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Oxidative Titrations of Reduced Cytochrome aa_3: Correlation of Midpoint Potentials and Extinction Coefficients Observed at Three Major Absorption Bands†

Nancy A. Schroedl and Charles R. Hartzell*

ABSTRACT: Anaerobic oxidative titrations of purified cytochrome aa_3 were monitored at three wavelengths (444, 604, and 820 nm), in both the absence and the presence of carbon monoxide. Computer simulation of each titration curve was utilized to ascertain the midpoint potentials of the four oxidation-reduction centers of the enzyme. For experiments performed under nitrogen, two components were found to titrate with low potential (heme a_L = 220 mV, Cu_L = 240 mV) and two with high potential (heme a_H, Cu_H = 340 mV), consistent with results obtained previously in reductive titrations. Unequal heme extinction coefficients were observed at 444 nm. Oxidation by either potassium ferricyanide or 1,1′-bis(hydroxymethyl)ferricinium ion showed that the low potential heme component contributed 75% of the absorbance change at 444 nm. At 820 nm, the entire absorbance change could be attributed to a single, low potential copper component. Midpoint potentials calculated for the carbon monoxide complexed enzyme agreed with previously reported values. The copper components retained the values observed under nitrogen, while the titratable heme group gave an apparent midpoint potential of 260 mV. These results enable us to assign absorbance changes at various wavelengths to specific redox components of cytochrome aa_3.

In a previous paper we presented midpoint potentials and extinction coefficients for the metal ion centers of cytochrome aa_3 observed at 604 nm (Schroedl and Hartzell, 1977). This absorbance band has been extensively studied and the absorbance changes accompanying oxidation and reduction have been correlated with those measured at other wavelenths and with changes in the electron paramagnetic resonance spectrum of the protein (Hartzell and Beinert, 1976). We report here an examination of direct chemical, oxidative titrations of reduced cytochrome aa_3, as monitored at the 444, 604, and 820 nm absorption bands, performed in the presence and absence of carbon monoxide atmosphere.

Experimental Procedure

Continuous anaerobic oxidative titrations were performed at ambient temperature on a purified, phospholipid-depleted preparation of cytochrome aa_3 (Hartzell and Beinert, 1974). A specially designed apparatus patterned after that of Foust et al. (1969) was used. Solutions contained 4-30 μM cytochrome aa_3 ($\Delta\epsilon_{604}^{R-O}$, 24 mM^{-1} cm^{-1}; $\Delta\epsilon_{444}^{R-O}$, 168 mM^{-1} cm^{-1} (Van Gelder, 1966); and $\Delta\epsilon_{820}^{R-O}$, 2.8 mM^{-1} cm^{-1} (Griffiths and Wharton, 1961) (all $\Delta\epsilon$ values are expressed as two heme *a* per cytochrome aa_3)), 0.1 M potassium phosphate buffer, pH 7.5, and either 0.2% (w/v) sodium cholate or 0.2% (w/v) Triton QS-30 as a solubilizing agent. The enzyme was reduced with a 5-15% equivalent excess of NADH (P-L Biochemicals) with 5 μL of 0.01% (w/v) phenazine methosulfate as mediator catalyst. Potassium ferricyanide and HMF (Strem Chemicals, Inc.) were standardized using ϵ_{420} = 1.02 mM^{-1} cm^{-1} (Ibers and Davidson, 1951) and titration of cytochrome *c* ($\Delta\epsilon_{550}^{R-O}$ = 21.1 mM^{-1} cm^{-1} (Van Gelder and Slater, 1962)) solutions, respectively. A more thorough description of the experimental procedure can be found elsewhere (Schroedl and Hartzell, 1977). Carbon monoxide (Matheson Gas Products) was freed of oxygen by passage over a bed of Ridox (Fisher Scientific Co.).

