COMPLETE ANALYSIS OF THE CYTOCHROME COMPONENTS OF BEEF HEART MITOCHONDRIA IN TERMS OF SPECTRA AND REDOX PROPERTIES

The c_1-Cytochromes

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ABSTRACT Using newer techniques for conducting and analyzing potentiometric titrations, we have studied the thermodynamic and spectral properties of cytochrome c_1 in beef heart mitochondria. We find two species of cytochrome c_1, both with $n = 2$ values for the number of electrons involved in their oxidation or reduction. One has an $E_m \sim 210$ mV and a spectral peak near 555 nm and the other has an $E_m \sim 255$ mV and a spectral peak nearer 553 nm. These E_m values are pH-independent in the range of pH 6 to 8. The E_m and n values of these two components are indistinguishable from those of two species of cytochrome aa_3 (i.e. spectral feature of 605 nm).

INTRODUCTION

Using techniques that enable the acquisition of a large number of complete optical spectra over a wide range of closely spaced solution potentials and newer methods of analysis that utilize this additional information, (see Shrager and Hendler, 1986) we have undertaken a reexamination of the spectral and thermodynamic properties of the cytochromes in beef heart mitochondria. The first paper of this series (Reddy and Hendler, 1983), examined the *b*-cytochromes and it was found that whereas the usual method of analysis employing a two-point ΔA indicate a single b_T and a single b_K species, both with $n = 1$ values, the more thorough analysis showed the b_T species to consist of two forms of cytochrome, one with $n = 1$ and the other with $n = 2$. The spectrum of the $n = 2$ ($E_m \sim 17$ mV)[1] species was close to the spectrum of the b_T-species which arises during the oxidant-induced reduction of cytochrome b_T. In two accompanying papers, (Reddy et al., 1985; Hendler et al., 1986), it is shown that cytochromes aa_3 display two $n = 2$ Nernstian components having E_m values similar to the two described in this paper.

We have found that cytochrome c_1, previously considered to be a simple $n = 1$ species with E_m 230 mV, is really a mixture of two species both with $n = 2$. The major species accounts for 60% of the total absorbance change during the titration and has an $E_m \sim 255$ mV and an absorption peak at 553 nm. The second species accounts for 30% of the absorbance change, has an $E_m \sim 210$ mV and a flatter absorbance maximum centered at ~555 nm. Using the traditional methods of analysis we find cytochrome c_1 to be a single $n = 1$ species with an $E_m \sim 230$ mV.

EXPERIMENTAL PROCEDURES

The mediators (and redox buffers) used in the present work were potassium ferricyanide (E_m = 435 mV), Merck; quinhydrone (E_m = 280 mV), Fisher, 1–2 naphthoquinone (E_m = 143 mV), phenazine methosulfate (E_m = 80 mV), Cal-Biochem; diaminodurene (E_m = 240 mV), Aldrich. Other chemicals used were MES (Sigma), sodium hydrosulfite (Fisher), and Tris buffer (Boehringer Mannheim, West Germany).

Bovine Heart Mitochondria

The mitochondria used and their method of preparation are described in our previous paper (Reddy and Hendler, 1983). Complex III prepared from bovine heart mitochondria was supplied by Dr. J. Rieske. The complex was suspended in 0.05 M Tris at pH 8.0 at a concentration of 2 mg protein/ml. The concentration of c_1 in the complex was 3 nmol/mg of protein (based on an extinction coefficient at 553 nm of the reduced minus oxidized spectrum of 17.1 mM^{-1} cm^{-1}.)

Titration Procedure

The titration procedure and apparatus used were essentially as described previously (Reddy and Hendler 1983) except for the following modifications. The mediators used were potassium ferricyanide and quinhydrone at 0.2 mM each, and diaminodurene, 1–2 naphthoquinone, and phenazine methosulfate at 0.1 mM each. The titration mixture contained 15 mg of protein for mitochondria and 1.0 mg of protein for complex III in a total volume of 3.0 ml. Titrations were performed by electrical reduction, chemical reduction, chemical oxidation, and combined electrical-chemical oxidation. Electrical reduction was performed after first raising the

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[1] *Abbreviations in this paper:* E_m denotes the midpoint potential at the pH specified and E, the solution potential, both referenced to the standard hydrogen electrode; ΔA is a difference in absorption; SVD is singular value decomposition.

voltage of 400 mV electrically and then up to 480 mV by addition of 2 to 4 μl 1 M $K_3Fe(CN)_6$. Diaminodurene was then added and the titration was carried out as previously described (Reddy and Hendler, 1983). In the case of chemical reduction, after raising the voltage of the solution as above, the titration was performed by the addition of 0.1 to 0.2 μl aliquots of freshly prepared sodium hydrosulfite in 1 M phosphate buffer at pH 9.2 followed by 3 to 4 min periods of equilibration, after which a spectrum was recorded. The solution potential was lowered about 5 mV by each addition. Chemical oxidation was performed by first lowering the voltage of the solution to ~100 mV by addition of 0.5 to 1 μl of sodium hydrosulfite, at which time diaminodurene was added. The titration was performed by addition of 1 M $K_3Fe(CN)_6$ (~0.1 to 0.2 μl) and the spectra were recorded after 2–3 min of equilibration. The solution potential was raised by an average of ~5 mV after each addition. A combined electrical oxidation-chemical oxidation was performed, first electrically up to 300 mV at which time there is resistance to further raising of the voltage. The titration was then continued by addition of $K_3Fe(CN_6)$ up to 450 mV. For each titration, 70–100 spectra were recorded in the voltage range of 100 to 450 mV.

The spectrometer, data collection and data analysis have been described (Reddy and Hendler, 1983). Protein concentration was estimated by the method of Lowry et al. (1951). The second derivative analysis for cytochrome c_1 was performed using 553 nm as the peak wavelength with six points (1.23 nm for each point) on either side of the peak. The two-point analysis for cytochrome c_1 was performed by taking the difference in absorbance between the peak wavelength at 553 nm and reference wavelength at 540 nm.

A description of all of the experiments is given in Table I. The table shows that three different preparations of mitochondria were examined as well as a fourfold range in mediator concentration. Other conditions examined were changes in pH, presence of inhibitors of cytochrome oxidase, the use of soluble complex III in place of mitochondria, and controls using an egg homogenate in place of a respiratory system.

