Oxidative Titrations of Reduced Cytochrome aa_3: Influence of Cytochrome c and Carbon Monoxide on the Midpoint Potential Values†

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ABSTRACT: Oxidative titrations were performed on the electrostatic complex formed between cytochrome c and cytochrome aa_3 at low ionic strength. Midpoint potentials of the redox centers in the proteins in 1:1 and 2:1 complexes were compared with those in *mixtures* of the cytochromes at high ionic strength. Computer simulations of all titrations yielded midpoint potentials for the components of cytochrome aa_3 which were consistent with literature values for isolated cytochrome aa_3 or mixtures of cytochromes c and aa_3. However, the unequal heme extinction coefficients observed previously (Schroedl, N. A., and Hartzell, C. R. (1977), *Biochemistry 16*, 1327) during oxidative titrations of cytochrome aa_3 became equal in magnitude under these experimental conditions. The binding of cytochrome c to cytochrome aa_3 changed the midpoint potentials of cytochrome aa_3 by 15–20 mV, while the midpoint potentials for cytochrome c were altered by 50–60 mV. Careful analysis of these titrations including computer simulation revealed that cytochrome c was able to bind to cytochrome aa_3 only after cytochrome a_L^{2+} had become oxidized. When bound to cytochrome aa_3, the midpoint potential of cytochrome c was 210 mV. Titrations performed under a carbon monoxide atmosphere revealed cytochrome aa_3 midpoint potentials unchanged from reported values. Cytochrome c again exhibited a midpoint potential of 210 mV *after* binding to cytochrome aa_3.

An analysis of the mechanism whereby cytochrome aa_3 accepts electrons from cytochrome c and transfers them to molecular oxygen requires an accurate investigation of the oxidation-reduction behavior of the complex formed between these proteins. The major interaction between isolated lipid-depleted cytochrome aa_3 and purified cytochrome c appears to be electrostatic in nature. The complex formation is highly dependent on ionic strength and on the types of ions present in the buffer system. In 0.01 M Tris[1]-cacodylate buffer containing 0.5% Tween-20, simple mixing of oxidized cytochrome c, and cytochrome aa_3 results in formation of a protein–protein complex which does not dissociate on gel filtration (Hartzell, manuscript in preparation; Ferguson-Miller et al., 1976).

Two binding sites have been characterized for the interaction of cytochrome c with cytochrome aa_3. A high affinity site ($K_D = 3 \times 10^{-8}$ M) has been correlated with the low K_m phase for cytochrome c oxidation by O_2 via cytochrome aa_3, while a low affinity site ($K_D = 1.2 \times 10^{-6}$ M) corresponded to the high K_m phase (Ferguson-Miller et al., 1976). The presence of 0.05 M phosphate eliminates the low K_m phase of the kinetic reaction. Consequently, all titrations of cytochrome aa_3, cytochrome c, and mixtures of these proteins performed in 0.1 M phosphate buffer (Fujihara et al., 1974; Tiesjema et al., 1973) or in other high ionic strength media (Leigh et al., 1974) exclude the existence of an electrostatic complex between these proteins.

To assess the effect of complex formation on the oxidation of reduced cytochrome aa_3 (cf. Schroedl and Hartzell, 1977a,b), anaerobic titrations were performed using the chemical oxidant, potassium ferricyanide. Complexes formed between 1 or 2 mol of cytochrome c and 1 mol of cytochrome aa_3 were compared with mixtures of the two enzymes at high ionic strength. The influence of carbon monoxide on the midpoint potentials of the cytochromes was also examined under binding and nonbinding conditions.

Experimental Procedure

Materials. Anaerobic titrations were performed using the preparation of cytochrome aa_3 developed by Hartzell and Beinert (1974). Cytochrome c was purchased from Miles Research Laboratory and purified according to Margoliash and Walasek (1967). NADH was purchased from P-L Laboratories, Milwaukee, Wis. Phenazine methosulfate and Tween-20 were obtained from Sigma Chemical Co., St. Louis, Mo. Potassium ferricyanide and tris(hydroxymethyl)aminomethane were reagent grade from Fisher Scientific Co., Pittsburgh, Pa. Cacodylic acid was purchased from Matheson Coleman and Bell. The concentrations of enzymes, NADH, and ferricyanide were determined spectrophotometrically as described previously (Schroedl and Hartzell, 1977a). The reduced minus oxidized extinction coefficient for cytochrome c at 604 nm was determined to be -1.4 mM^{-1} cm^{-1} and for cytochrome aa_3 at 550 nm as -2.4 mM^{-1} cm^{-1}. The reduced minus oxidized extinction coefficient of 1.2 mM^{-1} cm^{-1} was found for the CO complexed cytochrome aa_3 at 550 nm.

