COMPLETE ANALYSIS OF THE CYTOCHROME COMPONENTS OF BEEF HEART MITOCHONDRIA IN TERMS OF SPECTRA AND REDOX PROPERTIES. CYTOCHROMES aa_3

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ABSTRACT Using newer techniques of data collection that accumulate entire spectra at a series of discrete voltages and newer techniques of analysis that utilize the additional data, we have re-examined the redox behavior and corresponding difference spectra of redox centers responsible for the α absorbance features of cytochromes aa_3 in beef heart mitochondria. Our analysis reveals three Nernstian components with E_m values of 200, 260, and 340 mV with n values of 2, 2, and 1, respectively. The maximum α absorbance in the difference spectra for each of these species is located at 602, 605, and 607 nm respectively. Titrations in the presence of carbon monoxide led to the identification of the lowest voltage species as cytochrome a_3. The E_m of the carbon monoxide-liganded species was not raised. This is contrary to the result expected when a ligand has a much stronger affinity for the reduced form of a redox couple than the oxidized form. It is, however, consistent with a proton-pumping model of cytochrome oxidase in which the binding of ligand results in the dissociation of protons.

INTRODUCTION

Cytochromes aa_3 represent the most complicated members of the respiratory chain. Four known redox centers exist, namely, heme *a*, heme a_3, Cu_A, and Cu_B. In addition, there is a constant presence of extra copper (Cu_X) and as more recently shown, zinc (Einarsdóttir and Caughey, 1984) and magnesium (Einarsdóttir and Caughey, 1985) such that an active dimer may contain 13 metal centers (5 Cu, 4 Fe, 2 Zn, and 2 Mg atoms). Possibilities exist for cooperative interactions among these centers which could result in changes in E_m values, extinction coefficients and operational n values (n = number of electrons transferred) during the stages of complete oxidation or reduction (Caughey et al., 1976; Wikström et al., 1981). Previous attempts to define the spectral and thermodynamic properties of this enzyme have relied heavily on information provided by a ΔA between a peak wavelength of absorbance centered at either the α band position (605 nm) or the principal Soret absorbance (445 nm) and a reference point situated nearby. In an accompanying paper, we point out the deficiences in this approach (Shrager and Hendler, 1986). We have re-examined the problem with newer techniques that use much more of the spectral information and more rigorous analytical methods. We have found, instead of only two Nernstian species with E_m values of 230 and 340 mV, both with n values of 1, that there are three Nernstian species with E_m values of about 200, 260, and 340 mV. The n values of the lowest and highest voltage components are clearly indicated to be 2 and 1, respectively. The middle voltage component may have a fractional n value, but, if an integar value exists, it is most likely 2. Each of the species has a unique difference spectrum. The same conclusions have been arrived at in studies of the enzyme in situ in beef heart mitochondria (this paper) and of the isolated enzyme (Hendler et al., 1986).

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EXPERIMENTAL PROCEDURES

The mediators (and redox buffers), mitochondria, and titration procedures as well as the apparatus, spectrometer, and general procedures are described in an accompanying paper (Reddy and Hendler, 1986) and by Reddy and Hendler (1983). The methods of data analysis are described in an accompanying paper (Shrager and Hendler, 1986) and previous papers (Reddy and Hendler, 1983; Shrager and Hendler, 1982). These include the use of the second derivative of absorbance vs. wavelength at 604 nm, determined from a central point at 604 nm and six adjacent wavelengths spaced 1.23 nm apart on either side; and the SVD method of analysis, which utilizes either the full spectrum from 500 to 650 nm or the partial spectrum from 575 to 650 nm, in order to give greater emphasis to the α absorption peaks. Cytochrome *c* from horse heart was obtained from Sigma Chemical Co. (St. Louis, MO). For carbon monoxide experiments, the system was first made anaerobic with argon in the usual manner.

After making adjustments to the reaction cuvette and stirring apparatus, the gas was switched to carbon monoxide. The reaction cuvette was enclosed in a custom made hood. The gas was passed over the surface of the stirred suspension for 30 min prior to the start of the titration and continuously throughout the whole experiment. A description of all of the experiments and the composition of the incubation medium is given in Table I.

RESULTS

A series of twelve control titrations (Table I) consisting of five chemical reductions, three electrical reductions, one chemical oxidation and three combined electrical-chemical oxidations were analyzed both by the second derivative procedure and by SVD. In the second derivative analysis, the magnitude of the derivative at 604 nm wavelength is fit to a function of voltage specified by the Nernst Law. Computer fittings were performed with the traditional model using two components, both with $n = 1$ values, and with a variety of three-component models. A consistent feature in all of the fittings was the presence of an $n = 1$ component with an Em 340 mV. This limited the possible three-component models to 2,2,1; 2,1,1; 1,2,1; and 1,1,1; listing n values in order of increasing E_m values. One measure of the quality of a fit is the root mean square deviation between the data and fitted curve. For each separate titration experiment, the models were ranked from 1 to 5 based on this measure. Using the average ranking for all twelve titrations, the models are listed from best (top) to worst (bottom) in Table II. In addition to the composite results, the reductive and oxidative experiments are shown as separate groups. The agreement between the oxidative and reductive titrations especially for the 2,2,1 model, which we believe is most likely the correct one, shows that no gross alterations of the mitochondria occurred during the period of the titration and that the

TABLE I
DESCRIPTION OF THE EXPERIMENTS

Exp. no.	Type	Method*	Voltage range	Number of spectra analyzed	Exp. no.	Type	Method*	Voltage range	Number of spectra analyzed
			mV						
60‡	Control	CR	424–135	68	88	azide	CR	464–(−24)	111
61	Control	ER	447–136	79	89	azide	CR	477–2	101
63	Control	CO	100–440	73	90	azide	EO/CO	39–454	108
66	Control	EO/CO	115–498	93	91	azide	CR	466–101	91
67	Control	ER	473–100	102	92	azide	EO/CO	65–463	93
68	Control	EO/CO	123–397	50	65	mediator control	ER	440–234	56
84	Control	EO/CO	113–462	88	93	mediator control + azide	CR	456–100	80
102§	Control	CR	457–99	84					
103‖	Control	CR	466–98	82					
104¶	Control	CR	452–100	72					
105¶	Control	CR	460–100	73					
111	Control	ER	468–93	57	164	carbon monoxide	ER^P	447–102	49
64	pH 8.0	CR	444–75	74	165	carbon monoxide	ER^P	449–115	45
86	pH 8.0	CR	444–42	95					
87	pH 6.0	CR	436–101	78	167	carbon monoxide	CR	444–42	77
98	pH 6.0	CR	451–108	73					
101	pH 6.0	CR	444–60	84	79**	control	ER	448–105	122
99	cyanide	CR	443–98	71	80	pure aa_3	ER	468–107	93
100	cyanide	CO	124–449	64					