Spectra were recorded on a Cary 17 ratio recording spectrophotometer. Change in absorbance was plotted against oxidizing equivalents per cytochrome aa_3 (ΔA vs. equiv). All

† From the Department of Biochemistry and Biophysics, Paul M. Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received May 5, 1977.* This work was done during the tenure of C. R. Hartzell as an Established Investigator of the American Heart Association. Supported in part by grants from the Research Corporation, The American Heart Association, Berks County Pennsylvania Division, and United States Public Health Service, National Heart, Lung, and Blood Institute HL 17559. A preliminary report on part of this work has been presented (Schroedl and Hartzell, 1975).

[1] Abbreviations used: PMS, phenazine methosulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; HMF, 1,1′-bis(hydroxymethyl)ferricinium cation; $E^{o\prime}$, midpoint potential value; $\Delta\epsilon_\lambda^{R-O}$, reduced minus oxidized extinction coefficient at designated wavelength; NHE, normal hydrogen electrode; a_h, high potential heme *a*; Cu_H, high potential copper; a_L, low potential heme *a*; Cu_L, low potential copper; IR, infrared.

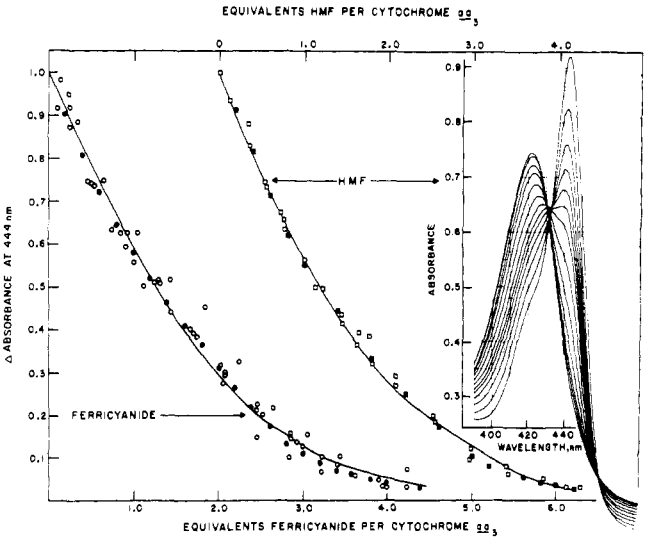

FIGURE 1: Oxidative titrations of cytochrome aa_3 under nitrogen atmosphere by potassium ferricyanide (left curve, abscissa scale on bottom) and HMF (right curve, abscissa scale on top) were monitored at 444 nm. Raw data (○, □) from five titrations, the average titration points (●, ■), and the simulated best fit (—) are given. Spectra, taken during a titration, are shown for the stepwise oxidation of 3.8 μM cytochrome aa_3 by 0.503 mM HMF (the first six spectra are 5-μL oxidant increments, while the remaining spectra are 10-μL oxidant increments). The $E^{\circ\prime}$ values and extinction coefficients found for the simulated curves are given in Table I.

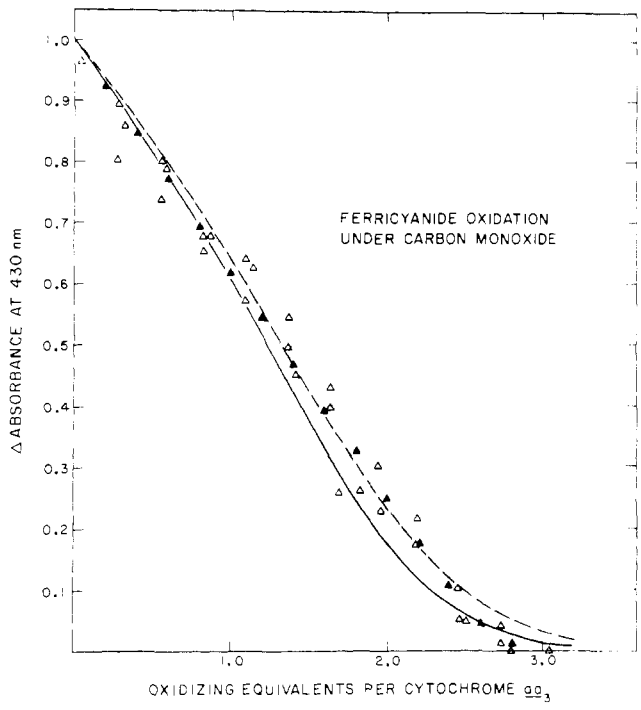

FIGURE 2: Oxidative titration of the cytochrome aa_3-CO complex was monitored at 430 nm. The raw data (△) is given for three titrations. The average titration curve (▲) is compared with two simulated curves. The midpoint potentials given in Table I for the titration under CO were used to generate both simulated curves. In the solid curve (—) only the low potential heme component was assumed to be as the absorbing species, while the dashed curve (- - -) included contribution from both heme and copper components (see Table I).

