Potentiometric Study of Cytochrome c_1aa_3 from *Thermus thermophilus*

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ABSTRACT

We have examined the redox behavior of the cytochrome c_1aa_3 complex from *Thermus thermophilus*. In potentiometric titrations the cytochrome c behaves as an independent center having $n = 1$ and $E = 205$ mV (NHE). Under the assumption that the individual centers equilibrate independently in this experiment, changes in the absorption band at 603 nm have been resolved into two components: cytochrome a ($n = 1$, $E_m = 270$ mV, 60% spectral contribution) and cytochrome a_3 ($n = 2$, $E_m = 360$ mV, 40% spectral contribution). The $n = 2$ process was attributed to strong chemical coupling between cytochrome a_3 and Cu_B. The enzyme was also titrated with a mixture of NADH and PMS, and the results are shown not to conform to a model of intramolecular equilibrium according to the equilibrium constants obtained from the potentiometric titration. It is suggested that a conformational equilibrium within the complex may control electron transfer between cytochromes a and a_3.

INTRODUCTION

The cytochrome c_1aa_3 complex from *Thermus* contains two heme A, two Cu, and one heme C(1). Over the past several years we have demonstrated that the spectral properties of this complex are strikingly similar to those of the extensively studied cytochrome aa_3 from eucaryotic sources. Thus, either in published [1–3] or unpublished work it has been shown that the aa_3 portion of the complex is similar to the eucaryotic enzyme in its optical, EPR, EXAFS [4], resonance Raman [5], and Mössbauer spectral properties [6]. The cytochrome c portion of the complex spectrally resembles the cytochrome c_1 from bovine mitochondria having $g_z = \sim 3.4$ in its EPR spectrum [7]. Examination of the protein portion of the complex has revealed that it is composed of only two subunits [2, 8] of ~33 kD (C-protein) and ~55 kD (A-protein). The smaller subunit has a covalently associated heme C

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and appears to have no other cofactors, suggesting [2] that the four cofactors of cytochrome aa_3 are associated with the larger subunit. This is a surprising result in view of the myriad protein subunits associated with the eucaryotic enzyme [9]. In general, bacterial cytochromes aa_3 have a simpler subunit structure than their eucaryotic counterparts [10].

The cytochrome c_1aa_3 complex was also shown to be an oxidase [3] toward the cytochromes c of horse heart mitochondria, yeast, and the cytochrome c_{552} found in the periplasmic space of *Thermus*. Indeed, when the latter protein was used as substrate for enzyme reconstituted into lipid vesicles, the enzyme was found to extrude ~ 1 H^+ per electron transferred to oxygen [3]; similar results were obtained by Sone et al. [11]. Taken together, the available data suggest that the single subunit enzyme of *Thermus* possesses the same essential properties of eucaryotic cytochrome oxidase, and whatever we learn from study of the bacterial enzyme will be generally transferrable to the eucaryotic system.

Understanding the mechanism of energy transduction by respiratory proteins remains an important goal of biochemical research. In the case of the terminal oxidase, cytochrome aa_3, it seems important in this quest to understand the thermodynamic distribution of electrons among the redox centers. In this short paper, written in honor of the memory of Eraldo Antonini, we present our preliminary results on the electrochemical properties of the bacterial cytochrome c_1aa_3 complex.

EXPERIMENTAL PROCEDURES

Thermus thermophilus cells were grown and cytochrome c_1aa_3 was prepared by the procedures of Yoshida et al. [2]. The complex was titrated under anaerobic conditions with a mixture of phenazine methosulfate (PMS) and NADH [12]. Oxygen was removed by repeated flushing of the cell with Ar purified through a Ridox (Fisher Scientific) column. The NADH solution was made in buffer bubbled with Ar and residual O_2 was removed by addition of protocatechuate dioxygenase and protocatechuic acid [13]. With such precautions, no lag in cytochrome aa_3 reduction was observed upon addition of NADH.