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-naphthoquinone and phenazine methosulfate at 0.1 mM each. The experiments with isolated cytochrome aa_3 (Yoshikawa et al., 1977) contained 5.3 μM of the enzyme plus 15 mg protein contributed by a total chicken egg homogenate added to protect the enzyme. The mediator controls contained the buffer and mediators plus 17.2 mg protein of the egg homogenate. For the experiments at pH 6.0, MES (66.5 mM) was present and the pH was adjusted with 1N HCl. For the experiments at pH 8.0, TRIS (66.5 mM) was present and the pH was adjusted with 1 M NaOH. Cyanide was present at 1 mM and azide at 10 mM. Carbon monoxide experiments were conducted under an atmosphere of the pure gas used in place of argon. Equilibrium of the medium with the gas phase was achieved by pre-gassing the stirred medium for at least 30 min.

*The methods of titration were CR (chemical reduction), ER (electrical reduction using a constant current generator), ER^P (electrical reduction using a potentiostat), EO (electrical oxidation using a constant current generator), and EO/CO (combined electrical chemical oxidation). These methods are more fully described in the references.

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

§Mitochondria obtained from Mr. A. D. Heindel of the Institute of Enzyme Research, University of Wisconsin, Madison, Wisconsin.

‖Double concentration of mediators used.

¶One-half concentration of mediators used.

**Cytochrome c (3.3 μM) was present.

TABLE II
SECOND DERIVATIVE ANALYSIS OF THE CONTROLS

n	All	Reductions	Oxidations
2	184 ± 10.0	178 ± 13.7	196 ± 11.5
1	258 ± 5.1	252 ± 4.3	272 ± 10.8
1	354 ± 3.8	347 ± 3.4	367 ± 2.7
Rank	1.7	1.9	1.5
1	144 ± 14.9Δ	136 ± 18.5	166 ± 23Δ
1	262 ± 7.6	252 ± 5.8	290 ± 14.8
1	357 ± 6.4	348 ± 3.3	384 ± 13.3
Rank	2.8	2.3	3.8
2	196 ± 7.8	192 ± 9.5	203 ± 14.7
2	259 ± 3.8	254 ± 4.7	269 ± 1.3
1	341 ± 3.6	336 ± 3.8	351 ± 5.4
Rank	3.0	3.5	2.0
1	182 ± 9.1Δ	181 ± 12.0	186 ± 12.3Δ
2	268 ± 8.9	255 ± 5.2	301 ± 21
1	342 ± 3.5	336 ± 2.2	357 ± 4.9
Rank	3.3	3.1	3.8
1	234 ± 5.5	236 ± 5.3	228 ± 13.8
1	343 ± 4.9	341 ± 4.3	348 ± 12.6
Rank	4.2	4.2	4.2

The magnitude of the α absorption peak was expressed in terms of the second derivative at 604 nm as described in the Methods and References. The voltage titration of the second derivative was analyzed according to models containing two or three Nernstian components with *n* values as shown. The analysis provided the E_m values in millivolts for each component. All 12 controls shown in Table I were examined. Averages ± the standard error are shown for the whole set and in individual groups for the eight reductive and four oxidative experiments. Each experiment was fit to the experimental model shown, using a DEC-10 main frame computer and the MLAB program. For each experiment, the root-mean-square deviation error was used to rank the fits from 1 to 5 in terms of closeness of fit (1 is best). The average value of the rankings is shown and based on this average for the total group of 12 titrations the different models are arranged from best (*top*) to worst (*bottom*). The "Δ" shows where one experiment of the group was not used because in two of the tested models (1,1,1 and 1,2,1) the desired fit could not be made in one of the oxidative titrations. The computer, in these cases, returned fits appropriate to one of the other models.

mediators were effective in bringing the cytochromes into equilibrium with the aqueous medium. The traditional two component model resulted in the worst fits. As will be shown below, this model can be eliminated on the basis of the more rigorous analyses by the SVD procedure. Among the three component models the 2,1,1 model produced the closest fits followed in order by the 1,1,1; 2,2,1; and 1,2,1 models.

Control experiments were also analyzed by the more powerful SVD procedure. This method is particularly well suited for distinguishing a three-component system from a two-component system, as described in an accompanying paper (Shrager and Hendler, 1986).

A brief synopsis of the key elements of the analysis is provided here as an aid to interpretation of the data that are presented. From all of the accumulated spectra taken at different voltages during the titration, the computer objectively selects from three to five fundamental spectra which together account for all of the systematic changes that occurred during the titration. These are not the spectra of individual components, but instead they contain contributions from all of the components which the computer sees as sharing common Nernstian properties. The magnitude of each of these fundamental spectra as a function of voltage is also provided by the computer. The most difficult part of the procedure to comprehend and yet its most powerful feature is the fact that both positive and negative slopes may occur in these titration curves of fundamental spectra. In a normal titration of cytochromes, the magnitude of the absorbance of a specific peak will always decrease with increasing voltage. Thus, two Nernstian components with close Em values present a blend of two sigmoid curves, which can be extremely difficult to distinguish from a single sigmoid. In the SVD titrations, the two will appear as a "V"-shaped or a "Λ"-shaped curve. A single titrating substance cannot produce such a result. Furthermore, in the SVD titrations, in addition to the type of curve described above, some titrations are found where only one of the two components is seen and in other titrations, the remaining component is seen without the first one being present. By observing all of these indications, the number of individually titrating components can be readily determined with no ambiguity.

These features of the SVD analyses of 15 experiments with mitochondria and 2 with purified cytochrome aa_3 are shown in Table III. The titration of each fundamental spectral component is summarized in the four columns. Titrations in the voltage ranges of the E_m values for the three Nernstian components observed are shown as having a negative ("−") slope or a positive ("+") slope if present, and as "0" if no Nernstian titration was present for the particular E_m component. There are numerous examples of separately titrating entities at 200 and 260 mV as evidenced by both "−" and "+" slopes in the same titration or of an absence of either the one or the other in a titration. The same kind of observations establish the presence of unique Nernstian components that titrate in the 260 and 340 mV regions. A system containing only two Nernstian components is incompatible with the behavior observed in these analyses.