ΔA values were normalized for ease of computation and comparison. Computer simulation of the titration curves used a modification of the approach outlined by Heineman et al. (1973), and Mackey et al. (1973) as described by Schroedl and Hartzell (1977).

Error analysis and theoretical fit evaluation were accomplished by the following procedures: Each experimental titration curve was plotted and adjusted for percentage of reduction and small concentration differences. At each 0.2 equiv of titrant, the normalized absorbance was recorded for each experimental curve. The recorded absorbance values at 0.2 equiv intervals were averaged to generate the average experimental curves shown in the figures as filled characters. The standard deviation was then determined for each 0.2 equiv increment. The average standard deviation in the experimental curve was determined and a standard deviation of the average standard deviation was calculated. Closeness of fit was determined by using the variance between the simulated curve and the average experimental curve for each 0.2 equiv increment. The average variance for all increments was calculated and the standard deviation of the average variance was calculated. In all cases agreement between the average variance and the average experimental standard deviation obtained was greater than 97%. The calculations were performed using a Digital Equipment Corporation PDP11/10 minicomputer.

Results

Soret Region Titrations. The oxidative titration of reduced cytochrome aa_3 followed at the Soret band is given in Figure 1. An isosbestic point was maintained at 433 nm during addition of approximately 1.2 equiv of ferricyanide per oxidase molecule. This isosbestic point abruptly changed position (to 431 nm) and again remained fixed for all further additions of oxidizing equivalents. This shift coincided with completion of 50% of the total change in absorbance observed for the titration at 444 nm. In excess of 4 equiv of ferricyanide was required to complete the absorbance change. Oxidation by HMF yielded an identical titration curve (Figure 1).

Reduction of cytochrome aa_3 in the presence of a carbon monoxide atmosphere gave the classical carbon monoxide complex (Lemberg, 1969). The wavelength maximum in the Soret region appeared at 430 nm for this complex with a distinct shoulder at 444 nm. Three oxidizing equivalents were required to complete the absorbance change monitored at 430 nm (Figure 2). The isosbestic point at 434 nm was maintained throughout the titration. Absorbance changes monitored at 444 nm instead of 430 nm in the presence of carbon monoxide gave a normalized titration curve identical with that exhibited in Figure 2.

604-nm Region Titrations. The absorbance change at 604 nm of reduced carbon monoxide complexed cytochrome aa_3 required nearly 3 oxidizing equiv. Isosbestic points were observed at 621 nm and 583 nm throughout the titration. A largely linear titration curve (Figure 3) was observed for the cytochrome a_3^{2+}-CO complex. HMF oxidation of the CO-complexed enzyme produced a similar ΔA vs. equivalents plot (Figure 3).

The Near-IR Band at 820 nm. Approximately 3 equiv of ferricyanide were required to complete the absorbance change monitored at 820 nm (Figure 4) during titration in an inert atmosphere. The sigmoidal shape of the titration curve reflected the distribution of electrons to components other than the absorbing species.

Under carbon monoxide atmosphere, a linear titration curve was observed at this wavelength (Figure 4). Two oxidizing equivalents was necessary to complete the absorbance change. The small initial absorbance change observed under nitrogen atmosphere disappeared. Reoxidation of the CO-complexed, reduced enzyme restored approximately 80% of the original

TABLE I: Summary of Calculated Midpoint Potential Values and Extinction Coefficients of Ferricyanide- and HMF-Oxidized Cytochrome aa_3 Components.