Methods. Oxidative titrations of the cytochrome c–cytochrome aa_3 complex and mixtures of the two enzymes were performed as described (Schroedl and Hartzell, 1977a). Nitrogen and carbon monoxide gases were treated for removal of oxygen by passage over hot copper turnings and BASF catalyst (BASF Corp., Carlstadt, N.J.), and "Ridox" (Fisher Scientific Co., Pittsburgh, Pa.) respectively. Titrations of $FMNH_2$ were performed with each ferricyanide solution used

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[1] Abbreviations used: $E^{\circ\prime}$, midpoint potential value; $\Delta\epsilon_\lambda^{R-O}$, reduced minus oxidized extinction coefficient; Tris, tris(hydroxymethyl)aminomethane; 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; MS-Mops, mannitol-sucrose-morpholinopropanesulfonate.

TABLE I: Midpoint Potential Values of Cytochrome c-Cytochrome aa_3 Mixtures and Complexes.

Titration conditions	Midpoint potential values (mV)						Precision of titrations	
	a_H	Cu_H	a_L	Cu_L	c_B[a]	c_F[b]	Cytochrome aa_3	Cytochrome c
Nitrogen atmosphere								
1:1 complex	360	365	225	240	210	255	0.007 ± 0.005[c]	0.006 ± 0.005[c]
Low ionic strength	(10.2)[d]		(13.8)[e]			(21.1)[f]	0.008 ± 0.005[g]	0.013 ± 0.008[g]
2:1 complex	365	370	230	230	220, 250	260	0.030 ± 0.028	0.021 ± 0.009
Low ionic strength	(12)		(12)				0.006 ± 0.005	0.011 ± 0.009
1:1 mixture	340	350	210	225	220	260	0.014 ± 0.004	0.014 ± 0.004
High ionic strength	(12)		(12)				0.010 ± 0.005	0.008 ± 0.004
Carbon monoxide								
1:1 complex	>424	360	245	210	220	255	0.016 ± 0.008	0.018 ± 0.009
Low ionic strength	(0)		(19.5)				0.010 ± 0.005	0.011 ± 0.008
1:1 mixture	>424	360	230	220	215	260	0.012 ± 0.008	0.018 ± 0.010
High ionic strength	(0)		(19.5)				0.010 ± 0.005	0.011 ± 0.008

[a] Bound cytochrome c. [b] Free cytochrome c. [c] Average standard deviation ± the deviation of the experimental data expressed as absorbance units on a 1.0 scale. [d] $\Delta\epsilon_{604}{}^{R-O}$ for a_H, expressed as mM^{-1} cm^{-1}. [e] $\Delta\epsilon_{604}{}^{R-O}$ for a_L, expressed as mM^{-1} cm^{-1}. [f] $\Delta\epsilon_{550}{}^{R-O}$ for the total cytochrome c in the system, expressed as mM^{-1} cm^{-1}. [g] Average variance of the simulated curve from the average curve ± the deviation in the variance.

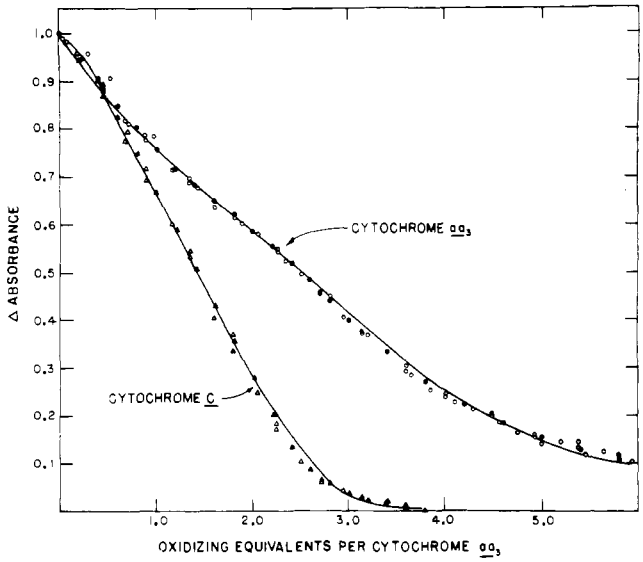

FIGURE 1: The oxidative titration of 17.9 μM cytochromes aa_3 and c in 0.01 M Tris-cacodylate buffer (pH 7.5) containing 0.5% Tween-20 under nitrogen atmosphere is given. Raw data (○, △) from four titrations, the averaged titration points (●, ▲), and the computer simulated best fit (—) are shown.

in the experiments to eliminate any possibility of oxygen contamination.

Titration experiments were performed in two buffer systems. A high ionic strength buffer contained 0.2 M Tris-cacodylate, pH 7.5, and 0.5% Tween-20 detergent and a low ionic strength buffer contained 0.01 M Tris-cacodylate, pH 7.5, and 0.5% Tween-20. The Tween-20 used in all experiments was pretreated by passage of a 20% (w/v) solution through a column (0.5 × 3 cm) of wetted neutral alumina (Bio-Rad Laboratories) and subsequently diluted to the desired concentration. Detergents, bound during the preparation of cytochrome aa_3 (loss of sodium cholate and Triton X-114 was determined by acid precipitation and 280 nm/420 nm absorbance ratios, respectively), were substantially removed by filtration of a concentrated solution of the enzyme (~300 μM) through a column (0.9 × 10 cm) containing G-25 Sephadex (Pharmacia Fine Chemicals) equilibrated with 1.0% Tween-20.