Independent evidence in favor of the three-component model as opposed to the traditional two-component model is provided by an examination of the difference spectra taken around the critical voltages specified by the three- and two-component models. The two-component model specifies that a single titrating entity with $E_m \sim 230$ mV is present and another with $E_m \sim 375$ (Wilson et al., 1972). The three component model specifies that instead of a single entity with E_m 230 mV, there are actually two different Nernstian species present with Em values near 200 mV and 260 mV. Fig. 1 shows difference spectra taken from the raw data of two experiments, around the four voltage regions specified in the two models for the expected

TABLE III

PRESENCE AND SLOPE OR ABSENCE OF THE THREE SPECIES IN THE TITRATIONS OF THE FUNDAMENTAL COMPONENTS OF PARTIAL* SVD ANALYSES OF MITOCHONDRIAL EXPERIMENTS

Type of exp.		Fundamental component 1			Fundamental component 2			Fundamental component 3			Fundamental component 4		
		E_m(mV)			E_m(mV)			E_m(mV)			E_m(mV)		
Control	Exp	200	260	340	200	260	340	200	260	340	200	260	340
	60	0	−	0	−	−	−	+	−	−	—	—	—
	61	−	−	−	−	−	−	+	−	−	0	−	0
	66	−	−	−	−	+	+	−	+	+	−	+	+
	67	−	−	−	−	−	+	−	0	+	—	—	—
	68	+	+	+	−	−	+	+	0	+	−	0	+
	84	+	+	+	−	−	0	0	+	+	−	+	0
	102	0	0	−	−	−	−	−	+	0	−	−	+
	111	−	0	−	−	−	−	−	+	+	+	−	−
pH 8.0	64	−	0	−	+	−	−	−	0	−	−	−	+
	86	0	0	−	−	−	−	0	+	−	−	−	+
pH 6.0	87	−	−	−	+	−	−	+	+	−	+	−	+
azide	89	+	0	+	0	−	+	−	−	+	−	+	0
	90	−	−	−	−	+	0	0	−	−	−	+	−
	91	−	−	+	−	−	−	+	+	−	−	+	+
cyanide	99	0	−	−	−	−	−	−	0	+	—	—	—
	100	+	+	+	0	−	+	−	0	−	−	+	+
cyt a, a_3	80	−	−	−	−	−	+	0	+	+	0	+	+
cyt a, a_3 + cyt$c$	79	−	−	0	+	−	−	+	−	−	+	0	+

0 means that the particular species is not present.

+ and − indicate positive and negative slopes of magnitude of the component vs. voltage.

*Wavelength range 575 to 650 nm.

Nernstian components. In the voltage range ± about 20 mV of the lowest E_m value specified by the three-component model, the spectral change displays a peak at 602 nm. This is obviously not a tailing of a titration occurring at 230 mV as specified by the two-component model, because the spectral change occurring around this voltage displays a peak at 605 nm. The spectral change occurring around the voltage region centered at 340 mV shows a maximum at near 607 nm. The spectral change seen to occur at 250–260 mV is most frequently at or near 605 nm. These observations provide direct confirmation of the presence of three different titrating species in the voltage regions specified by both the SVD and second derivative methods of analysis for the three-component models. They are not consistent with the two-component model. The SVD results, combined with the direct difference spectra plus the decided preference of the second derivative method for the three-species model over that of the traditional two-species model is so strong as to exclude the simpler model from further consideration.

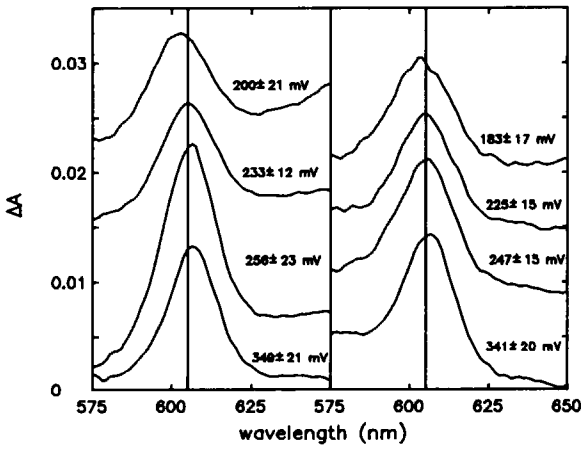

FIGURE 1 Difference spectra are shown from two experiments listed in Table I. The four on the left are from experiment 111 and the four on the right from experiment 61. The voltage ranges were chosen to be near the three titrations indicated by the three component model (i.e. 200, 250, and 340 mV) and near the two voltages indicated by the two component model (i.e. 230 and 340 mV). The vertical lines were drawn at 605 nm wavelength.

The existence of a 5-nm separation between the absorption peaks of the titration occurring at about 200 mV and the one at about 340 mV is always seen. This includes control experiments with mitochondria, experiments where some condition was varied such as pH, and in experiments with pure cytochrome aa_3. Fig. 2 shows a representative sampling of such experiments. The average positions for the absorption maxima of the 200 mV, 260 mV, and 340 mV species in a group of five experiments were 601.9, 606, and 606.6 nm, respectively, in SVD reconstructed spectra and at 601.9, 604.8, and 606.6 nm in the difference spectra.

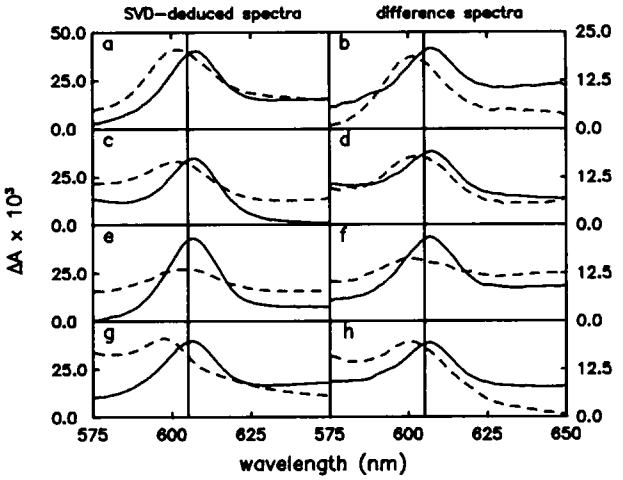

FIGURE 2 SVD *(left)* and direct difference spectra *(right)* are shown for four experiments listed in Table I. In each case, the dashed curve is the spectrum of the low voltage component (~200 mV) and the solid line is that of the high voltage component (~340 mV). The experiments from *top* to *bottom* were No. 66 (control, electrical/chemical oxidation), No. 67 (control, electrical reduction), No. 87 (pH 6.0, chemical reduction), and No. 79 (pure cytochrome aa_3 plus cytochrome *c*, electrical reduction). The difference spectra covered a range of ± 16 to 22 mV of the E_m value specified in the SVD analyses. The vertical lines were drawn at 605 nm wavelength.