	Nitrogen atmosphere											
Wavelength:	Soret				604 nm				820 nm			
Component:	a_H	Cu_H	a_L	Cu_L	a_H	Cu_H	a_L	Cu_L	a_H	Cu_H	a_L	Cu_L
Ferricyanide												
$E^{\circ\prime}$, mV vs. NHE	340	350	220	230	340	340	220	240	340	340	210	230
$\Delta\epsilon^{R-O}$, mM^{-1} cm^{-1}	42	0	126	0	3.6	0	20.4	0	0	0	0	2.8
HMF												
$E^{\circ\prime}$, mV vs. NHE	340	340	220	240	340	340	220	240				
$\Delta\epsilon^{R-O}$ (mM^{-1} cm^{-1})	42	0	126	0	4.8	0	19.2	0				
	Carbon monoxide											
Component:	a_3·CO	Cu_H	a	Cu_L	a_3·CO	Cu_H	a	Cu_L	a_3·CO	Cu_H	a	Cu_L
Ferricyanide												
$E^{\circ\prime}$ (mV) vs. NHE	>424	320	255	240	>424	360	260	240	>424	350	260	220
$\Delta\epsilon^{R-O}$, mM^{-1} cm^{-1}	0	0	29.6[a]	0	0	0	19.5	0	0	0	0	2.25
HMF												
$E^{\circ\prime}$ (mV) vs. NHE					>465	320	255	240				
$\Delta\epsilon^{R-O}$ (mM^{-1} cm^{-1})					0	0	19.5	0				

[a] Extinction coefficient values of 26.6 mM^{-1} cm^{-1} for cytochrome a and 3.0 mM^{-1} cm^{-1} for Cu_H yielded a better fit to the experimental curve during simulation.

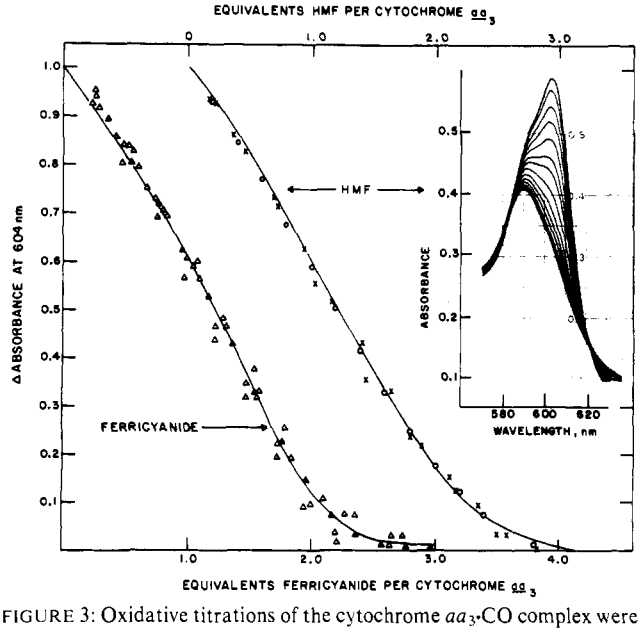

FIGURE 3: Oxidative titrations of the cytochrome aa_3·CO complex were monitored at 604 nm. The raw data (△, X) for five ferricyanide titrations (left curve, abscissa scale on bottom) and two HMF oxidations (right curve, abscissa scale on top) are given. The calculated average titration points (▲, O) are compared with the simulated best fit (—). Spectra, taken during a titration, are shown for the oxidation of 12.75 μM cytochrome aa_3 by 1.68 mM HMF (10-μL increments). The $E^{\circ\prime}$ values and extinction coefficients corresponding to the simulated curve can be found in Table I.

