (*Top*) Cytochrome aa_3 (6 μM heme A) was incubated for 4 h in an open cuvette containing 125 mM KCl, 62.5 mM potassium phosphate (pH 7.0). Spectra were taken at 1, 2, 3, and 4 h. The difference spectra are shown relative to the starting spectrum for the resting enzyme. (*Middle*) At the end of the 4-h aerobic incubation described above, the cuvette was closed and subjected to a 300 cm^3/min flow of argon for 30 min. $Na_2S_2O_4$ (0.1 μM) was then added and the spectrum taken after 10 min. The solid line shows the difference spectrum resulting from the exposure to $Na_2S_2O_4$. At the end of the experiment the cap was removed, a few crystals of solid $Na_2S_2O_4$ were added, and a final spectrum was taken after 2 min. The dashed line shows the spectral change resulting from the exposure to the crystalline $Na_2S_2O_4$, but the magnitude was cut by a factor of 3 for illustration purposes. (*Bottom*) After a 25-min pregassing of the KCl/KPO_4 buffer (described above) with flowing argon at 300 cm^3/min, cytochrome aa_3 (6 μM heme A) was added and the anaerobic incubation continued for 4 h. The spectra from bottom to top show the differences occurring after 1, 2, 3, and 4 h incubation.

manuscript in preparation). Fig. 8 (bottom) shows the results of an anaerobic incubation of the resting enzyme under slightly reducing conditions. The reducing equivalents were supplied by a stream of argon passed through a scrubbing bottle containing $Na_2S_2O_4$ in 0.2 M sodium phosphate (pH 9.6) to remove any traces of O_2 that may be present. There are two potential sources for these reductants. One is in the possible transport of minute amounts of $Na_2S_2O_4$ in microdroplets of an aqueous aerosol formed by the sintered glass bubbler and carried along by the gas stream. The other is in the form of SO_2 which is a natural product of the reduction of O_2 by $Na_2S_2O_4$ in the scrubbing bottle. Evidence of the reduction is seen in the rise of the reduced peaks for cytochrome *a* at ~446 and ~606 nm in the 1-, 2-, 3-, and 4-h difference spectra (bottom to top in the lower panel). The amount of reduction of cytochrome *a* after 4 h was 5% of the maximum achievable with crystalline $Na_2S_2O_4$ based on the amplitude of the α peak. The disappearance of the peak at ~428 nm under these anaerobic and reducing conditions is consistent with the proposed existence of a redox center that interacts anticooperatively with that of cytochrome a_3.

Under both the oxidizing conditions depicted in the top panel of Fig. 8 and the reducing conditions of the bottom panel, the net loss of the peak at ~428 nm relative to the resting enzyme is seen. If in both cases the loss is due to the oxidation of a reduced high potential form of cytochrome a_3, then no loss should be seen when the reduced high potential form is not present. The explanation of the trough at ~428 nm in the top panel is that the reduced high potential cytochrome a_3 was oxidized by O_2. If this is true there may be little or no reduced cytochrome a_3 present after 4 h of this aerobic incubation, and consequently little or no further development of a trough at ~428 nm when reducing equivalents are provided, in contrast to what was seen in the incubation depicted in the lower panel. When the cuvette was closed at the end of the air incubation, made anaerobic, and then exposed to 0.1 μM $Na_2S_2O_4$, cytochrome *a* reduction resulted but no trough was seen at ~428 nm (solid line curve in middle panel of Fig. 8). In fact, the relative amplitude at 428 nm compared with the peak at ~444 nm was higher than in the case of the total reduction of the enzyme caused by addition of crystalline $Na_2S_2O_4$ (dashed line at 1/3 magnification in the same panel). This is consistent with the interpretation that the low level of $Na_2S_2O_4$ would reduce the high potential cytochrome a_3 with its Soret peak near 428 nm, whereas the excess $Na_2S_2O_4$ would reduce the free low potential species of cytochrome a_3 with its Soret peak near 446 nm (Sidhu and Hendler, 1990).