Electrochemical potentiometric titration was carried out in the presence of a mixture of mediator dyes as described by Prince et al. [14] using a Bioanalytical Systems apparatus. The anaerobic spectroelectrochemical cell employed was a slightly modified version of Stankovich [15]. It contained a large platinum working/measuring electrode, a Ag/AgCl auxiliary electrode, a standard calomel reference electrode (SCE = 242 mV vs. NHE), and the solution was stirred constantly while in the optical path of the spectrophotometer. The cell was degassed thoroughly before insertion of the electrodes, and the residual O_2 was consumed by electrochemical reduction until current passing through the cell was stabilized at a low value. The oxidative titration was started from the condition described above, while the reductive titration was carried out after electrochemically bringing the solution to the fully oxidized state. All reactions were carried out at 23 °C. Optical absorption spectra were recorded with a Perkin-Elmer model 320 spectrophotometer. EPR spectra were recorded at 20 K under nonsaturating conditions using a Varian E-112 spectrometer equipped with a home-built cryogenic system. Near infrared spectra were obtained using a Zeiss DMR-21 recording spectrophotome-

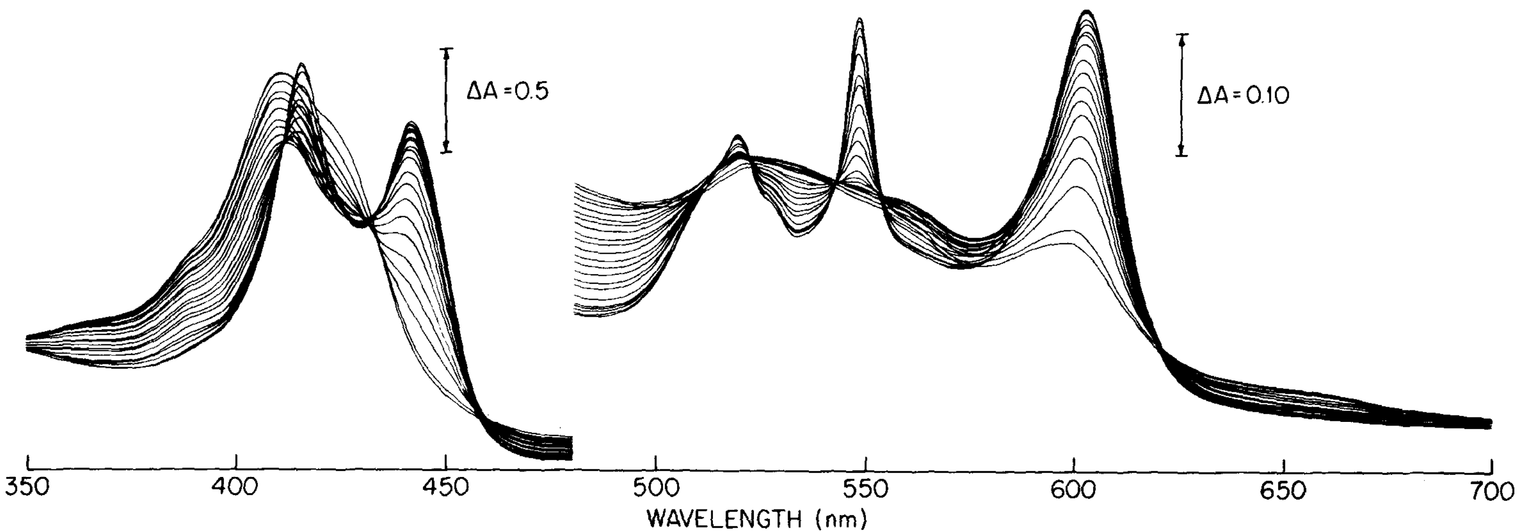


FIGURE 1. Anaerobic titration of cytochrome c_1aa_3 with NADH in the presence of PMS. The reaction solution contained 21 μM cytochrome c_1aa_3, 23 μM PMS, 50 mM tris-HCl pH 7.8, 0.2 mM EDTA, and 0.2% lauryl maltoside. The solution was degassed in an anaerobic titration cell (0.4 cm path length) and aliquots of 2.1 mM NADH were added anaerobically.