RESULTS

The results of a two-point ΔA analysis (553–540 nm) in a standard E vs. log [OX]/[RED] plot for a titration performed by chemical reduction are shown in Fig. 1 *a*. The points fall most closely around a theoretical $n = 1$ curve with E_m 220 mV. The data are not close to the theoretical $n = 2$ curve and the absence of a clear inflection indicates that one, rather than two or more components is present. This picture and analysis are very close to previously published analyses for cytochrome c_1 or cytochromes ($c + c_1$) (e.g., Dutton et al., 1970, Fig. 3). The same data represented as ΔA vs. E, very closely fall along a theoretical $n = 1$ plot and are easily distinguished from the theoretical $n = 2$ curve (Fig. 1 *b*). Although the $n = 1$ fits are clearly superior to the $n = 2$ fits, closer examination of the points in relation to the $n = 1$ theoretical curves shows systematic rather than random deviations. As discussed elsewhere (Reddy and Hendler, 1983; Shrager and Hendler, 1986), a two-point ΔA analysis, especially in particulate preparations, is subject to a variety of interferences from other cytochromes, and background effects related to light scattering. More definitive information about a discrete transition centered at a particular wavelength (i.e. 553 nm) is obtained in a second derivative vs. E analysis.

In Fig. 2 *a* the raw data expressed as the magnitude of the 2nd derivative at 553 nm are shown as large points and a best fit curve for a single $n = 1$, Nernstian component as

TABLE I
DESCRIPTION OF THE EXPERIMENTS

Exp. No.	Type	Method*	Voltage range mV	Number of spectra analyzed
60‡	Control	CR	425–135	68
61	Control	ER	447–136	79
62	Control	EO	133–432	52
63	Control	CO	100–440	73
66	Control	EO/CO	115–498	93
67	Control	ER	473–100	102
68	Control	EO/CO	123–397	50
83	Control	EO/CO	109–526	97
84	Control	EO/CO	113–462	88
102§	Control	CR	457–99	84
103‖	Control	CR	466–98	82
104¶	Control	CR	452–100	72
105**	Control	CR	460–100	73
74‡‡	complex III	CR	334–(−254)	130
64	pH 8.0	CR	444–75	74
86	pH 8.0	CR	444–42	95
87	pH 6.0	CR	436–101	78
98	pH 6.0	CR	451–108	73
101	pH 6.0	CR	444–60	84
99	cyanide	CR	443–98	71
100	cyanide	CO	124–449	64
88	azide	CR	464–(−24)	111
89	azide	CR	477–2	101
90	azide	EO/CO	39–454	108
91	azide	CR	466–101	91
92	azide	EO/CO	65–463	93
65	mediator control	ER	440–234	56
108	mediator control	CR	477–110	97
93	mediator control + azide	CR	456–100	80

Control experiments contained 15 mg protein of mitochondria in 3 ml of a medium containing 125 mM KCl, 62.5 mM potassium phosphate, and mediators at pH 7.0. Unless otherwise noted, mitochondria prepared by the procedure of Blair (1967) were used. The usual mediator mix contained potassium ferricyanide and quinhydrone at 0.2 mM each, and diaminodurene, 1,2 napthoquinone and phenazine methosulfate at 0.1 mM each. The experiment with complex III contained 1 mg protein. For the experiments at pH 6.0 M (66.5 mM) was present and the pH was adjusted with 1 N HCl. For the experiments at pH 8.0, Tris (66.5 mM) was present and the pH was adjusted with 1 N NaOH. Cyanide was present at 1 mM and azide at 10 mM.

*The methods of titration were CR (chemical reduction), ER (electrical reduction), EO (electrical oxidation), CO (chemical oxidation), and EO/CO (combined electrical, chemical oxidation). These techniques are fully described in methods and in Reddy and Hendler (1983). In the mediator controls, 17.2 mg of protein of a raw egg homogenate was used in place of mitochondria.

‡Mitochondria prepared by the procedures of Löw and Vallin (1963).

§Mitochondria obtained from the Institute of Enzyme Research, Wisconsin.

‖Double concentration of mediators used.

¶One half concentration of mediators used.

**In addition to the standard mediator mix, additional mediators present were 2 hydroxy naphthoquinone at 0.1 mM, duroquinone at 50 μM and anthroquinone sulfonates, which included the 1-sulfonate, the β-sulfonate, the (1,5) disulfonate, and the (2,6) disulfonate at 20 μM each.

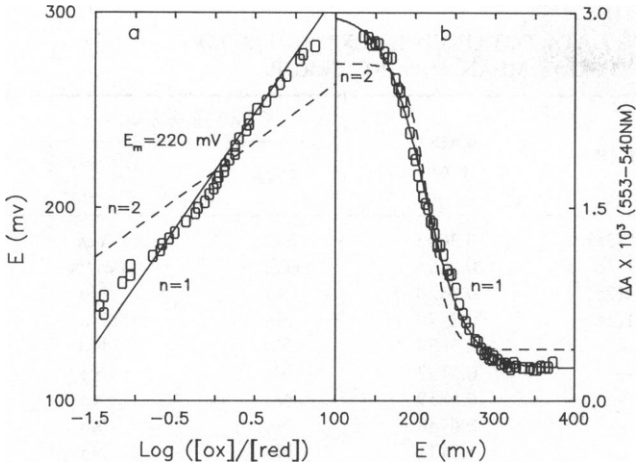

FIGURE 1 The data are from experiment 60, shown in Table I and performed by chemical reduction. The ΔA for the points at 553 and 540 nm are shown as E vs. log[OX]/[RED] according to the method of Wilson and Dutton (1970) in panel *a* and as ΔA vs. E for panel *b*. Theoretical curves for single $n = 1$ and single $n = 2$ components using the E_m values and totals selected by the computer are shown.

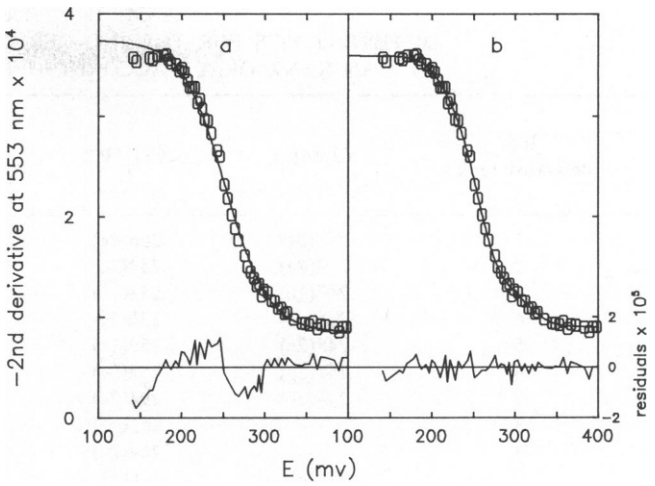

FIGURE 2 The data from experiment 60 are represented by the negative 2nd derivative of thirteen successive points centered at 553 nm. The line in panel a shows the theoretical behavior for a single $n = 1$ Nernstian component with an $E_m = 253$ mV. The same data fit by three $n = 2$ Nernstian components with E_m's of 215 mV, 256 mV, and 319 mV in approximate proportions of 1:3:0.5 are shown in panel *b*. The residuals between the experimental data and the computer fits are shown in the bottom parts of the figures using the scale on the vertical axis to the right.