DISCUSSION

We have characterized a high potential form of cytochrome a_3 with an E_m near 770 mV (Hendler and Sidhu, 1988) and two low potential forms with E_m values <200 mV (Sidhu and Hendler, 1990). When the resting, oxidized enzyme is added to an anaerobic medium at 450 mV, a shift in its Soret location occurs that is consistent with the reduction of cytochrome a_3. As the voltage of the solution is raised to 780 mV, the Soret peak shifts back towards its oxidized position. The interpretation of these spectral shifts in terms of changes in redox state assumes that no ligands other than electrons are present.

One possibility, which had not been seriously considered in the earlier work was suggested to us by Peter Nicholls, is the main inspiration for the studies reported in this paper. Perhaps cyanide could be liberated in sufficient amount by photolysis of the metallocyanide redox mediators used in the potentiometric titrations to allow formation of the ferric cytochrome a_3–cyanide complex (Waino, 1959; Yonetani, 1960; Lemberg et al., 1964; Yoshikawa and Orii, 1973), and thus account for the spectral shift we observed at 450 mV. The K_d for the oxidized cytochrome a_3–cyanide complex formed from the resting enzyme is 10^{-4} M (Yoshikawa and Orii, 1973). To have 50% of the cytochrome a_3 liganded, an equilibrium concentration of 100 μM cyanide would be needed. From the fact that the entire Soret peak was observed to shift from ~420 nm (oxidized position) to ~428 nm (reduced position under the experimental conditions), rather than a growth of a shoulder at the higher wavelength, at least 50% complex formation would be required. The main objective of the current studies was to consider this possibility.

Our results reported in this paper do not support the CN complex explanation of the spectral shifts. We find that the shift in position of the Soret peak occurs in the dark, where the measured free cyanide concentration is 0–2 μM (Figs. 5 and 6), whereas incubation of the resting enzyme in 2 μM cyanide does not cause the shift (Figs. 5 and 6). Incubations in the light for 1 h where the cyanide concentration is <40 μM leads to a ~50% greater amount of shift than occurs in the dark. The extent of shift in the presence of added 50 μM cyanide is far less than that which occurs in regular incubations in the light and in the dark. Alternating 60-min light and dark incubations does not lead to much of a difference in the rate or extent of the shift (Fig. 5) even though the level of cyanide dramatically drops when an incubation in the light is followed by a dark period (Fig. 3). Therefore, the shift in position of the Soret peak does not require or depend on free cyanide in the medium. The increase in the extent of the shift that

occurs in the light could be due to the ability of the irradiated mediators to supply electrons for the reduction of cytochrome a_3 and possibly to some formation of the oxidized cytochrome a_3–cyanide complex.

Having established that the spectral transition we observe when the resting enzyme is placed into a medium at 470 mV is not due to cyanide complex formation, we proceeded one step further. Cyanide is actually the reduced member of a redox couple that includes cyanogen (C_2N_2). The standard redox potential of the C_2N_2/HCN couple has been quoted as 370 mV (Corain et al., 1981). In fact, a commercial method for forming $(CN)_2$ involves the oxidation of cyanide by $CuSO_4$ (Janz, 1957). If the binding affinity of cytochrome oxidase for C_2N_2 is extremely high, if cyanide can be efficiently converted to C_2N_2 under the experimental conditions, and if the spectral change occurring upon cytochrome a_3–C_2N_2 formation is the same as the one we have observed, then an alternative explanation to the change in redox state we have advanced may still be relevant. To test these possibilities (i.e., that the spectral changes may be due to the binding of C_2N_2), we obtained a tank of C_2N_2 and developed a chemical assay for the substance. We find that cyanide is not converted to C_2N_2 in any detectable amount under the conditions of incubation in either the presence or absence of the enzyme. Furthermore, when amounts of C_2N_2 up to 100 μM are added to the enzyme, the spectral change seen is for the reduction of cytochrome *a* rather than a shift of Soret peak location from ~420 to ~428 nm. This could be due to small amounts of cyanide formed from the C_2N_2 that we find in the aqueous solution. At 1,000 μM spectral evidence of complex formation of cytochrome a_3–C_2N_2 was seen, but its difference spectrum was not the same as seen either in our reported spectral shift or in formation of the enzyme–cyanide complex.