Spectral changes during oxidative titrations were recorded on a Cary 17 ratio recording spectrophotometer. The absorbance readings taken after each 10-20-μL addition of potassium ferricyanide were corrected for dilution before any additional data treatment. For each experiment, the average of three or four titrations was calculated and the standard deviation in the raw data computed (Schroedl and Hartzell, 1977b). Computer simulations were performed on averaged titration curves, using the approach outlined previously (Schroedl and Hartzell, 1977a). Corrections were made in the simulated curves for the absorbance contribution of cytochrome c at 604 nm and for cytochrome aa_3 at 550 nm. Midpoint potentials are expressed vs. the NHE.

For clarity, cytochrome c will be discussed in three contexts: (1) cytochrome c free in buffered solution, participating in neither hydrophobic nor electrostatic interactions is termed *"free" cytochrome c;* (2) cytochrome c which interacts loosely with membranes, submitochondrial particles, or electron-transport particles is termed *"membrane-bound" cytochrome c;* and (3) the cytochrome c which interacts strongly in an electrostatic manner with cytochrome aa_3 is termed *"bound" cytochrome c.* The latter interaction constitutes the cytochrome c-cytochrome aa_3 complex as described by Ferguson-Miller et al. (1976).

Results

Oxidative Titrations under Nitrogen Atmosphere

Low Ionic Strength. The oxidative titration of an equimolar complex of cytochromes c and aa_3 is presented in Figure 1. Changes in absorbance were monitored at 550 and 604 nm for each addition of potassium ferricyanide. Cytochrome c and cytochrome aa_3 initially titrated simultaneously. After addition of 6 oxidizing equiv of ferricyanide, the cytochrome c was fully oxidized while the cytochrome aa_3 was restored to 90% of the original oxidized absorbance level.

Computer simulation of these titration curves is shown as the solid line in Figure 1 and is summarized in Table I. The iron and copper centers were split into a low potential pair and a high potential pair of redox components and the extinction coefficients of the heme prosthetic groups were nearly equal in magnitude. For simulation of the cytochrome c curve, midpoint potentials established for the "membrane bound" protein ($E^{\circ\prime}$ = 225 mV, Dutton et al., 1970), or for cytochrome c "free" in solution ($E^{\circ\prime}$ = 262 mV; Heineman et al., 1975) were used in the initial calculations. Figure 2, curves 2 and 3, shows that neither midpoint potential alone was manifest in the titration of the cytochrome c-cytochrome aa_3 complex.

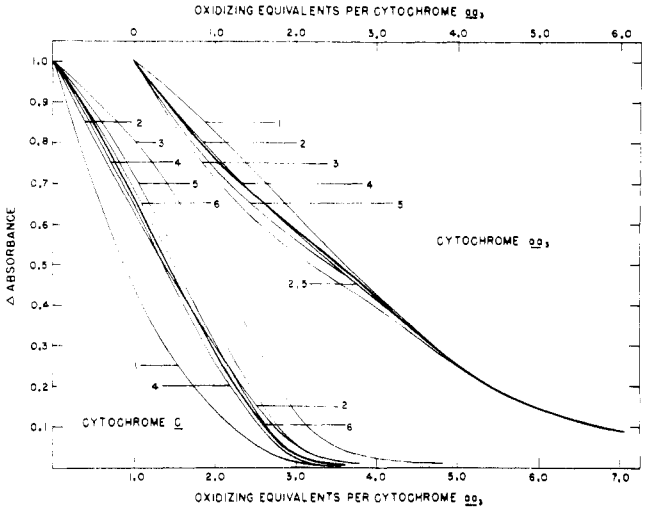

FIGURE 2: Theoretical titration curves were generated assuming 15 μM cytochromes aa_3 and c. The midpoint potential values were heme a_H = 360 mV, Cu_H = 365 mV, heme a_L = 225 mV, and Cu_L = 240 mV in all cases. The cytochrome c midpoint potentials were: (curve 1) only free cytochrome c, $E^{\circ\prime}$ = 200 mV; (curve 2) only free cytochrome c, $E^{\circ\prime}$ = 230 mV; (curve 3) only free cytochrome c, $E^{\circ\prime}$ = 260 mV; (curve 4) two cytochrome c potentials, one based on the oxidation of heme a_L, $E^{\circ\prime}$ = 200 mV, the second portion being free cytochrome c, $E^{\circ\prime}$ = 260 mV; (curve 5) two potentials as in curve 4, the heme a_L dependent $E^{\circ\prime}$ = 230 mV, free cytochrome c, $E^{\circ\prime}$ = 260 mV; (curve 6) two potentials as in curve 4, one based on the oxidation of Cu_L^{1+}, $E^{\circ\prime}$ = 200 mV and the second was free cytochrome c, $E^{\circ\prime}$ = 260 mV. The heavy curve (—) is the simulated best fit from Figure 1. Note: The cytochrome aa_3 and c curves are off-set to avoid overlap.