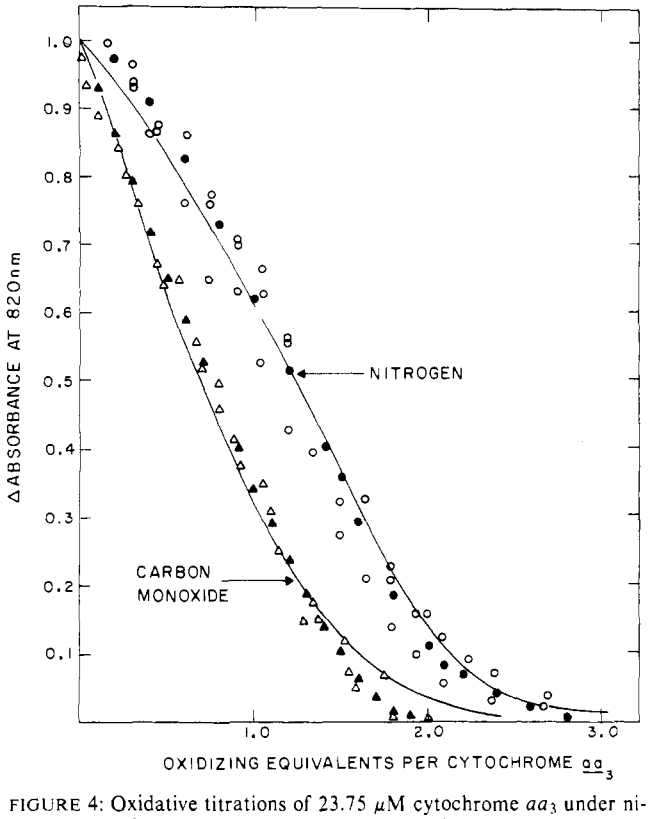

FIGURE 4: Oxidative titrations of 23.75 μM cytochrome aa_3 under nitrogen and CO atmosphere are presented. Raw data (O, △) from three titrations, the calculated average titration points (●, ▲) and the simulated best fit (—) for the ferricyanide titrations, are given. The $E^{\circ\prime}$ values and extinction coefficients corresponding to the simulated curves are given in Table I.

absorbance value. No unique near infrared spectral characteristics analogous to the 430 and 590 nm bands were induced by carbon monoxide.

Simulation of Titration Curves. Each figure includes a theoretical curve (solid line) calculated by using the $E^{\circ\prime}$, and $\Delta\epsilon$ values for each oxidase component. The $\Delta\epsilon$ value at the wavelength under investigation is expressed as the reduced minus oxidized change per mole of cytochrome aa_3 and is fixed at a maximum value in each case (Van Gelder, 1966). These values for $\Delta\epsilon$ are listed in Table I. For simulations of titrations performed under CO atmosphere, the $\Delta\epsilon$ values have been calculated from the known concentration of cytochrome aa_3 under nitrogen atmosphere (see Table I).

Simulations are necessary in order to gain information about $E^{\circ\prime}$ and $\Delta\epsilon$ values from experimental data. However, the term *simulation* inherently implies a non-unique solution to the problem. To maximize the reliability of our calculations, boundaries were placed on the computer simulations so that

the $E^{\circ\prime}$ values were limited by the thermodynamic region cytochrome aa_3 functionally requires and well-established (i.e., literature) $E^{\circ\prime}$ and $\Delta\epsilon$ values. In addition, internally consistent midpoint potentials were required to fit all three wavelengths investigated for both oxidants employed. Variations of the simulated curves from the average experimental curves were calculated and were required to be within the standard deviation of the experimental data itself. The error in $E^{\circ\prime}$ values is placed conservatively at ± 15 mV for all wavelengths investigated with considerable improvement in accuracy and precision for the wavelengths where larger absorbancy changes occur.

It is difficult to assess the exact starting point of each oxidative titration. Utilization of a small excess of the reducing agent is required in order to consume small but variable amounts of oxygen remaining in the anaerobic cuvette. It appears from our data that the lowest $E^{\circ\prime}$ value (in N_2) belongs to a heme component of the oxidase. This heme component then oxidizes first in titrations monitored at either 604 or 444 nm after all excess reductant is consumed. The point where the heme initially titrates is utilized to indicate where total depletion of excess reductant occurs. Careful corrections are then made, particularly at the 820 nm band, where it appears that copper ($E^{\circ\prime}$, 240 mV) is the only absorbing species.