The SVD method was also useful in deciding the question of n values for the three Nernstian titrations. The ability of this procedure to provide titrations with one component isolated from another or for the two to be present with different slopes, provided further opportunities for testing $n = 1$ vs. $n = 2$ for a given component. By this means, it was found that $n = 2$ was clearly preferred for the value of the titration occurring with an E_m 200 mV. For the titration occurring around 260 mV, $n = 2$ was generally more acceptable than $n = 1$, but the preference was not so marked as to exclude $n = 1$ as the correct value. This is especially true in view of the results obtained with the second derivative analyses (Table II), which showed that the 2,1,1 model produced closer fits than the 2,2,1 model. Nonetheless, the fact that $n = 2$ provided better fits in general with the SVD procedure for the 260 mV titration and the fact that when $n = 2$ was used, the standard error for the fitted E_m value of this component was less than when $n = 1$ was used (Table IV) called for a more stringent test for the *n* value of this component. Table IV shows a series of control experiments analyzed by SVD with the n value of the middle component held at either 1 or 2. In addition to the greater uncertainty found for the fitted E_m value already noted, when *n* was fixed at 1, Table

TABLE IV
COMPARISON OF 2e 2e 1e VS. 2e 1e 1e SVD FITS

Exp	2e 2e 1e			2e 1e 1e		
	2e	2e	1e	2e	1e	1e
			(A) Mitochondria (full SVD)			
60	195 ± 5.5	262 ± 5.2	339 ± 14.6	197 ± 4.4	256 ± 10.5	339 ± 13.3*
66	191 ± 4.4	256 ± 5.6	332 ± 9.0	193 ± 2.9*	264 ± 11.9	332 ± 15.9*
67	190 ± 3.7	248 ± 4.6	349 ± 14.7	188 ± 4.7*	225 ± 15.6	321 ± 14.0
84	221 ± 1.5	268 ± 6.0	329 ± 11.5	198*	265 ± 11.9	345 ± 23.7
102	217 ± 3.4	268 ± 5.7	351 ± 8.6	213 ± 1.8*	273 ± 11.0	347 ± 17.4
Avs. ± S.E.	203 ± 6.7	260 ± 3.8	340 ± 4.4	197 ± 4.2	257 ± 8.3	337 ± 4.7
			(B) Mitochondria (partial SVD)			
60	198 ± 0.5	243 ± 9.0	339 ± 2.5	194 ± 2.0*	262 ± 16.0	348 ± 1.5
61	183 ± 3.1	247 ± 5.5	337 ± 5.3	170 ± 10.2	247 ± 7.5	364 ± 12.5*
66	194 ± 2.3	255 ± 6.9	331 ± 7.0	194 ± 4.7	231 ± 3.0	336 ± 11.5
67	190 ± 2.3	239 ± 1.5	343 ± 10.5	185 ± 4.6*	217 ± 2.0	327 ± 5.0
68	181 ± 3.7	264 ± 11.0	338 ± 5.5	178 ± 5.1*	227 ± 2.5	333 ± 11.0*
84	225 ± 1.3	267 ± 5.9	353 ± 8.1	213 ± 7.8*	257 ± 6.2	369 ± 10.2
102	203 ± 10.1	263 ± 10	326 ± 6.0	200 ± 10.3*	215 ± 31.5*	317 ± 4.4
111	190 ± 4.0	267 ± 13.7	329 ± 10.1	203 ± 13.9	254 ± 16.0	354 ± 19.6
Avs. ± S.E.	196 ± 4.9	256 ± 4.0	337 ± 3.0	192 ± 4.9	245 ± 7.8	344 ± 6.5
			(C) Cytochrome oxidase (partial SVD)			
79	214 ± 7.2	263 ± 6.7	337 ± 15.0	213 ± 8.8*	234 ± 8.5*	340 ± 20.5
80	215 ± 6.2	265 ± 9.9	342 ± 8.0	215 ± 10.0	283 ± 1.2*	327 ± 8.0
Avs. ± S.E.	215 ± 0.5	264 ± 1.0	340 ± 2.0	214 ± 1.0	259 ± 25	333 ± 6.0

SVD analyses were performed according to a 2e 2e 1e or a 2e 1e 1e model in which the *n* values of the titrations occurring near 200, 260, and 340 mV respectively, were held fixed as indicated. Full SVD used the wavelength range from 500 to 650 nm. Partial SVD used a wavelength range concentrated on the α absorption peak (575 to 650 nm).

The numbers in the blocks alongside a particular experiment represent E_m values and were averaged among the several ΔA vs. V plots of the fundamental spectra generated in that experiment for the indicate *n*-value component.

The numbers shown as AVS ± S.E. were averaged from all of the average values obtained for the indicated component in the experiments listed.

*The spectrum reconstructed for the component did not correspond to the difference spectrum taken ±20 mV of the indicated E_m value.

IV also shows another failure associated with this assignment. The SVD procedure is supposed to provide difference spectra for actual changes that occur in the voltage region around the E_m values it has deduced. Such spectral changes should be recognizeable in the difference spectra taken around the indicated voltage. This similarity of the actual difference spectrum with that of the SVD deduced spectrum lends support to the validity of the SVD reconstruction and results. On the other hand, the absence of similarity indicates the SVD solution is not the correct one. In all cases with the 2,2,1 model a close similarity was observed between the SVD deduced spectrum and the different spectrum taken around the SVD-deduced E_m (e.g., Fig. 3). In Table IV, an asterisk is used to mark cases where the difference spectrum in the specified voltage range did not agree with the SVD deduced spectrum. This discrepancy was seen to occur in all three components when the 2,1,1 model was imposed. Table IV shows this test applied to the 2,1,1 SVD solution for the same experiment analyzed by the 2,2,1 model in Fig. 3. In this case, the poor agreement was found for the component with highest E_m, which the 2,1,1 solution placed at 364 mV. It also should be noted that the three E_m values fit by the SVD analysis using the 2,2,1 model (i.e. 183, 247, and 337 mV) agree with the three E_m values found in the second derivative analysis (i.e. 180, 243, and 334 mV). This was not true for the fitted E_m values in the analyses using the 2,1,1 model which were 170, 247, and 364 mV in the SVD analysis (see Fig. 4) and 150, 237, and 343 mV in the second derivative analysis. For three SVD experiments in the wavelength range 575 to 650 nm, the n values were fitted as well as the E_m values and quantities. The fitted n values returned were 2.0 ± 0.2 for the low E_m component, 1.9 ± 0.1 for the middle E_m component, and 0.99 ± 0.14 for the high E_m component. Based on the SVD analyses, we feel that the 2,2,1 model has more support than the 2,1,1