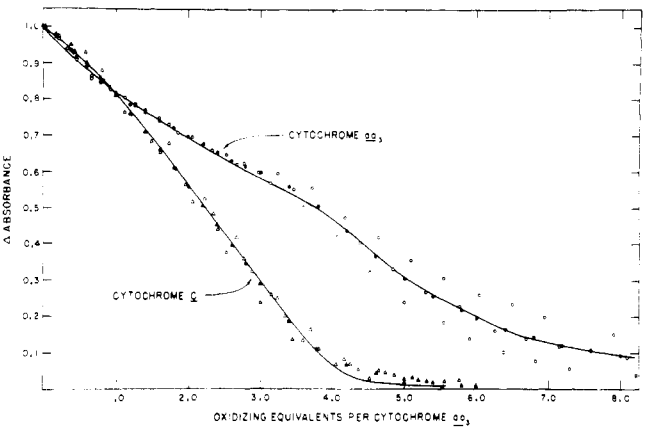

FIGURE 3: The oxidative titration of 17.3 μM cytochrome aa_3 and 34.6 μM cytochrome c in 0.01 M Tris-cacodylate containing 0.5% Tween-20 under nitrogen atmosphere is shown. Raw data (○, △) from three titrations, the averaged titration points (●, ▲), and the computer simulated best fit (—) are shown.

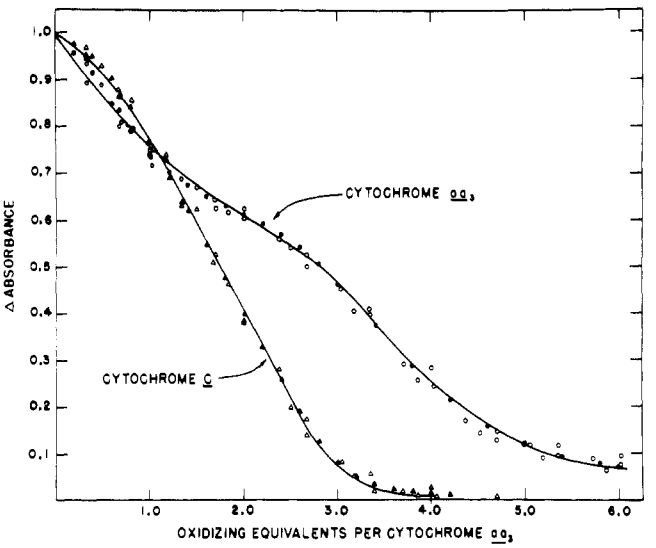

FIGURE 4: The oxidative titration of 12.7 μM cytochromes aa_3 and c in 0.2 M Tris-cacodylate containing 0.5% Tween-20 and under nitrogen atmosphere is given. Raw data (○, △) from four titrations, the averaged titration points (●, ▲), and the computer simulated best fit (—) are presented.

To fit the data, two cytochrome c midpoint potentials were necessary. The protein was divided into two pools and a corresponding Nernst expression for each pool was included in the calculations. The "free" protein was found to titrate with an $E^{\circ\prime}$ value of 255 mV, while the "bound" cytochrome c exhibited a midpoint potential of 210 mV.

Under low ionic strength conditions, 2 mol of cytochrome c are bound per mol of cytochrome aa_3 (Ferguson-Miller et al., 1976; Hartzell, manuscript in preparation). Oxidative titrations were performed using 17.3 μM cytochrome aa_3 and 34.6 μM cytochrome c in 0.01 M Tris-cacodylate plus 0.5% Tween-20, and monitored at 604 and 550 nm, respectively. Figure 3 indicates that the 2:1 complex was oxidized in a manner similar to the 1:1 complex shown in Figure 1. A significant plateau region was observed in the cytochrome aa_3 curve due to the titration of the additional cytochrome c. After 8 oxidizing equiv was added, the cytochrome c appeared fully oxidized while 90% of the cytochrome aa_3 was in the oxidized state.

Results of computer simulation of this titration are indicated in Figure 3 and Table I. The heme and copper centers exhibited the paired high and low midpoint potentials observed for the 1:1 complex, while the extinction coefficients of the heme groups absorbing at 604 nm were identical. To computer simulate the cytochrome c titration curve, it was necessary to include two pools of "bound" protein and a pool of "free" cytochrome c. Midpoint potentials of 220 and 250 mV were found for the "bound" cytochrome c while the "free" protein exhibited a midpoint potential of 260 mV.