a solid line. At first glance, the data seem to cluster closely around the theoretical curve. However, a plot of the residuals between the two curves (at the bottom of the panel) shows systematic deviations. This indicates that the model is not a correct representation of the data. In panel 2 *b*, the data are shown in relation to a theoretical curve for three $n = 2$ Nernstian components. A plot of the residuals at the bottom of the panel shows a much better fit with random deviations representing the noise level in the experiment. The statistics of the fit (Table II) show low standard errors and dependency values that indicate the reality of three independently titrating Nernstian species. A variety of other possible fits were tried. Using the root mean square deviation as a guide, these are listed in rank order from best to worst in Table III. The best fit obtained by use of singular-value decomposition (SVD) (Shrager and Hendler, 1982; Reddy and Hendler, 1983; Shrager and Hendler, 1986) is also shown. It is seen that the best fits obtained by the second derivative and SVD analyses are very close to each other. As the root mean square deviation from the second-derivative analysis increased, the agreement with SVD deteriorated. The most prominent species in terms of the amount of absorbance it contributes, is the central species with $E_m \sim 256$ mV. A very critical question is "could this component be an $n = 1$ species"? Attempts to force a fit for $n = 1$ by manipulating the n values of the neighboring components were generally unsuccessful in that there was no convergence on a minimum for the sum of squares, the standard errors became infinite, or the dependency values reached their limits of 1.000000 with these restrictions (rows 10–13). The best fit for $n = 1$ is shown in row 5. This fit required an $n = 2$ component with $E_m = 248$. If these two species are actually present, they should be resolved by SVD. As will be shown below, SVD did not see these species but did resolve the same three species seen in row 1 of the Table. Direct analysis of the raw spectral data by taking difference

TABLE II
STATISTICS FOR COMPUTER FIT SHOWN IN FIGURE 2 *B*

Parameter	Value	S.E.	Number of electrons	Dependency
*T*1	0.5181×10^{-4}	0.7121×10^{-5}	2	0.985733
*T*2	0.1819×10^{-3}	0.6546×10^{-5}	2	0.991047
*T*3	0.3536×10^{-4}	0.2624×10^{-5}	2	0.966174
*E*1	214.7 mV	3.6		0.932286
*E*2	256.0 mV	1.3		0.943433
*E*3	318.7 mV	3.3		0.770912

*T*1, *T*2, and *T*3 represent the magnitudes of the negative second derivatives for three components. *E*1, *E*2, and *E*3 represent the corresponding E_m values. The significance of dependency values in indicating necessary components to the fit has been previously discussed (Reddy and Hendler, 1983; Shrager and Hendler, 1986; Hendler et al., 1986).

TABLE III
DIFFERENT FITS FOR THE 2ND DERIVATIVE DATA OBTAINED IN EXP. 60 LISTED IN RANK ORDER ACCORDING TO THE ROOT MEAN SQUARE ERROR

2nd derivative rank	COMP 1	COMP 2	COMP 3	RMS* error	Compatible with	
					SVD	Difference spectra
1	215(2e)	256(2e)	319(2e)	0.3003	Yes	Yes
2	213(2e)	254(2e)	301(1e)	0.3235	Yes/No	Yes/No
3	247(1e)	253(2e)	324(2e)	0.3420	No	No
4	245(1e)	252(2e)	311(1e)	0.3479	No	No
5	248(2e)	259(1e)	—	0.3674	No	No
6	251(1e)	252(2e)	—	0.5193	No	No
7	243(2e)	294(2e)	—	0.5859	No	No
8	—	253(1e)	—	0.6788	No	No
9	—	254(2e)	—	1.1771	No	No
10	—(1e)	—(1e)	—	—	—	—
11	—(2e)	—(1e)	—(1e)	—	—	—
12	—(2e)	—(1e)	—(2e)	—	—	—
13	—(1e)	—(1e)	—(1e)	—	—	—
SVD	197(2e)	256(2e)	325(2e)			

The table shows E_m values in millivolts for up to three components that transfer the number of electrons shown in parentheses.
—No fit obtained with these components.
*Units = 10^{-4}.

spectra across limited voltage ranges was also compatible with the best fits obtained by second derivative and SVD analyses and not with the fits that had an $n = 1$ value for the component with E_m 256 mV. There was a general consistency in all of the titrations in that three $n = 2$ components provided the best fit and that fits using an $n = 1$ for the central component were either inferior or not possible. As shown in Fig. 1, the same data analyzed by the procedures commonly used, namely the two point ΔA, graphically analyzed as a function of E vs. log (OX/RED) indicate a model with a single $n = 1$ species. This important point is even more strongly shown in the data of another experiment illustrated in Fig. 3. As is characteristic of the E vs. log [OX]/[RED] technique, data beyond log = ±1 are particularly subject to noise. The central points fall closely on a theoretical curve for a single $n = 1$ component. The $n = 2$ curve is far from the data and no hint of heterogeneity is seen. These data analyzed by the second-derivative method are shown in Table IV along with the results of an SVD analysis, and the compatibilities of the second derivative, SVD, and difference spectra analyses. Whereas the analysis based on the two-point ΔA and using the E vs. log [OX]/[RED] technique shows a single $n = 1$ component, the more discriminating analyses based on the 2nd derivative at 553 nm finds the presence of three $n = 2$ species.

The conclusion from the analysis of all data sets by the second derivative procedure is that the major species of cytochrome c_1 is a two-electron-transferring component with $E_m \sim 255$ mV. It accounts for 60% of the total absorbance change (expressed as the second derivative) during the titration. A second two-electron-transferring species is always present. It has an $E_m \sim 210$ mV and accounts for ~30% of the total absorbance change. A third component with a weak absorbance (<10%) is usually required to obtain the closest fits of the data. This component is not always distinguishable above noise levels.