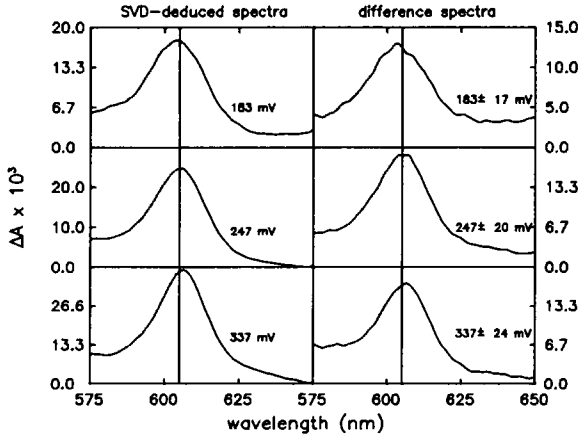

FIGURE 3 SVD-deduced spectra for the 2,2,1 model for experiment No. 61 (Table I and Table IV) are shown on the *left*. Corresponding difference spectra taken around the voltages specified by the SVD analysis are shown on the *right*. The vertical lines are drawn at 605 nm wavelength.

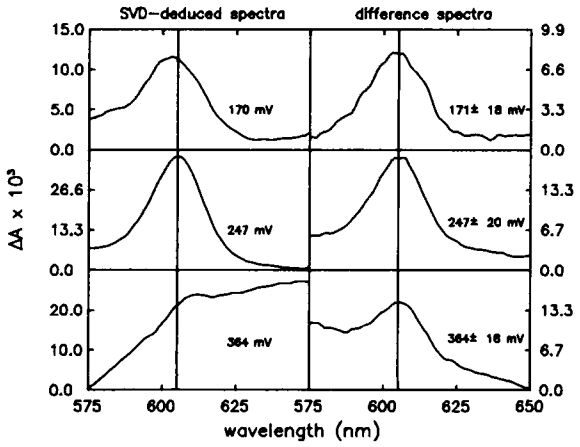

FIGURE 4 SVD-deduced spectra for the 2,1,1 model for experiment No. 61 (Table I and Table IV) are shown on the *left*. Corresponding difference spectra taken around the voltages specified by the SVD analysis are shown on the *right*. The vertical lines are drawn at 605 nm wavelength.

model. A more rigorous test of the two models in studies with pure cytochrome aa_3 in which the Soret absorbances have been examined in addition to the α peak absorbances, also led to the selection of the 2,2,1 model (Hendler et al., 1986).

A similarly rigorous analysis applied to experiments in which the pH was varied or cyanide or azide was present during the titration led to the conclusion that the 2,2,1 model was also applicable to these conditions. Table V shows a summary of results for titrations of controls and various other conditions analyzed in terms of the 2,2,1 model by both the second-derivative and SVD procedures. Raising the pH one unit to pH 8 lowered the E_m of the 340 mV component by 30 mV. Effects on the two lower E_m components were less apparent. Lowering the pH one unit to pH 6.0 produced more variability in the titrations. There is an indication that a marginal increase in the E_m of the high voltage component might have occurred. The two lower E_m components were effected little, if at all. Cyanide lowered the values of the two lower E_m components by ~10 to 30 mV. Azide had no effects on reductive titrations (i.e., added to the oxidized form) but there is an indication in the oxidative titrations analyzed by SVD, that some lowering of all Em values may have occurred. None of the treatments had profound effects on the E_m values of any of the three components. Neither were the shapes of the respective spectral changes across the voltage ranges appropriate for the three voltage transitions altered.

Replacing the argon atmosphere with carbon monoxide led to the appearance of a new α peak located at ~594 nm. This has long been recognized as characteristic of the carbon monoxide complex of heme a_3. The spectra and E_m values of the 250 and 340 mV components as analyzed by SVD were not changed. However, the α peak at 602 nm, characteristic of the 200 mV component was not seen (Fig. 5). In its place was the 594 nm feature of the carbon

TABLE V
EFFECTS OF pH AND LIGANDS ON E_m VALUES

		2nd derivative E_m	SVD E_m	Change from control: 2nd derivative	Change from control: SVD
		mV	*mV*	*mV*	
Control	2	196 ± 7.8	207 ± 4.4		
	2	259 ± 3.8	261 ± 2.4		
(pH 7.0)	1	341 ± 3.6	340 ± 2.9		
		(12)	(10)		
pH 8.0	2	186 ± 1	195 ± 3.0	−10	−12
	2	246 ± 9	252 ± 2.0	−13	−9
	1	313 ± 3	310 ± 3.5	−28	−30
		(2)	(2)		
pH 6.0	2	203 ± 15.2	205 ± 7.8	—	—
	2	267 ± 9.8	264 ± 8.2	—	—
	1	350 ± 5.1	347 ± 16.4	+9	—
		(3)	(3)		
cyanide	2	164 ± 6	192 ± 2.0	−32	−15
(1 mM)	2	234 ± 1	250 ± 3.0	−25	−11
	1	333 ± 4	342 ± 8.5	—	—
		(2)	(2)		
azide	2	192 ± 20	186 ± 13	—	−20
(10 mM)	2	246 ± 15	249 ± 4.0	—	−12
oxidative	1	330 ± 16	314 ± 4.0	—	−26
		(2)	(2)		
azide	2	190 ± 2.3	202 ± 8.0	—	—
(10 mM)	2	263 ± 11.5	263 ± 0.3	—	—
reductive	1	338 ± 2.7	346 ± 4.3	—	—
		(3)	(3)		
carbon	*	158 ± 5.0	136 ± 13.3	no	no
monoxide	*	198 ± 4.0	195 ± 8.4	comparison	comparison
	2	243 ± 7.1	260 ± 7.1	−16	—
	1	335 ± 2.4	335 ± 6.8	−6	—
		(3)	(3)	(3)	(3)