High Ionic Strength. A comparison was drawn between the 1:1 complex of cytochrome c–cytochrome aa_3 and an equimolar mixture of the two proteins under conditions where separation upon gel filtration occurred (Hartzell and Zook, unpublished observation). Figure 4 illustrates the ferricyanide oxidation of a 12.7 μM mixture of cytochromes c and aa_3 in 0.2 M Tris-cacodylate buffer containing 0.5% Tween-20. In the early portion of the titration only cytochrome aa_3 was oxidized until the addition of nearly 1 equiv of ferricyanide. While the cytochrome c was oxidized, a large plateau region was observed in the cytochrome aa_3 curve (cf. Figure 1). The addition of 6 oxidizing equiv restored 93% of the cytochrome aa_3 to the original oxidized absorbance level.

The results of computer simulations are depicted in Figure 4 and summarized in Table I. While the redox components of cytochrome aa_3 remain split into two pairs, the high potential heme–copper pair is 20–25 mV lower in value than midpoint potentials observed under binding conditions. Likewise, the low potential heme–copper centers are 5–20 mV lower than the corresponding values in low ionic strength buffer. The extinction coefficients of the heme groups were equal. Although from gel filtration studies it appears that cytochrome c does not bind to cytochrome aa_3 under these experimental conditions (Hartzell and Zook, unpublished observations), midpoint potentials of 260 and 220 mV were found for cytochrome c.

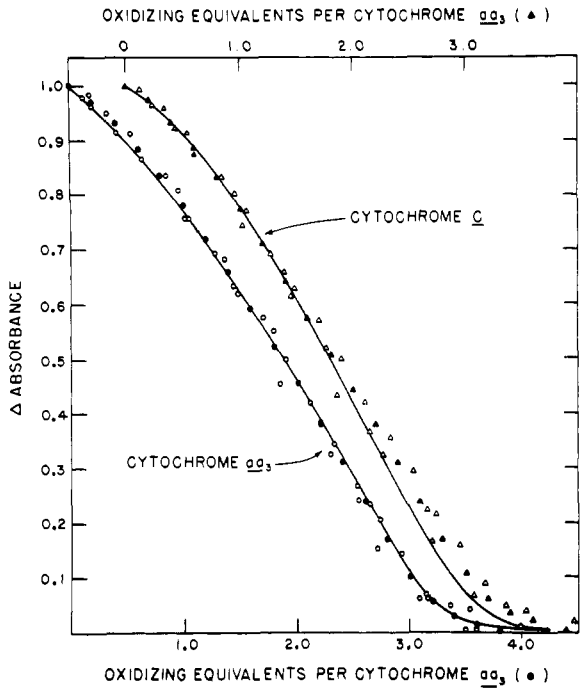

FIGURE 5: The oxidative titration of 16 μM cytochrome aa_3 and 18 μM cytochrome c under CO atmosphere is shown. Buffer conditions are listed in Figure 1. The curves are staggered for clarity; the curve on the left (bottom scale) describes cytochrome aa_3. Raw data (○, △) from three titrations, the averaged points (●, ▲), and the computer simulated best fit (—) are given.

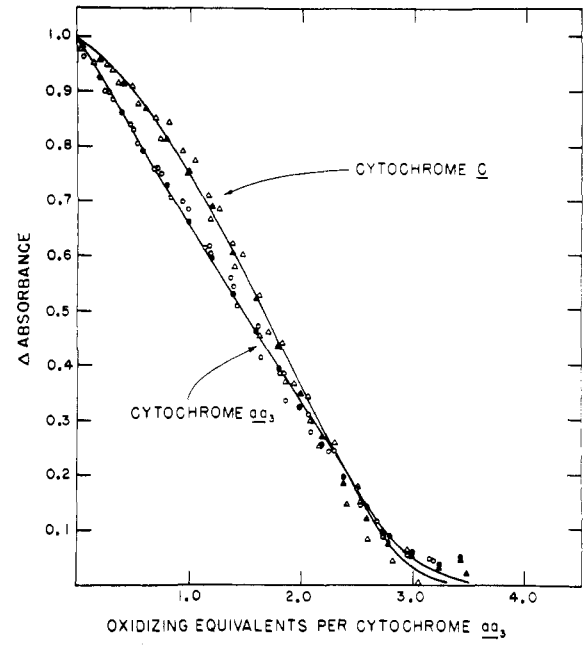

FIGURE 6: The oxidative titration of 14.75 μM cytochromes aa_3 and c under CO atmosphere is presented. Buffer conditions were used as in Figure 4. Raw data (○, △) from three titrations, the averaged titration points (●, ▲), and the computer simulated best fit (—) are shown.

Oxidative Titrations under Carbon Monoxide Atmosphere

Low Ionic Strength. Figure 5 illustrates the oxidative titration of an equimolar complex of cytochromes c and aa_3 under a carbon monoxide atmosphere. The two proteins were oxidized simultaneously over the entire course of the titration. Approximately 3.5 equiv of ferricyanide were required to titrate components that absorbed at 550 and 604 nm.