The characterization of c_1-cytochrome species was further pursued by SVD analysis. The spectral information in the experiment represented in Fig. 4 has been resolved into four fundamental spectra (panels a, c, e and g). The first point of interest revealed in these spectra is that the characteristic absorbance for cytochrome c_1 at 553 nm is

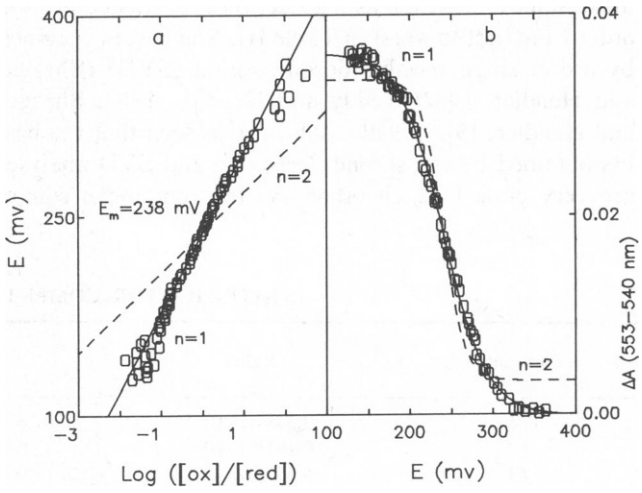

FIGURE 3 The data are from experiment 67 shown in Table I and performed by electrical reduction. The ΔA between the points at 553 and 540 nm was used as the indicator of reduction. An E vs. log [OX]/[RED] plot is shown in panel *a* and the ΔA vs. E plot is shown in panel *b*. Theoretical $n = 1$ and $n = 2$ curves using the E_m values and totals selected by the computer are drawn.

TABLE IV
DIFFERENT FITS FOR THE 2ND DERIVATIVE DATA OBTAINED IN EXP. 67 LISTED IN RANK ORDER ACCORDING TO THE ROOT MEAN SQUARE ERROR

2nd derivative rank	COMP 1	COMP 2	COMP 3	RMS* error	Compatible with	
					SVD	Difference spectra
1	222(2e)	262(2e)	307(2e)	0.7724	Yes	Yes
2	223(2e)	262(2e)	281(1e)	0.7919	Yes/No	Yes/No
3	236(2e)	258(1e)	—	0.8929	No	No
4	229(2e)	278(2e)	—	1.0720	No	No
5	—	251(1e)	—	1.0893	No	No
6	—	251(2e)	—	3.4043	No	No
7	—(1e)	—(2e)	—(2e)	—	—	—
8	—(1e)	—(2e)	—(1e)	—	—	—
9	—(2e)	—(1e)	—(1e)	—	—	—
10	—(2e)	—(1e)	—(2e)	—	—	—
11	—(1e)	—(2e)	—	—	—	—
12	—(1e)	—(1e)	—	—	—	—
13	—(1e)	—(1e)	—(1e)	—	—	—
SVD	209(2e)	250(2e)	300(2e)			

The table shows E_m values in millivolts for up to three components which transfer the number of electrons shown in parentheses.
—No fit obtained with these components.
*Units = 10^{-5}.

always accompanied by a prominent absorbance feature at 605 nm. Absorbances in the region of 605 nm are attributed to cytochrome aa_3 (i.e. cytochrome *c* oxidase). The titration behavior for each of these fundamental spectra is shown in the corresponding right panels. The main question that we want to answer is which of two quite different views of cytochrome c_1 is correct. The prevailing view is that cytochrome c_1 is a single entity with E_m 220 to 240 mV and $n = 1$. The second derivative technique found two principal species with E_m values near 200 mV and 250 mV and both with $n = 2$. The results of the SVD analysis represented in Fig. 4 are unequivocally in support of the conclusions reached by the second derivative analysis. Two vertical lines at 200 mV and 250 mV in the right hand panels are used to mark titrations occurring at these voltages. The marked change of slope in the voltage regions around these values, indicating two separate components, is clearly evident in the titration of the first fundamental spectrum in panel b. In panel d only the component titrated at 200 mV is present. The flatness of the curve in the region

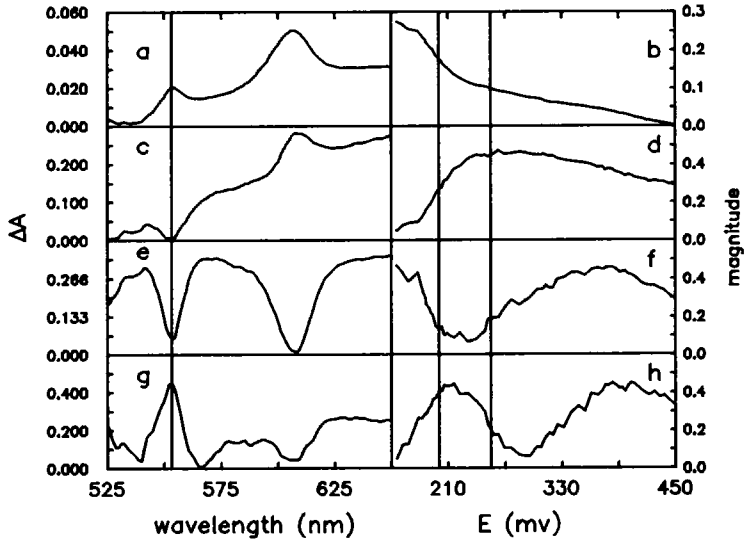

FIGURE 4 Data from experiment 66 shown in Table I and performed by combined electrical, chemical oxidation. Analysis was by the technique of SVD (Shrager and Hendler, 1982; Reddy and Hendler, 1982; Shrager and Hendler, 1985). The first four principal spectral components are shown in the *left-hand* panels. The titration behavior for each of these components is shown in the corresponding *right-hand* panels. The vertical line through the spectra on the left is at 553 nm. The two vertical lines through the titrations on the right are at 200 and 250 mV.

TABLE V
TITRATIONS OF PRINCIPAL SPECTRAL COMPONENTS OBTAINED BY SVD ANALYSIS OF EXP. 66

E_m		No. of electrons	COMP 1 12.4*	COMP 2 0.44*	COMP 3 0.12*	COMP 4 0.034*
mV	Av.					
196			0.174099‡			
199		2		−0.418326		
	191					
190					0.507272	
179						−0.479556
267			0.0240749			
248	256	2			−0.259316	
254						0.547755
327			0.0441543			
359		1		0.131548		
	332					
317					−0.245269	
327						−0.564993

*Singular value.
‡The decimal numbers are the computer-fitted amounts for each titrating species as identified by the first two columns.

around 250 mV means that the spectrum shown in panel c is a component of the spectrum for the species with E_m 200 mV and not part of the spectrum for the species with E_m 250 mV. The presence of two separate components at ~200 mV and ~250 mV is clearly shown in panels f and h. In these panels, the slopes of the titration curves are of the opposite sign for the two components. A single component with $n = 1$ and E_m 235 mV could not produce these results. Even though two, rather than a single species are required to account for the results of the SVD analysis, the question of whether the main species is a one-or two-electron-transferring component still requires further consideration. The data from the best fits for the four titration curves shown in Fig. 4 are presented in Table V. It can be seen that a two-electron component in the region near 200 mV was present in all four titrations of the fundamental spectra. A two-electron component near 250 mV was present in three of the four titrations.