The average E_m value ± standard error is given for the number of experiments shown in parentheses.
Data are given for both the second derivative analysis at 604 nm and for the SVD analysis using spectra from 500 to 650 nm. Changes which are less than the larger standard error of either the control or the tested condition are not listed separately.
In the case of the carbon monoxide experiments, the spectral range used for SVD analysis was 575 to 650 nm.
*The n value was not unambiguously established. It is 1 or 2 for the lower E_m component and 2 or 4 for the higher E_m component. The second derivative analysis of these components was at 594 nm.

monoxide complex. This peak when analyzed both by the second-derivative procedure at 594 nm and the SVD procedure was seen to titrate as two Nernstian components, the major one with an E_m near 200 mV and a minor one with an E_m of 145 mV (Table V). This behavior in the presence of carbon monoxide is also seen with the isolated enzyme where, in addition to SVD and second-derivative analyses at 594 nm, a second-derivative analyses at 435 nm can be performed. The characteristic features of the carbon monoxide complex were seen to titrate as two components with E_m values of 200 and 115 mV (Hendler et al., 1986). With the isolated enzyme, the n value in the SVD analysis required a 4 for fitting. A fit with $n = 2$ was quite inferior. In the case of the mitochondria, either a 2 or 4 can be accepted. This ambiguity may reflect the fact that, in the mitochondria, other components such as cytochromes b and c_1 may be contributing absorbances to this spectral region which can not be totally isolated, even by SVD. With respect to the n value of the titration occurring at 145 mV, we have not established whether the n value is 1 or 2. What is known with certainty in both the studies with the mitochondrial and isolated enzyme is that only the spectrum associated with the component that has an E_m value of 200 is effected. In place of this spectrum we see the typical carbon monoxide spectrum of heme a_3. The titration behavior of this spectrum is that of two Nernstian components with E_m values of 200 and 145 mV in the case of mitochondria and 200 and 115 mV in the case of the pure enzyme.

To provide continuity with earlier work on the thermo-

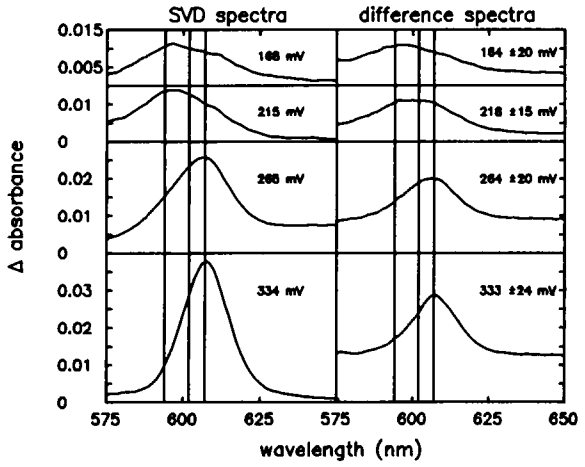

FIGURE 5 SVD-deduced spectra from a chemical reduction (Exp. 167, Table I) performed under a carbon monoxide atmosphere are shown on the *left*. Corresponding difference spectra obtained from the raw data are in the panels on the *right*. The vertical lines are drawn at 594, 602, and 607 nm.

dynamic properties of cytochrome aa_3, we have analyzed one data set using the two-point ΔA graphic technique of Wilson and Dutton (1970) (see Shrager and Hendler, 1986), as is commonly done. Fig. 6 shows the typical sigmoid curve, with extremes at ± log (oxidized/reduced) = 1.5, approximating theoretical $n = 1$ slopes. The figure also shows the resolution of this graphic two-point data into two $n = 1$ components with E_m values of 377 and 230 mV. This may be compared to Fig. 1 of Wilson et al., 1972 who found two $n = 1$ components with E_m values of 375 and 230 mV using the same two-point ΔA and pigeon

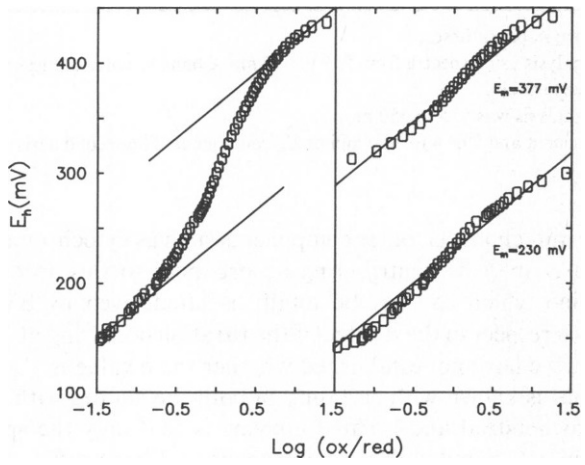

FIGURE 6 An electrical reduction experiment (Exp. 61, Table I) was analyzed using the ΔA between 605 and 630 nm as the measure of reduction. The panel on the *left* used the ΔA observed in the experiment. The two lines drawn at the extremes are theoretical $n = 1$ curves. The panel on the *right* shows the resolution of the data on the left into two components with slopes very close to the theoretical $n = 1$ lines that are shown and E_m values of 377 and 230 mV. The resolution technique is that of Wilson and Dutton (1970, and personal communication).

heart mitochondria. The experiment analyzed in Fig. 6 is the same one that showed conclusive three-component behavior by difference spectra (Fig. 1), SVD (Fig. 3) and second derivative analyses. A detailed discussion of the weaknesses of the two-point graphic technique is presented in an accompanying paper (Shrager and Hendler, 1986).