Discussion

I. *Relationship of the Redox Components of Cytochrome aa_3 to Specific Visible Absorption Bands.* The pioneering work of Keilin and Hartree (1938) launched an intense investigation of the mitochondrial cytochrome system, with the ultimate goal being the understanding of respiration in the cell. Visible spectral band assignments by Keilin and Hartree (1938) have remained relatively unchallenged to this day. We will now discuss each of the cytochrome aa_3 spectral regions with special emphasis on band assignments based on our data and literature information.

Soret Band. The Soret region of the visible absorption spectrum is usually considered to arise from the iron porphyrin groups of this enzyme (Lemberg, 1969). Oxidative titrations of reduced cytochrome aa_3 reveal a hyperbolic titration curve, suggesting unequal heme *a* extinction coefficients or significant deviation in the midpoint potential values of the four redox centers.

Computer simulation of these titrations, performed under nitrogen atmosphere, support the contention that two heme groups contribute to the absorbance change at 444 nm (cytochrome a^{2+} absorbs at 447 and 442 nm, and cytochrome a_3^{2+} at 444 nm (Gilmour et al., 1967)). However, the extinction coefficient of the low potential heme component accounts for 75% of the observed absorbance change. The change observed in isosbestic point position after 1.2 oxidizing equiv has been added indicates either the formation of an intermediate with independent spectral characteristics or a change in the electron distribution among the four redox components.

Under carbon monoxide atmosphere, three components appear to be oxidized in the reduced cytochrome aa_3·CO complex. Titrants such as potassium ferricyanide cannot oxidize the CO-complexed cytochrome a_3 when used at stoichiometric levels (Anderson et al., 1976). We have therefore assumed that the absorbance change at 430 nm arises from oxidation of cytochrome *a* alone. However, computer simulations indicate an increase in copper absorption with oxidation at 430 nm is a possibility (Figure 2). The cytochrome aa_3·CO absorbance maximum occurs at this wavelength, and the oxidation of the uncomplexed heme was monitored here as its band maximum moved from 444 to 426 nm. The small extinction coefficient change ($\Delta\epsilon_{430}$ = 29.6 mM^{-1} cm^{-1}) observed under CO atmosphere would be more sensitive to a small copper contribution at this wavelength when the data were simulated. In addition, it was found that the initial slope of the titration was smaller for the CO complexed enzyme than was observed under nitrogen atmosphere. An increase in the midpoint potential of the titratable heme group (Table I) is suggested by our simulations. Consumption of three oxidizing equivalents supports the contention that one CO molecule binds per mole of cytochrome aa_3 (Anderson et al., 1976) and that the CO molecule is bound only to ferrocytochrome a_3. This position is further supported by a recent report (Choc et al., 1977) where infrared spectroscopy shows only one CO bound per cytochrome aa_3 and where no evidence for interaction with metal ions other than one heme iron was found.

The 604-nm Band. The band located at 604 nm is attributed primarily to the iron porphyrin system (Lemberg, 1969). The assignment of heme extinction coefficients under nitrogen atmosphere has been considered (Schroedl and Hartzell, 1977); therefore, the discussion here is limited to the titration of the CO-complexed cytochrome aa_3.

Controversy exists over the number of reducing equivalents required by cytochrome aa_3 in order to form the CO complex. Greenwood et al. (1974) reported that approximately 3 oxidizing equiv were required to form the mixed valance complex (all electron-accepting components oxidized except those binding CO) from the fully reduced CO-complexed cytochrome aa_3. They also found that addition of 1 reducing equiv to cytochrome aa_3 in the presence of CO formed the mixed valance compound. Additionally, this complex could be formed within a few minutes by making CO-saturated-oxidase solution anaerobic at room temperature. Cyclic coulometric titrations were performed by Anderson et al. (1976), who found that 3 oxidizing equiv were required to form the mixed valence compound from the fully reduced CO-complexed cytochrome aa_3. They also found that three electrons must be added back to regain the fully reduced state. These results are contrasted with those reported by Lindsay and Wilson (1974) and Lindsay et al. (1975). This latter group presents evidence, based on potentiometric titrations, that two electrons must be accepted by cytochrome aa_3 in order for carbon monoxide to bind. They interpret their results in terms of a Nernstian *n* value equal to 2, thus implicating a heme-CO-copper species. Our results presented herein do not support the model presented by Lindsay and Wilson (1974).