Computer simulation of the two curves is shown in Figure 5, and the quantitative midpoint potential values are given in Table I. The titratable heme component was much higher in midpoint potential (245 mV) than the low potential copper center (210 mV). The heme complexed to carbon monoxide was not oxidized by stoichiometric amounts of ferricyanide, while the high potential copper component was similar in midpoint potential to the value observed for the 1:1 complex under nitrogen atmosphere. Both "bound" and "free" cytochrome c exhibited midpoint potentials consistent with values found in the absence of carbon monoxide.

High Ionic Strength. When an equimolar mixture of cytochromes c and aa_3 in 0.2 M Tris-cacodylate, pH 7.5, and 0.5% Tween-20 was titrated in carbon monoxide, the curves shown in Figure 6 resulted. In the early portion of the titrations the components of cytochrome aa_3 titrated more rapidly than cytochrome c. However, the components of both proteins monitored at 604 nm and 550 nm were oxidized after the addition of 3.5 equiv of ferricyanide.

Table I and Figure 6 indicate the results of computer simulation of the titrations. Although the titratable heme group exhibited a higher midpoint potential value (230 mV) than the low potential copper center (220 mV), this heme $E^{\circ\prime}$ value was 15 mV and 30 mV lower then observed either in low ionic strength or for cytochrome aa_3 in the absence of cytochrome c, respectively. The high potential copper ion maintained an $E^{\circ\prime}$ value of 360 mV, while the CO-complexed heme was not oxidized. as was observed under nitrogen atmosphere, the cytochrome c exhibited two midpoint potential values.

Extinction Coefficients of Cytochrome c and Cytochrome aa_3 under Binding Conditions

To ascertain if binding of cytochrome c to cytochrome aa_3 influenced the reduced minus oxidized extinction coefficient of either protein, differences were determined as follows: 1.5 mL of stock ~15 μM cytochrome c and 1.5 mL of stock ~15 μM cytochrome aa_3 in 0.01 M Tris-cacodylate buffer containing 0.5% Tween-20 were placed in a 3-mL cylindrical cuvette. In a cylindrical tandem cell (Pyrocell, Westwood, N.J.) 1.5 mL of the same stock cytochrome c and 1.5 mL of the stock cytochrome aa_3 were placed in each chamber. Low ionic strength buffer (1.5 mL) was added to each chamber so the concentrations of cytochromes c and aa_3 in the tandem and 3-mL cylindrical cells were identical. Absolute spectra and difference spectra were recorded in the oxidized and reduced states. Five determinations showed no significant differences in extinction coefficients for either protein.

Discussion

Midpoint Potentials of Cytochrome aa_3 and Cytochrome c under Nitrogen Atmosphere. In the presence of cytochrome c, most researchers have found a high potential pair and a low potential pair of heme and copper redox centers for cytochrome aa_3 (Table II). Our values are consistent with those reported for both oxidative and reductive titrations performed using a variety of techniques. In our titrations using low ionic strength buffer, we find an increase in $E^{\circ\prime}$ values of the high potential pair of components, similar to values reported for these components in submitochondrial particles (Leigh et al., 1974). The low potential pair of redox centers exhibits quantitatively lower values at higher ionic strengths. The unequal extinction coefficients of the heme components observed for oxidative titrations of isolated cytochrome aa_3 (Schroedl and Hartzell, 1977a) have been eliminated. Equivalent extinction values (12 mM^{-1} cm^{-1}/heme a) are observed under binding and non-

TABLE II: Midpoint Potentials of Cytochrome aa_3 in the Presence of Cytochrome c.

Investigator	$E^{\circ\prime}$ (mV) Heme a	Cu	Heme a	Cu	Cytochrome c	Type of experiment
			Nitrogen Atmosphere			
Fujihara et al. (1974)	340 ± 15		209 ± 15		254 ± 10	Indirect electrochemical titrations in 0.1 M phosphate
Heineman et al. (1973)	340–350		210–225		250	Coulometric titrations in 0.3 M phosphate
Leigh et al. (1974)	360		210		None given	Potentiometric titration in MS-Mops buffer
Tiesjema et al. (1973)	370	280	230	280	None given	Potentiometric titration in 0.1 M phosphate
			Carbon Monoxide Atmosphere			
Anderson et al. (1976)	>465	340 ± 20	260 ± 10	190 ± 20	255	Indirect electrochemical titrations in I = 0.15 phosphate