DISCUSSION

Current understanding of the Nernstian behavior of cytochromes has been based on data acquisition and analysis techniques that are not able to resolve cytochromes in situ in light-scattering structures or in isolation when more than a single redox center is present (Shrager and Hendler, 1986; Reddy and Hendler, 1983). We have developed new techniques that acquire and utilize much more of the relevant data (Reddy and Hendler; 1983; Shrager and Hendler, 1982; Shrager and Hendler, 1986). Characterization of the redox properties of cytochromes aa_3, based on the previous inadequate procedures, has led to a confusing picture that is still not resolved. Data has been collected as a two point ΔA centered at 605 nm and referenced to 630 nm. Analysis has been by the graphic method of plotting E vs. log "OX/RED" followed by a subjective replotting procedure (see Shrager and Hendler, 1986). Wilson and collaborators (1972) have described two Nernstian titrations, each accounting for 50% of the ΔA, with E_m values of 230 and 375 mV and n values of 1.0. Another group of workers represented by Wikström (see Wikström et al., 1981) point to observations based on the effects of ligands of heme a_3 on the optical absorbance at 605 nm to dispute Wilson's interpretation of the data. When heme a_3 is liganded, the absorbance at 605 decreases only by a small amount placed from 2 to 20% by various workers. If 80 to 98% of the absorbance at 605 nm belongs to heme a, then how could Wilson et al. see two distinct E_m components, each accounting for 50% of the absorbance? Wikström and others attribute the observed behavior to heme-heme interaction resulting in a negative cooperativity so that after the first electron has been added, the second is much more difficult to add. This view, based on an earlier suggestion by Nicholls (Nicholls and Petersen, 1974) has been called the "neo-classical" model. The two hemes when oxidized, are able to receive an electron with an Em of 375 mV, and this electron is shared between the two centers. The presence of the first electron then interferes with the acquisition of the second electron so that a new and lower E_m is established, accounting for the second observed Em at 230 mV. Wilson et al., reconcile their findings with a different view of heme-heme interaction, one that involves extinction coefficients rather than E_m values. In their view, the finding that ligands such as carbon monoxide result in only a slight decrease in the absorbance at 605 nm is because the binding of a ligand to heme a_3 causes an increase in extinction of heme a so that only a small loss in total absorbance is seen.

In our approach, we are spared these problems, because our analysis is based on information over broad spectral regions and we are not forced to deduce the behavior of both heme *a* and heme a_3 from a single ΔA whose signficance in terms of the separate hemes has been questioned. Thus, we find three distinct titrations characterized by different spectral changes. The titration with E_m ~200 mV has its α peak centered at 602 nm, the titration with E_m ~340 mV has its α peak centered at 607 nm and the titration with E_m ~260 mV has its primary α absorption located at 605 nm. In an accompanying paper (Hendler et al., 1985), using purified cytochrome aa_3, we have been able to add unique Soret absorbances to the three titrations characterized here by only their α absorbance features. Thus, the component with E_m at 200 mV and α peak at 602 nm has a Soret peak at 429 nm; the component with E_m at 340 mV and α peak at 607 nm has a Soret peak at 448 nm; and the component with an E_m at 260 mV and α peak at 605 nm has a Soret peak at 446 nm. It is of interest to note that although the two point ΔA approach has resolved only two Nernstian titrations with E_m values at 230 and 375 mV, titrations monitored by EPR by Wilson et al. (1976) show three titrations that correspond in E_m values to the three titrations that we find. The $g = 3$ signal and a portion of the $g = 6$ signal titrate with an apparent E_m between 300 and 400 mV. The major titration of the $g = 6$ signal titrates with an E_m of 190 mV, and a titration is seen at ~245 mV in a $g = 2$ signal appropriate to copper. The main difference between the conclusions of our studies and those based on the optical two-point ΔA analysis is that the component described as a single $n = 1$ entity with an E_m of 230 mV has been resolved into two $n = 2$ components with E_m values near 200 and 260 mV. In an accompanying paper (Shrager and Hendler, 1986) we point out that the methods previously used are incapable of analyzing the mixture of Nernstian components which each of our two newer methods has resolved. It is of some interest that the resolution of a single $n = 1$ component with an E_m of 230 mV into two $n = 2$ components with E_m values near 200 and 260 mV applies both to the potentiometric behavior of cytochrome c_1 (Reddy and Hendler, 1986) and to cytochrome oxidase.

Another point of difference between the results of our current analyses and existing views is in regard to the effects of carbon monoxide and the identity of cytochrome a_3. Cytochrome a_3 is defined as the cytochrome that binds ligands such as carbon monoxide and is thereby altered in its properties. The characteristic spectrum of the carbon monoxide complex of cytochrome a_3 has an α absorbance near 595 nm and a Soret peak at ~435 nm. We have found that the appearance of the characteristic carbon monoxide spectrum at the α band in the current work and both the α and γ bands in the studies with pure enzyme (Hendler et al., 1986) is at the expense of the component which normally is seen with an α band at 602 nm (present work) and a γ band at 429 nm. This component is the one with an E_m of ~200 mV rather than the high E_m component (>300 mV) previously identified as the a_3 component. The carbon monoxide-cytochrome a_3 complex as reported by the characteristic α absorbance at ~594 nm showed Nernstian behavior for a predominant species at E_m ~200 mV and for a minor component at ~160 mV. These findings are contrary to the commonly accepted view that carbon monoxide addition to heme a_3 must result in an increase in the E_m of this redox center. The belief is based on the fact that when a ligand binds more strongly to the reduced form of a redox couple, the E_m of the liganded form relative to that of the free form will be raised according to the expression

$$E_{mL} = E_{mF} + \frac{60}{n} \log \frac{K_0}{K_R},$$

where the subscripts L and F refer to the liganded and free couples and K_0 and K_R are the dissociation constants for the liganded oxidized and reduced members, respectively. It must be pointed out that the model leading to this relationship ignores the possible role of cytochrome oxidase as a proton pump. If the reduced liganded (with O_2 or CO) form of the enzyme binds protons on the inside surface of the membrane and, upon oxidation, discharges the protons to the outer surface, it can be shown that

$$E_{mLH} = E_{mF} + \frac{60}{n} \log \frac{K_0}{K_R} - \frac{60}{n} \log \frac{K_{HO}}{K_{HR}},$$

where K_{HO} and K_{HR} represent the dissociation constants for a proton from the oxidized and reduced liganded forms, respectively. The difference in proton dissociation constants resulting from such a Bohr effect expected of a pumping enzyme would be in the direction of reducing the E_m of the liganded form. If more than a single proton is involved per molecule of cytochrome aa_3, the magnitude of the effect would be greater, although not in linear proportionality. Our data therefore are in support of a proton pumping form of cytochrome oxidase. In view of our findings, we looked for direct potentiometric data in the literature in support of the idea that the carbon monoxide-liganded, species does in fact have an elevated E_m. The most relevant work is that of Lindsay et al. (1975) and Lindsay and Wilson (1974). In both of these papers, the technique of mediated anaerobic titrations in the presence of carbon monoxide is used, just as in our studies. The conclusion reinforced by both papers is that the E_m of the carbon monoxide liganded species increases 30 mV per decade change in carbon monoxide concentration. This conclusion is based on the use of the wrong equation of Clark (Clark, 1960) which has been cited by these authors (Lindsay, 1974; Wilson et al., 1972). The specific equation cited is Eq. 10 on page 211 of Clark's book. This equation assumes that there is a dissociation constant for both the oxidized and reduced forms of a simple redox couple. The voltage specified by the form of the equation cited by