Many attempts were made to obtain fits with the component near 250 mV held fixed at $n = 1$. This led to a variety of incompatibilities that included: 1) lack of agreement between the SVD and 2nd derivative analyses; 2) high standard errors, root-mean-square deviations, and dependency values; 3) lack of agreement between SVD reconstructed spectra and direct difference spectra. The correspondence of spectra deduced by SVD with actual difference spectra obtained over the same voltage region is essential in validating the SVD-derived results.

Fig. 5 shows SVD reconstructed spectra (solid lines) along with difference spectra (dashed lines) taken across narrow voltage regions around the SVD-determined E_m values. From top to bottom the panels show the components titrating near 190, 250, and 330 mV, respectively. The agreement between the SVD-deduced and actual difference spectra is seen to be quite close. An additional argument for the existence of two separate forms of cytochrome c_1 is obtained from the fact that the spectra for the two compounds are different. The location of the sharp peak for the more significant of the two (E_m 256 mV) shown in the middle panel is very close to 553 nm. The location of the broader peak for the compound at E_m 191 mV shown in the top panel is closer to 555 nm. The $n = 1$ component titrating with E_m 332 mV shown in the bottom panel is due to cytochrome *c* oxidase and it will be discussed in full, in separate papers dealing with this cytochrome (Reddy et al., 1986; Hendler et al., 1986). The difference in shape and location of the peaks for the

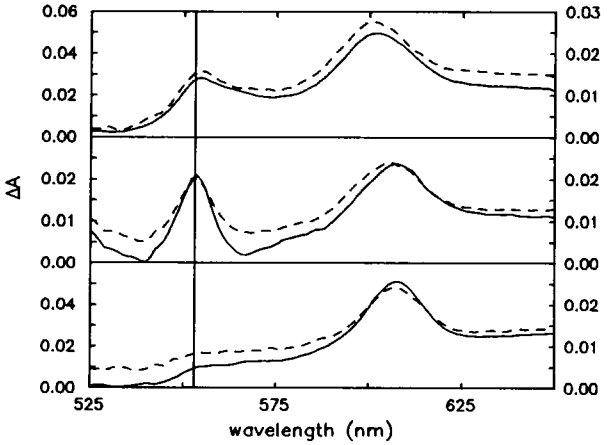

FIGURE 5 A comparison of SVD-deduced and actual difference spectra from the titration in experiment 66. SVD-deduced spectra are shown with solid lines that use the scales on the left axis and difference spectra obtained from the raw data are shown as dashed lines using the scales on the *right* axis. The top panel shows the SVD spectrum for the component with E_m = 191 mV and n = 2 and for the difference spectrum taken at 193 ± 16 mV. The *middle* panel shows the SVD spectrum for the component with E_m = 256 mV and n = 2 and for the difference spectrum at 257 ± 18 mV. The *bottom* panel shows the SVD spectrum for the component with E_m = 332 and mV and n = 1 and for the difference spectrum at 331 ± 20 mV. The vertical line is at 553 nm.

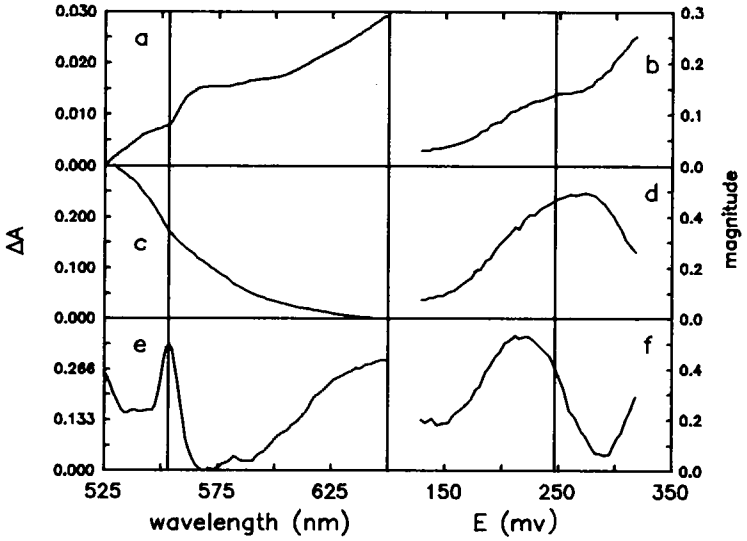

FIGURE 6 Data from experiment 74 using soluble complex III. Analysis by SVD yielded the three principal fundamental spectra shown in panels *a*, *c*, and *e* and the corresponding titrations shown in panels *b*, *d*, and *f*. The vertical line on the left shows the 553 nm position. The vertical line on the right shows the 247 mV position where titrations are seen to occur in panels *d* and *f* indicating the involvement of spectral components *c* and *e* in the full spectrum of the titrated component.

absorbance feature near 553 nm as shown in Fig. 5 is consistently seen under a variety of conditions which include, oxidations, reductions, change of pH and presence of cyanide (not shown).

In regard to cytochrome oxidase, it is important to point out that SVD sees its titration (the peak near 605 nm) to be simultaneous with, and indistinguishable from the titration of cytochrome c_1. With reference to the characterization of cytochrome c_1, two considerations of the titration characteristics of cytochrome oxidase become germane. Does the finding of $n = 2$ for titrations involving both cytochrome c_1 and cytochrome oxidase indicate a tight coupling between the two cytochromes, so that two $n = 1$ redox centers titrate as a unit of $n = 2$? If cytochrome oxidase titrates with $n = 2$ by itself and has the same E_m value as an $n = 1$ cytochrome c_1, species, could SVD be confused and mistakenly find a single $n = 2$ behavior for the mixture? This is unlikely because SVD is designed to resolve mixtures of this kind, and because of the results of the second derivative technique, which concentrates specifically on the behavior of the cytochrome c_1 absorbance. Nonetheless, we have specifically addressed these questions in two ways. Titrations and analyses were performed using purified complex III, which contains cytochrome c_1 and no cytochrome oxidase. In addition, we have examined the titrations obtained with mitochrondria in the restricted wavelength region of 525 to 585 nm where the spectral contributions characteristic of cytochrome oxidase are absent. In Fig. 6 are shown the fundamental spectra and their titrations derived from an experiment with isolated complex III. In contrast to the spectra obtained with whole mitochondria these spectra show no evidence of the presence of cytochrome oxidase. Best fits of the 553 nm feature by the second derivative procedure found an $n = 2$ and $E_m = 252$ mV for the major component. Fixing $n = 1$ produced a much inferior fit with the E_m at 259 mV. Best fits of the SVD titration data were for an $n = 2$ component at 247 mV, that was present in the titrations of the 2nd and 3rd fundamental spectral components. Forcing an $n = 1$ for this feature allowed a fit only in the case of the 3rd (i.e. least significant) spectral component and the E_m was placed at 285 mV. The second derivative analysis did not see any component at 285 mV.