The oxidase preparation utilized in our studies *will not* spontaneously form the CO mixed-valence-state complex under anaerobic conditions. This preparation was also used by Anderson and co-workers (1976) in their experiments. Therefore, this cytochrome aa_3 preparation, unlike all others tested, appears to contain no endogenous electron donors that can reduce cytochrome a_3 and then form a CO complex. A second noteworthy point should be indicated concerning equilibrium. In many of our preliminary studies on the stoichiometry of the oxidative reaction we often found that only two electrons or less were required to "completely oxidize" fully reduced CO-complexed cytochrome aa_3. These results were obtained *only* in nonequilibrium conditions.

Computer simulation of our oxidative titration data indicated that the low potential heme component has an increased midpoint potential value, similar to the value first reported by Tzagoloff and Wharton (1965), but that its extinction coefficient is the same or smaller than that found for our titrations under nitrogen atmosphere (Schroedl and Hartzell, 1977). Wikström et al. (1976) concur with this observation. We then assign 85% of the absorbance change at 604 nm to the low

potential heme *a* in either the presence or absence of CO. The remainder of the change in nitrogen atmosphere is assigned to the high potential heme *a*. Under no circumstances was copper found to contribute to the absorbance change at this wavelength.

The 820-nm Band. Absorption at 820 nm had been attributed originally to copper (Lemberg, 1969). Controversy remains over how many copper atoms contribute. Suggestions have been made that one of the heme components absorbs in this region (Caughey and McCoy, 1966; Tiesjema et al., 1973; Greenwood et al., 1973, Malmström, 1974). Titrations of cytochrome aa_3 monitored in the near-IR region indicate that two components of low potential titrate initially. Contribution to this absorbance band by the lower potential component cannot be detected at this wavelength, while the component of slightly higher potential dominates the absorbance change (Figure 4).

Mackey et al. (1973) reported that the 820–830 nm band titrated concurrently with the 604 nm band during indirect electrochemical titrations. Direct chemical titrations (Tiesjema et al., 1973) show that the absorbance change at 830 nm occurs only with the second and third *reducing* equivalents of NADH. Potentiometric titrations by a number of research groups do not appear to resolve these differences.

Under CO atmosphere only 2 oxidizing equiv are required to complete the titration observed at 820 nm (Figure 4). The titratable heme group is higher in potential than the copper atom; thus, the initial absorbance change during the reoxidation in the presence of CO would arise from a copper center. Since only 80% of the original oxidized absorbance level is recovered upon completion of the titration, contribution by cytochrome a_3 to the absorbance at this wavelength cannot be completely eliminated. This decrease more likely arises from the diminished extinction coefficient of the 655 nm band under CO. A lack of complete restoration of this absorbance band was also observed by Wharton (1964) for ferricyanide oxidation of cytochrome aa_3.

Computer simulation of the oxidative titrations indicated that the 820 nm band observed in cytochrome aa_3 arises from a single, low potential copper component in either nitrogen or CO atmospheres. Neither heme *a* nor high potential copper absorbancy contributions could improve the curve fit.

It is now apparent that cytochrome aa_3 has paired heme *a*-copper oxidation-reduction components under anaerobic conditions even in the absence of cytochrome *c*. One pair has midpoint potentials between 200 and 250 mV, while the high potential pair has $E°'$ values between 340 and 360 mV. The low potential heme *a* component contributes 85% to the reduced-minus-oxidized absorbancy change observed at 604 nm and this heme's $E°'$ value is increased 50–60 mV in the presence of CO. General agreement also appears to be emerging that the low potential copper is the only absorbing species at 820 nm. The overall significance of paired redox carriers and unequal heme *a* and copper extinctions must not be lost in defining a mechanism for electron transfer from cytochrome *c* to dioxygen via cytochrome aa_3.

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