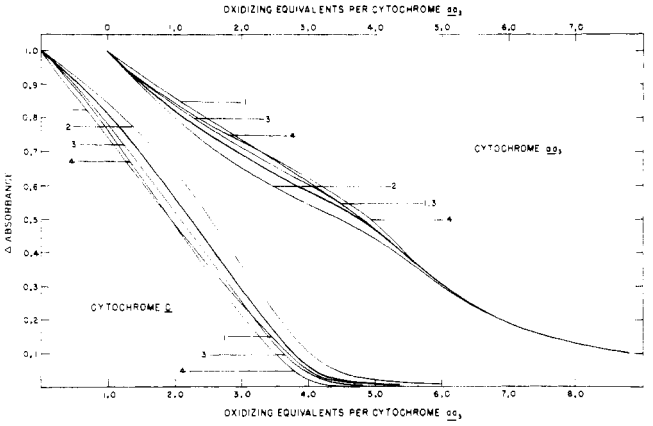

FIGURE 7: Theoretical titration curves for 15 μM cytochrome aa_3 and 30 μM cytochrome c are shown. Midpoint potentials for cytochrome aa_3 were: heme a_H = 365 mV, Cu_H = 370 mV, heme a_L = 230 mV, and Cu_L = 230 mV. Midpoint potentials for cytochrome c were: (curve 1) only free cytochrome c, $E^{\circ\prime}$ = 230 mV; (curve 2) only free cytochrome c, $E^{\circ\prime}$ = 260 mV; (curve 3) three midpoint potentials were used, one based on the oxidation of heme $a_L{}^{2+}$, $E^{\circ\prime}$ = 200 mV, one based on the oxidation of $Cu_L{}^{+}$, $E^{\circ\prime}$ = 250 mV, and the remainder of the cytochrome c was free in solution, $E^{\circ\prime}$ = 260 mV; (curve 4) $E^{\circ\prime}$ = 200 mV for two pools of cytochrome c, one dependent on the oxidation of heme $a_L{}^{2+}$, the second dependent on the oxidation of $Cu_L{}^{+}$, the remaining free cytochrome c, $E^{\circ\prime}$ = 260 mV. The heavy curve (—) is the simulated best fit depicted in Figure 3.

binding conditions using the same preparation of cytochrome aa_3 employed in earlier studies (Schroedl and Hartzell, 1977a). The small difference observed between the heme extinctions in the 1:1 complex could easily arise from a mixed population of cytochrome aa_3 molecules containing zero, one, or two molecules of bound cytochrome c.

The binding of cytochrome c to cytochrome aa_3 has a strong influence on the midpoint potential(s) of cytochrome c. Theoretical titration curves are shown in Figure 2 for a 1:1 complex and Figure 7 for a 2:1 complex. Several possible cytochrome c midpoint potentials are included. When the midpoint potential was fixed at a single value (curves 1–3, Figure 2; curves 1–2, Figure 7), the deviation from best fit was significant. A simple mixture of two midpoint potentials, one for the "bound" cytochrome c and a second for the "free" protein, was then included in the simulation procedure (not illustrated). However, no improvement in fit was found. When the "bound" cytochrome c was considered to titrate *only* after the low potential heme component had oxidized, we obtained an excellent fit to the titration data. Our results indicate that reduced cytochrome c, "free" in solution ($E^{\circ\prime}$ = 260 mV) binds to cytochrome aa_3 *after* cytochrome $a_L{}^{2+}$ has become oxidized, and at that point the cytochrome c midpoint potential becomes equivalent with cytochrome a (210–220 mV). This observation reduces the thermodynamically unfavorable condition where reduction of cytochrome a^{3+} (205–220 mV) is required by cytochrome c^{2+} with a midpoint potential of 260 mV.

Our experiments have been performed in low ionic strength (0.01 M Tris–cacodylate, pH 7.5) and high ionic strength (0.2 M Tris–cacodylate, pH 7.5) conditions; yet two cytochrome c midpoint potentials were found for both cases. Under low ionic strength conditions, the cytochrome c is assumed to be bound (with an $E^{\circ\prime}$ value = 210–220 mV) predominantly to the high affinity site in the 1:1 complex and to both sites in the 2:1 complex. The apparent midpoint potential of the low affinity site (250 mV) observed in the 2:1 complex may be an average potential value between bound (220 mV) and free (260 mV) cytochrome c. Surprisingly, the high ionic strength experiment showed an oxidation–reduction equilibrium dependent interaction of cytochrome c with cytochrome aa_3 that induced a drop in the cytochrome c midpoint potential, in spite of the fact that direct binding studies do not indicate strong interaction (Hartzell, manuscript in preparation).

A marked similarity between oxidation of a cytochrome c–cytochrome aa_3 mixture by molecular oxygen (Heineman et al., 1973) and our titration of an equimolar mixture of the two proteins with ferricyanide is noted. A Minnaert-type plot of our high ionic strength titrations compared with those of Heineman and co-workers (1973) shows a remarkable degree of overlap. Heineman and co-workers (1973) postulated that cytochrome c is *only* oxidized by cytochrome aa_3 when O_2 is the oxidant. We suggest that cytochrome c interacts with cytochrome aa_3 at the electron exchange loci even in high ionic strength medium. This interaction may be facilitated by the presence of Tween-20. We further suggest that ferricyanide, like oxygen, oxidizes bound cytochrome c through cytochrome aa_3 in spite of the fact that at equilibrium ferricyanide should oxidize both proteins.