Lindsay and Wilson is the voltage at which the sum of both free and liganded oxidized species equals the sum of both free and liganded reduced species. It is not the E_m of either the free or the liganded species. In this model each species would have its own E_m determined by the energy required to remove the electron (or electrons). The E_m of the liganded species would not be a function of the concentration of the ligand, if the same number of ligands per molecule is added to the reduced and oxidized species. Naturally, the voltage at which the sums of free and liganded species are equal will be a function of ligand concentration as predicted by the equation. If instead of solving for the voltage at which the sums are equal, the equation is developed for the voltage at which the concentration of the oxidized and reduced liganded species are equal (i.e., the E_m of the liganded species), the simple equation involving only the log of the ratios of the two dissociation constants is obtained (given above). In the 1975 paper of Lindsay et al., verification of the conclusion that the E_m of the liganded species rises 30 mV per decade change in carbon monoxide concentration was achieved by a different method of analysis, which is flawed for additional reasons. The voltage in this work is not plotted as a function of log oxidized/reduced according to the Nernst equation but as a function of the log unbound/bound. The voltage at which this log is zero is taken as the E_m of the liganded species. If their techniques for arriving at this ratio is correct, they have defined the voltage at which the total of all unliganded species involving permutations of electron distributions on one or more of four metal centers, equals the total of all forms of liganded species which has been identified as two (Greenwood et al., 1974). This voltage is not the Em for any liganded species. It would be expected to be a function of ligand concentration. Another flaw in this approach is the experimental technique used to determine the ratio unbound/bound. The ΔA at 590–624 nm for some low voltage is taken to represent the ΔA of the totally liganded species. The difference between this value and the value at higher values of E_h is taken as the total due to all unbound species. This requires the unlikely assumption that all of the many possible unbound forms contribute absolutely nothing to the ΔA used for monitoring. This work does not establish or confirm that the E_m of the liganded species as measured by mediated anaerobic titrations was raised relative to the unliganded species.

Another kind of observation leading to the conclusion that the E_m of the carbon monoxide liganded heme a_3 is in fact raised can not be so easily dismissed. If fully reduced enzyme is treated with carbon monoxide and then exposed to a strongly oxidizing concentration of $K_3 F_e (CN)_6$, the liganded heme a_3 center remains reduced (see Clore et al., 1980). This can be true only if the E_m of the liganded heme was dramatically raised. Proof that the heme a_3 center remained reduced is provided by the observations that the spectrum was that of the reduced liganded form, the liganded form could be photolyzed to release the carbon monoxide and the enzyme was then able to bind oxygen. There is a way in which our observations and these can be reconciled. An important difference between the two experiments is that under the conditions of the anaerobic titration, heme *a*, and both copper atoms were reduced when heme a_3 was being titrated in the presence of carbon monoxide whereas under the conditions of the formation of the mixed valence liganded enzyme, all of the non liganded centers were oxidized. If the redox potential of heme $a_3 \cdot$ CO is influenced by the redox state of one or more of the other centers, such an apparent difference in results could be made compatible. This same thought might also reconcile two other findings. Our low voltage species has an α peak at 602 nm (this work) and a Soret at 429 nm (Hendler et al., 1986). This is the spectrum attributed to the oxygenated form of cytochrome oxidase, and it appears when freshly reduced enzyme is exposed to oxygen. In our work, we have identified the spectrum as that of reduced heme a_3 based on our carbon monoxide studies. If the spectrum of the oxygenated enzyme is really the same and due to the same chromophore, then perhaps in the oxygenated enzyme, heme a_3 is still reduced even under oxidizing conditions. There is evidence to support this view (Davison and Waino, 1968; Brunori et al., 1979). It is also possible that the chromophores are not the same even though the spectra are quite similar, in which case the speculation just developed is irrelevant.

It has not escaped our notice that the sum of *n* values we have assigned to cytochrome oxidase is 5 and not 4. One explanation of this is that cytochrome oxidase is present in more than a single form and that for each separate species the sum of the *n* values is 4. If this is true, the same mixture of species must be present in the intact mitochondria and in the pure enzyme since we see the same picture with both preparations (see Hendler et al., 1986). Bickar et al. (1982) have concluded that several stable forms of cytochrome oxidase exist. One possible form of heterogeneity is the presence of monomer and dimer. An alternative possibility is that although only four electrons are needed to reduce oxygen to water, a fifth redox center is needed to handle the required intermediate stages during the complete process. In this connection, there have been suggestions that a redox site in addition to the four known centers may be present in the enzyme. Such a center has been proposed to be either a ferryl iron (+4) or a cationic free radical (Clore et al., 1980; Seiter and Angelos, 1980; Wikström et al., 1981; Hagen et al., 1984). We, at the moment, are presenting the data and analyses and are not prepared to offer a particular point of view. A definitive answer as to whether a fifth redox center is present might be obtained by careful coulometry with the pure enzyme. Such an approach requires a way to deconvolute successive spectral changes as known amounts of electrons are added to a known amount of enzyme. An accuracy much more stringent than ± 12.5% must be obtained to distinguish the $n = 4$ from the $n = 5$ case. Techniques for achieving these

results are currently under development in our laboratory. A related question is what mole fraction can be assigned to each of the three Nernstian titrations we have described. The coulometric approach should also resolve this question. It is not enough to quantify relative spectral changes that we are using, since no one, ourselves included, has determined extinction coefficients for either the second derivatives or reconstructed spectra from the SVD procedure. It may or may not be meaningful to note that the total change in the magnitude of the second derivative at 604 nm is due 50% to the $n = 1$ component at 340 mV and 25% each to the two $n = 2$ species.

In addition to the novel findings of our studies discussed above, we have determined the effects of changes in pH or the presence of other ligands on the redox properties of the enzyme. These effects are listed in Table V. In brief, the high voltage component showed a 30 mV decrease in E_m for a 1 pH increase from pH 7 in pH 8. The components with E_m values of 260 and 200 mV showed about one-third this change. Decreasing the pH from 7 to 6 showed no detectible changes. Effects of cyanide and azide on the E_m values were quite small but some decreases were noted in many of the test situations.

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