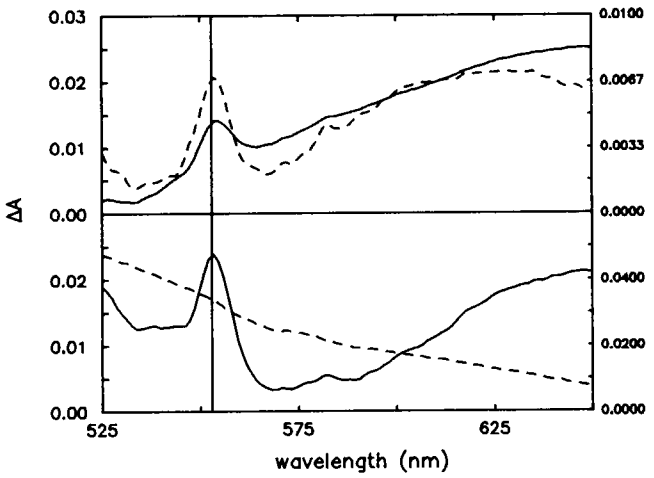

FIGURE 7 Comparison of SVD-deduced spectra from experiment 74 and analyzed in Fig. 6, with the actual difference spectra. The *top* panel shows the best-fit spectrum deduced by SVD for a component with $E_m = 247$ and $n = 2$ (solid line). A difference spectrum from the raw data taken across 244 ± 21 mV is shown in the same panel (dashed line). The only $n = 1$ component that could be fit in the SVD analysis is seen in panel *f* of Fig. 6 with an E_m at 285 mV and is shown as a solid line in the *lower* panel. The difference spectrum taken across 286 ± 17 mV is shown as a dashed line in the same panel. The vertical line is at 553 nm. The lack of correspondence in the spectra in the *lower* panel indicates that the $n = 1$ component at 285 does not exist.

The SVD-deduced spectrum for the major cytochrome c_1 component with $n = 2$ at 247 mV is shown in Fig. 7 (top panel, solid line) with the difference spectrum obtained from the original data across the voltage range 244 ± 21 mV (top panel, dashed line). The reconstructed spectrum for a component with a forced $n = 1$ at 285 mV is shown in Fig. 7 (bottom panel, solid line) along with the difference spectrum over the range of 284 ± 17 mV (bottom panel, dashed line) It is obvious that the SVD-deduced spectrum with $n = 2$ corresponds to the actual difference spectrum whereas the deduced spectrum obtained by fixing $n = 1$ does not. Experiments with purified cytochrome oxidase reveal that the feature near 605 nm titrates as $n = 2$ in the absence of cytochrome c_1 (Hendler et al., 1986). Treating the data obtained with intact mitochondria in the limited wavelength range of 525 to 585 nm by the SVD procedure yields the fundamental spectra and their titrations shown in Fig. 8. These data are enriched with the contribution of cytochrome c_1 and impoverished with respect to cytochrome oxidase. The best fits of the titration data yielded three $n = 2$ components with E_m values of 200, 257, and 323 mV. This corresponds to the best fit by the 2nd derivative method for three $n = 2$ components at 212, 260, and 308 mV. The SVD-deduced spectra with corresponding difference spectra obtained from the raw data are shown in Fig. 9. The agreement between the deduced and actual difference spectra is quite close. Fixing the central component at $n = 1$ split the $n = 2$ component with $E_m = 257$ mV into two components: an $n = 1$ at 215 mV and an $n = 1$ at 277 mV. The standard errors and dependency values were higher for these fits than for the fits with a single $n = 2$ component. The E_m values obtained for the $n = 1$ components were not seen by the 2nd derivative method which when forced to use $n = 1$, found a single

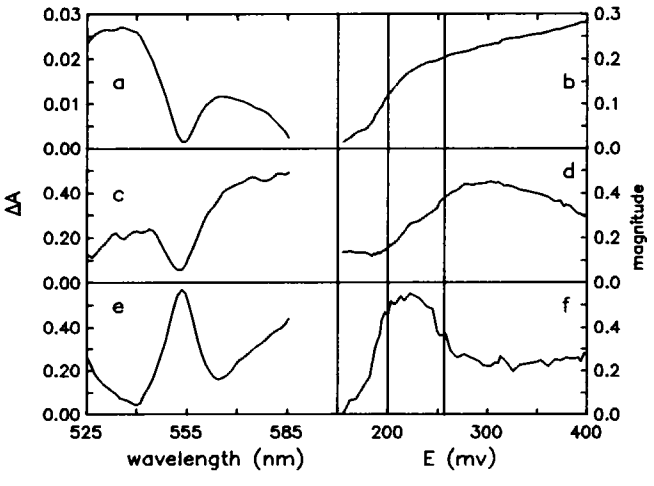

FIGURE 8 Data from experiment 66 analyzed by SVD in the restricted wavelength region 525 to 585 nm. The three principal fundamental spectra are shown in panels *a*, *c*, and *e* and the corresponding titrations are shown in panels *b*, *d*, and *f*. The vertical lines denote the 200 and 257 mV positions. Titration activity is seen in all three panels for both of these components.

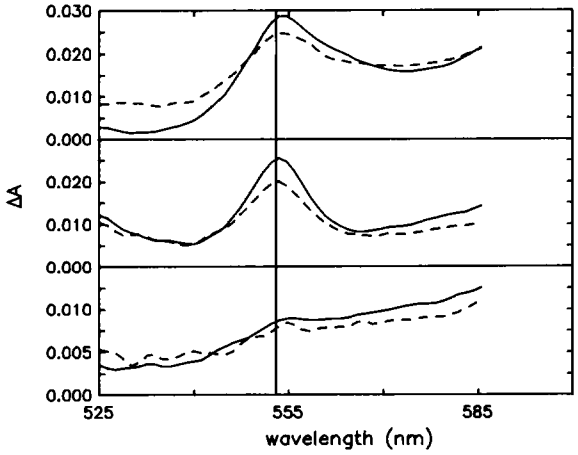

FIGURE 9 The SVD-deduced spectra from the analysis shown in Fig. 8 are shown here as solid lines. Difference spectra are shown as dashed lines. The *top* panel shows the SVD-deduced component with $E_m = 200$ mV and $n = 2$ and the difference spectrum across 200 ± 18 mV. The *middle* panel shows the SVD-deduced component with $E_m = 257$ mV and $n = 2$ and the difference specrum across 257 ± 18 mV. The *bottom* panel shows the SVD-deduced component with $E_m = 323$ and $n = 2$ and the difference spectrum taken across 20 mV. The vertical line is at 553 nm.

component at 240 mV. Furthermore, in other experiments using SVD where the best fits were consistent with $n = 2$ for the main cytochrome c_1 component, fixing $n = 1$ for this component gave solutions which were not consistent among the different experiments. Taking all of the many titrations analyzed by 2nd derivative and SVD procedures in relation to difference spectra across specific voltage regions using the raw data, the best picture is for a predominant c_1, cytochrome having an $n = 2$, an E_m ~255 mV, and a peak absorbance at 553 nm. A second c_1-cytochrome is present with an $n = 2$, an E_m ~205 mV, and a peak absorbance near 555 nm. A third c_1-cytochrome with $n = 2$ and E_m ~320 mV may also be present but its absorbance is too low for further characterization at this time.