Midpoint Potentials of Cytochrome aa_3 and Cytochrome c under Carbon Monoxide Atmosphere. The midpoint potentials of the carbon monoxide complex of cytochrome aa_3 were influenced slightly by the presence of cytochrome c. As had been observed for the isolated cytochrome aa_3 (Schroedl and Hartzell, 1977b), the midpoint potential value of the titratable heme group was elevated with respect to the low potential copper center. However, both low potential centers exhibited numerically lower $E^{\circ\prime}$ values than were observed in the absence of cytochrome c (Schroedl and Hartzell, 1977b). The high potential copper ion maintained the midpoint potential observed in the absence of CO.

Cytochrome c again displayed the duality in midpoint potential in the presence of CO. The oxidation state of heme a_L dictated the binding and subsequent decrease in midpoint potential (215–220 mV) of free cytochrome c (255–260 mV).

In conclusion we find that the measured $E^{\circ\prime}$ values for bound cytochrome *c* differ significantly from the $E^{\circ\prime}$ for cytochrome *c* in solution. The decrease of 50 mV found in the $E^{\circ\prime}$ value when cytochrome *c* is bound makes cytochrome *c* equal-potential with the low potential heme *a* and copper components of cytochrome aa_3. It is not obvious the role this cytochrome *c* interaction with cytochrome aa_3 has in either the mechanism of electron transfer or energy conservation; however, it now appears quite certain that cytochrome *c* indeed binds to cytochrome aa_3 and each cytochrome recognizes the presence of the other.

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Use of Specific Lysine Modifications to Locate the Reaction Site of Cytochrome *c* with Cytochrome Oxidase†

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ABSTRACT: The reaction of cytochrome *c* with trifluoromethylphenyl isocyanate was carried out under conditions which led to the modification of a small number of the 19 lysines. Extensive ion-exchange chromatography was used to separate and purify six different derivatives, each modified at a single lysine residue, lysines 8, 13, 27, 72, 79, and 100, respectively. The only modifications which affected the activity of cytochrome *c* with cytochrome oxidase (EC 1.9.3.1) were those of lysines immediately surrounding the heme crevice, lysines 13, 27, 72, and 79, and also lysine 8 at the top of the heme crevice. In each case, the modified cytochrome *c* had the same maximum velocity as that of native cytochrome *c*, but an increased Michaelis constant for the high affinity phase of the reaction. This supports the hypothesis that the cytochrome oxidase reaction site is located in the heme crevice region, and the highly conserved lysine residues surrounding the heme crevice are important in the binding.

There are numerous lines of evidence suggesting that the binding of cytochrome *c* to both its reductase and oxidase is largely facilitated by ionic interactions involving positively charged lysine ϵ-amino groups on the surface of the cytochrome *c* molecule. It is not known, however, whether these lysines are involved only in favorable interactions at the binding interfaces, or whether they are also involved in the actual mechanism of electron transfer to and from the iron. Further, although recent evidence (Dickerson and Timkovich, 1975) suggests that both the reductase and oxidase bind in the general area of the exposed heme edge of the cytochrome *c* molecule, it is not clear what specific regions are involved, whether there is any overlap of the two binding sites, or whether in fact the oxidase and reductase bind to the same site on cytochrome *c*.

Selective chemical modification of single lysine residues on the cytochrome *c* molecule offers an attractive approach toward determining the involvement of these residues in the reactions with the reductase and oxidase. Dickerson and Timkovich (1975) review the effects of a number of lysine derivatives. More recently, Staudenmayer et al. (1976, 1977) reported on the preparation of five derivatives with single trifluoroacetylated lysine residues at positions 13, 22, 25, 55, and 99. In a preliminary report, Brautigan and Ferguson-Miller (1976) obtained six mono-4-carboxy-2,6-dinitrophenyl (CDNP)[1] derivatives, three of which were identified as CDNP-lysine 13, 60, and 72 cytochromes *c*.

We report here on the reaction of *m*-trifluoromethylphenyl isocyanate with horse cytochrome *c* to produce a variety of trifluoromethylphenylcarbamoyl (TFC) derivatives. Six of these have been identified as containing singly modified ϵ-amino groups at residues 8, 13, 27, 72, 79, and 100. Their characteristics, ^{19}F NMR properties, and reactivities with beef heart cytochrome oxidase are presented.

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[1] Abbreviations used are: TFA, trifluoroacetyl; TFC, trifluoromethylphenylcarbamoyl; CDNP, 4-carboxy-2,6-dinitrophenyl; Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TMPD, *N,N,N′,N′* tetramethyl-*p*-phenylenediamine dihydrochloride; Mops, 4-morpholinepropanesulfonic acid; TNP, trinitrophenyl; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.