If cytochrome a_3 has one E_m near 770 mV and another below 200 mV, then at 450 mV the reduced form of the high potential species should predominate. As the voltage is lowered to 200 mV, the reduced high potential form should be converted to the oxidized low potential form. Our evidence supports this conversion (Hendler et al., 1986; Hendler and Sidhu, 1988). In the voltage range 450–200 mV, concomitant with the apparent oxidation of cytochrome a_3, the reduction of two species of cytochrome *a* is seen with E_m's at 260 and 340 mV (Hendler et al., 1986). Our potentiometric data indicate $n = 2$ for the lower and $n = 1$ for the higher E_m conversions. Redox transitions at 200, 260, and 340 mV have been seen by others (Nicholls and Wrigglesworth, 1988; Steffens and Buse, 1988). According to the Nernst equation, if cytochrome a_3 becomes more oxidized as cytochrome *a* becomes more reduced, another center must be involved if the two cytochromes exhibit the same *n* values. We attribute the $n = 2$ titration of cytochrome a_3 to coupling between the heme and Cu_B. If so, Cu_B cannot be the other center. A likely candidate for the other center involved in the $n = 2$ titration of cytochrome *a* is Cu_A. If this is so, then Cu_A cannot be the other center. For this reason we have speculated another center, X (Cu_X?), to be the controlling center. Mathematical models that involve another center, X, can simulate all of our experimental findings (Hendler and Westerhoff, manuscript in preparation). At this point, however, the existence of the proposed controlling center is without any direct experimental support. It is a speculation that can account for a number of experimental observations which cannot be explained by current concepts of the enzyme, including the neoclassical model. Below we list several observations that are consistent with our proposed model. It is important to note that these observations are not compatible with the alternative explanation based on CN complex formation.

1. In the voltage range 750–790 mV the Soret peak shifts from ~428 nm (reduced position) back toward 420 nm (oxidized position) (Hendler and Sidhu, 1988).

2. In the voltage range 450–200 mV the Soret peak at ~428 nm disappears concomitantly with the rise of peaks at ~ 446 and ~606 nm (Hendler et al., 1986). According to the cyanide complex explanation there is no change in the cytochrome a_3–cyanide complex which is "locked" in the oxidized form. The trough at ~428 nm is proposed to be due to the loss of the oxidized Soret peak for cytochrome *a*. This explanation requires that the reduced minus oxidized difference spectrum for cytochrome *a* shows a trough at ~428 nm and peaks at ~446 and 606 nm. That the trough at ~428 nm is modulated independently of the peaks at ~446 and 606 nm, however, is shown in observations 3 and 4 below, and similarly in Ludwig and Gibson (1981).

3. When resting enzyme is incubated in an open cuvette in KCl/KPO_4 buffer, a marked trough occurs at ~428 nm with no changes at ~446 and ~606 nm (Fig. 8, top). If the E_m of high potential cytochrome a_3 is ~770 mV, then some reduced high potential cytochrome a_3 should be present in the stored resting enzyme. In an air-saturated medium this reduced cytochrome a_3 should be oxidized by O_2, resulting in the loss of absorbance at ~428 nm. This spectral change is independent of the reduction of cytochrome *a* and the rise of a peak at ~446 nm. Neither cyanide nor metallocyanide mediators are present during these incubations.

4. In a continuation of the air incubation experiment above, after 4 h the cuvette was closed and made anaerobic with argon. Very small amounts of $Na_2S_2O_4$ in argon-saturated buffer were then added. Cytochrome *a* was reduced as evidenced by peaks at ~446 and ~606 nm,

but no trough was seen at ~428 nm (Fig. 8, middle). Instead, a shoulder was seen at ~428 nm which would result from the reduction of high potential cytochrome a_3. Just as observation 3 showed that the trough at ~428 nm can develop independently of the peaks at ~446 and ~606 nm, this observation shows that the peaks can develop in the absence of the trough. These observations are inconsistent with the cyanide complex explanation of observation 3 above.