A summary of results from both second derivative and SVD analysis of all experiments is shown in Table VI. The control series consisted of thirteen experiments including five chemical reductions, four electrical/chemical oxidations, two electrical reductions, one chemical oxidation, and one electrical oxidation. In this series, three different preparations of mitochondria were used as well as double the usual mediator concentration and one-half the usual concentration. All of the controls are shown in Table VII. The average values for the seven reductive titrations were the same as the average values for the six oxidative titrations. The variations for the percent of each species from experiment to experiment were greater than the variations for E_m values. Thus, little significance can be attached to the apparent alteration of relative amounts of the two major species of cytochrome c_1 seen at pH 8 (Table VI). It is possible, however, that the low potential species is more difficult to mediate at pH 8. Evidence for a greater difficulty in mediating (less accessibility?) is further sug-

TABLE VI

COMPLETE SUMMARY OF E_m VALUES OBTAINED BY 2ND DERIVATIVE AND SVD TECHNIQUES

	No. of Exp.	COMP 1		COMP 2		COMP 3	
		Second derivative results					
		E_m (mV)	%	E_m (mV)	%	E_m (mV)	%
Control	13	214 ± 3.1	32.0 ± 4.6	259 ± 2.4	58 ± 4.6	319 ± 5.3	12.0 ± 1.6
pH 8.0	2	211 ± 0	19 ± 6.4	255 ± 4.5	67.2 ± 6.6	305 ± 5.0	13.7 ± .9
pH 6.0	3	230 ± 7.0	32.7 ± 7.6	267 ± 6.0	51 ± 5.5	315 ± 9.0	16.2 ± 3.1
CN	2	220 ± 4.0	36.1 ± 14.5	264 ± 10.5	47.1 ± 5.5	339 ± 2.8	14.4 ± 7.2
Azide Oxida.	2	219 ± 6.0	53 ± 14.7	255 ± 12.0	44 ± 11.9	—	—
RED	3	248 ± 1.9	58 ± 5.3	280 ± 5.7	40.2 ± 6.8	—	—
		SVD results full range (500 to 650 nm)					
Control	10	207 ± 4.4		261 ± 2.4			
pH 8.0	2	195 ± 3.0		252 ± 2.0			
pH 6.0	3	205 ± 7.8		263 ± 8.4			
CN	2	192 ± 2		250 ± 3.0			
Azide Oxida.	2	186 ± 13		249 ± 4.0			
RED	3	202 ± 8		263 ± .3			
		SVD partial range (500 to 585 nm)					
Control	4	205 ± 3.9		257 ± 1.1		318 ± 6.0	

The table shows E_m values and percent values for each component ± S.E. for the number of experiments indicated. A fuller description of the individual experiments is given in Table I. The full range SVD experiments show component 3 as an $n = 1$ cytochrome oxidase component rather than the very minor $n = 2$ cytochrome c_1 component indicated by the 2nd derivative and partial range SVD analyses.

gested by the fact that at one-half mediator concentration, this component was not seen. Variations in individual experiments where different mitochondria were used (i.e. exps. 60 and 102) or different mediator concentrations were used (i.e. exps. 103 and 104) were no greater than the variations seen in the bulk of the experiments using the standard mitochondria and mediator concentration. The different techniques for performing the oxidations or reductions also did not prejudice the results. The experiments at pH 6 and pH 8 were all chemical reductions. The cyanide experiments were done by chemical oxidation and chemical reduction. The azide experiments consisted of

TABLE VII

DETAILED 2ND DERIVATIVE DATA FOR THE CONTROL EXPERIMENTS

Exp. No.	Mitocondria	Mediator conc.	Titration method	COMP 1 E_m	COMP 1 %	COMP 2 E_m	COMP 2 %	COMP 3 E_m	COMP 3 %
				mV		mV		mV	
60	LV	Usual	CR	211	19	256	68	319	13
102	ER	Usual	CR	225	37	265	50	315	13
103	B	Double	CR	220	42	265	51	334	7
104	B	Half*	CR	—	—	264	85	324	15
105	B	Half*	CR	—	—	272	90	329	10
66	B	Usual	EO/CO	213	50	260	42	307	7
68	B	Usual	EO/CO	216	40	254	53	300	7
83	B	Usual	EO/CO	197	7	248	93	—	0
84	B	Usual	EO/CO	230	21	260	73	361	6
61	B	Usual	ER	204	21	244	54	290	25
67	B	Usual	ER	222	37	262	48	306	14
62	B	Usual	EO	211	42	244	42	311	17
63	B	Usual	CO	200	22	267	63	326	14
AV.*	7 REDUCTIONS			216 ± 3.9	31 ± 4.7	261 ± 3.4	54 ± 3.6	317 ± 5.6	13.8 ± 2.1
	6 OXIDATIONS			211 ± 4.9	30 ± 6.6	256 ± 3.5	61 ± 8.0	321 ± 10.8	8.5 ± 2.5
	ALL EXPERIMENTS			214 ± 3.1	32 ± 4.0	259 ± 2.4	58 ± 4.6	319 ± 5.3	12.0 ± 1.6

*Because the low potential component was not seen at this concentration of mediators, neither an E_m value for this component nor the percent values for the two major c_1 components were included in the averages. The E_m values for the other two components, however, were in line with all of the other E_m values and were used in averaging.