5. When resting enzyme is incubated anaerobically under flowing argon, a trough develops at ~428 nm accompanied by very small peaks at ~446 and ~606 nm (Fig. 8, bottom). The argon gas is scrubbed in a bottle containing $Na_2S_2O_4$ at pH 9.6 to remove traces of O_2, if any are present. The small amount of reduction seen is due to some reducing equivalents carried along with the scrubbed gas stream as described above. The amount of cytochrome *a* reduction, as measured by the amplitude of the α peak, was ~5% in 4 h of exposure to the argon gas compared with full reduction using crystalline $Na_2S_2O_4$.

It is important to note that the trough at ~428 develops under both oxidizing (observation 3) and reducing conditions (here). That the oxidation of reduced high potential cytochrome a_3 should be provoked both by an oxidant and a reductant is a unique prediction of the redox cooperativity explanation.

6. In a reductive titration from 200 to 100 mV, the Soret peak at ~428 nm rises (Hendler et al., 1986) in accordance with the reduction of low potential cytochrome a_3. According to the cyanide complex explanation, when the voltage is titrated to 200 mV from 400 mV, oxidized cytochrome a_3–cyanide complex and reduced cytochrome *a* are present (see observation 2). The reduction, therefore, must be of the complex. But then this should have led to the disappearance of the peak near 428 nm, which is characteristic of the oxidized complex, and to the rise of a peak in the 440–445 nm range, which is characteristic of the reduced complex (Waino, 1959; Yonetani, 1960; Lemberg et al., 1964; Yoshikawa and Orii, 1973).

Our findings of a high potential form of cytochrome a_3 (Hendler et al., 1986; Hendler and Sidhu, 1988; this paper) are consistent with other studies indicating high voltage transitions involving cytochrome a_3. In 1981 Wikström reported on studies of the high voltage behavior of cytochrome oxidase (Wikström, 1981). These studies were stimulated by the findings of effects of high ΔG_{ATP} on the spectrum of the enzyme (Erecinska et al., 1972; Wilson and Brocklehurst, 1973; Wilson et al., 1975). Wikström's evidence supported the idea that energy-dependent elevation of the ambient voltage at the cytochrome a_3–Cu_B binuclear center could shift the redox equilibrium between the center and H_2O such that electrons would flow from the latter to the former. In subsequent publications additional evidence was provided in support of this interpretation (Wikström, 1987, 1988). There are many significant differences between the work of Wikström and the studies described in this paper. Nonetheless, both approaches are based on the same thermodynamic reasoning suggesting that high voltage redox transitions for cytochrome a_3 are likely, and both studies provide experimental evidence for the existence of these transitions.

We would like to close the discussion with a consideration of the possible physiological significance of our results. Oxygen is the final electron acceptor in respiration and its direct electron donor is reduced cytochrome a_3. We have demonstrated that there exists a redox potential for cytochrome a_3 below 200 mV (Hendler et al., 1986; Hendler and Sidhu, 1990). The redox potential for cytochrome *a* is either ~260 mV ($n = 2$) or ~340 mV ($n = 1$) (Hendler et al., 1986; Nicholls and Wrigglesworth, 1988; Steffens and Buse, 1988). It would not be reasonable to consider an electron route to proceed from a donor with E_m >260 mV to one with an E_m of ~185 mV and then directly in a single step to the final acceptor (O_2) with an E_m of 815 mV, or to O_{22-} with an E_m of 1.3 V. A cooperative interactive system, however, which went through a stage where the E_m of the donor cytochrome a_3 would be raised to nearer that of its acceptor, would have the final electron transfer step take place between better matched E_m's of donor and acceptor. This system would have thermodynamic and kinetic advantages and the conformational transition implied in the change of the E_m value may be related to proton translocation. A possible mechanism based on these ideas has been presented (Hendler and Sidhu, 1988).

The authors are grateful to Dr. Hans Westerhoff for a thorough reading of the manuscript and many helpful suggestions.

Received for publication 2 August 1989 and in final form 25 May 1990.

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