three chemical reductions and two combined electrical-chemical oxidations. Experiments which involved inhibitors of cytochrome *c* oxidase were included because these experiments were more complex than just being dedicated to cytochrome c_1. Data relating to the titration behavior of cytochrome oxidase was obtained from these titrations and these analyses are presented in a separate paper devoted to this subject (Reddy et al., 1986). Complete information relating to cytochrome c_1 was also obtained in these experiments and these are the data analyzed in Table VI. Very little confidence is attached to component three at this time because of the relatively small contribution it provided to the overall optical changes. Including it in the second derivative fittings generally improved the quality of the fit. When the spectra from 500 to 650 nm were analyzed by SVD, component 3 assumed the identity of an $n = 1$ species of cytochrome oxidase. A third component of cytochrome c_1 was not seen. This component, if real, was lost in the background noise. When several of the data sets were examined by SVD in the limited wavelength range of 500 to 585 nm, which excluded the contribution of the characteristic cytochrome oxidase species at 605 nm, a weak absorbance for an $n = 2$ cytochrome c_1 species at 318 mV was frequently seen. The major cytochrome c_1 species in terms of its contribution to both the second derivative and SVD analysis was the $n = 2$ component with E_m 255 mV. Both the 2nd derivative and SVD analyses placed the E_m of this cytochrome c_1 species at 255 to 260 mV. The SVD analysis fixed the E_m of the less significant c_1 species closer to 205 mV whereas the 2nd derivative analysis saw this titration nearer to 215 mV. Very little change, if any, was seen in these values by varying pH or by the addition of cyanide. Although azide did not change the 2nd derivative values in an oxidative titration, it appears to have raised them in a reductive titration. In addition, both inhibitors may have slightly decreased the amount of the major component. A likely explanation of these apparent effects is that azide, when added to mitochondria in the oxidized state, produces a pronounced spectral change with a broad peak at 555 nm (Fig. 10). This spectral change is not seen when azide is added to the reduced mitochondria, or to the oxidized mediator mix in the absence of mitochondria, or when cyanide is added to oxidized or reduced mitochondria. The reductive experiments were performed after the mitochondria were first oxidized and then azide was added leading to the large azide-induced spectral change. This large background spectrum decreased with voltage and it undoubtedly contributed to the changes seen by the second derivative technique. The oxidative experiments apparently were not subject to this influence and the results of these experiments are more in line with all of the other titrations. Support for this general interpretation of the azide effect comes from the SVD analysis; it was able to separate out this unique spectral phenomenon, which it found to be an $n = 2$ component with an E_m at 430 mV. The

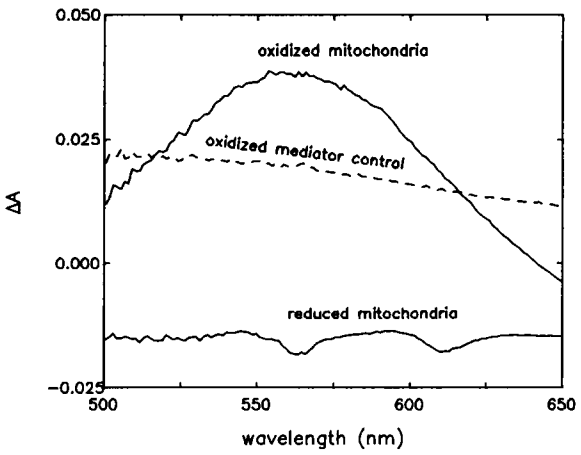

FIGURE 10 Spectral changes caused by addition of 10 mM azide to oxidized mitochondria, reduced mitochondria, and to the oxidized mediators in the absence of mitochondria. The usual preparation of 15 mg protein of mitochondria in 3 ml of 125 mM KCl, 62.5 mM potassium phosphate at pH 7.0 was used. The oxidized mitochondria and mediator mixture were raised to 460 mV by the combined mitochondria were prepared by adding freshly dissolved sodium dithionite to bring the voltage to 100 mV. Spectra were recorded just before addition of 10 mM sodium azide and then 8 min after the addition. The spectra shown are difference spectra. During the course of the 8 min interval a change of 3 to 4 mV occurred. This accounts for the slight oxidation of the cytochromes indicated in the case of the addition to the reduced mitochondria.

cytochrome c_1 components were clearly seen as isolated components with undistorted n and E_m values. From the fact that azide caused these effects and cyanide did not and the finding that whereas cyanide was able to inhibit 100% of the succinoxidase activity, azide could maximally inhibit only 80%, we believe these data indicate that azide and cyanide attack the system at two quite different loci. Additional control experiments where an egg homogenate replaced the mitochondria showed that all of the titrated features discussed in the paper are due to the mitochondria or complex III and not to the mediators in either aqueous or hydrophobic environment.

DISCUSSION

It should be stressed that the $n = 2$ values found in the present work with cytochrome c_1, in the previous analyses with one of the b_T cytochromes (Reddy and Hendler, 1983) and in the accompanying papers devoted to cytochromes aa_3 (Reddy et al., 1986; Hendler et al., 1986) are due entirely to the newer methods of analysis (Shrager and Hendler, 1986). These consist of two separate techniques. The second derivative technique uses thirteen absorbance values to determine the magnitude of the second derivative at a central wavelength, which for the cytochrome c_1 analysis is 553 nm. The SVD technique uses the entire spectrum. When we examine our experimental data using only two points at 553 and 540 nm for cytochrome c_1, we find in agreement with previous work (Dutton et al., 1970; Nelson and Gellefors, 1974; Van Wielink et al., 1982;

Rich, 1983) that only a single $n = 1$ species with $E_m \sim 230$ mV is present. Although Van Wielink et al. (1982) did use a Gaussian analysis, their technique would most likely have missed the $n = 2$ values we have found because: (a) Their model fixed all n values at 1. (b) The base-line was assumed to be a straight line. (c) Their analysis included full contributions from four cytochrome b's in addition to cytochrome c_1. (d) A 474 mV region was covered with only 19 spectra, which on average would separate the spectra by ~25 mV. This would allow about three spectra for a voltage range during which an $n = 2$ component is titrated from 90% oxidized to 90% reduced. We have tried to force fits of our data using the 2nd derivative and SVD analyses to either a single $n = 1$ species or at least a major $n = 1$ species with whatever else is necessary to obtain a fit. The results of these efforts either failed to produce a fit of the data or produced inferior fits or indicated the existence of species which could not be confirmed by direct difference spectra of the raw data. The finding of $n = 2$ for a species of cytochrome b_T and for cytochrome c_1 and for cytochromes aa_3 is compatible with the operational facts that two electrons are removed from a substrate undergoing oxidation and two electrons are needed to reduce one atom of oxygen. The functional value of $n = 2$ is also compatible with structural studies that indicate that complex III exists in nature as a dimer (Rieske and Ho, 1985). It is also observed that cytochrome oxidase occurs as a dimer (Georgevich et al., 1983). An alternative explanation for the $n = 2$ value is that the reduction of a single cytochrome center such as in c_1 is strongly coupled to the reduction of some other $n = 1$ center.

An electron transport chain that operates on the simultaneous transfer of two electrons rather than one would pose serious problems for current formulations of the coenzyme Q cycle (Trumpower, 1981). These schemes require the transfer of a single electron from QH_2 to cytochrome c_1 or the Rieske iron sulfur center in order to form the one-electron semiquinone thought to be the direct reductant for cytochrome b_T. Clearly if cytochrome c_1 requires two electrons for reduction, the Q-cycle system would have to be more complex. The double Q-cycle recently formulated (Devries et al., 1982) does not correct this problem. These and related problems are discussed in more detail elsewhere (Hendler et al., 1985).

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