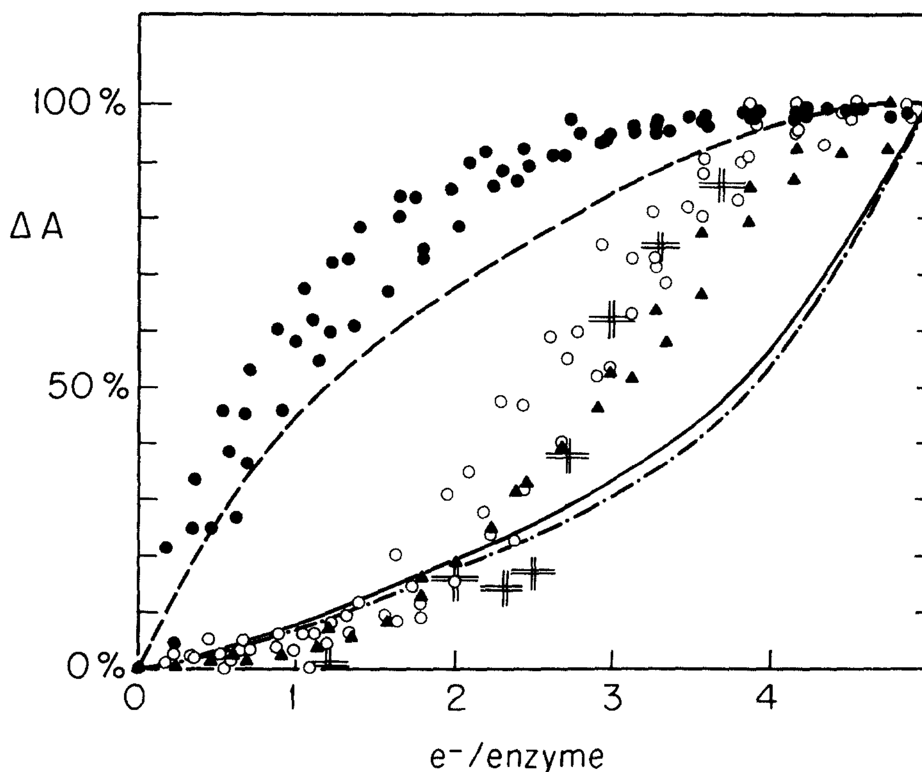
ter. All chemicals were of the best commercial grade and water subjected to purification with a Milli-Q system was used throughout.

RESULTS

Figure 1 shows the changes in the optical spectrum which occur when the enzyme is titrated with NADH in the presence of the mediator PMS. These are characterized by increases at 603 and 400 nm due to reduction of the cytochromes aa_3 and by increases at 548 and 418 nm due to the reduction of the cytochrome *c*. Fairly good isosbestic points occur near 620, 553, 543, 522, 514, 458, and 432 nm. The A hemes are titrated first followed by the cytochrome *c*.

Figure 2 shows a summary of percent changes of the individual signals observed during the reductive titration of cytochrome c_1aa_3. The intensity of the Cu_A^{2+} signal was obtained from EPR titrations; the primary data are not shown. The first electron-equivalent added to the complex appears to reside largely on cytochrome *a*, although a small amount of Cu_B must also have been reduced in order to account for the appearance of the small amount of high-spin heme ($< 1\%$) observed in the

FIGURE 2. Summary of signal changes during electron titration of cytochrome c_1aa_3. Data from experiments similar to Figure 1, as well as the near-ir, and EPR titrations (not shown) are plotted. [•], cytochrome aa_3 (602 nm); [○], cytochrome c_1 (548 nm); [△], Cu (800–980 nm); [+], Cu_A EPR signal ($g = 2$); [- - -], calculated line for cytochrome aa_3; [——], calculated line for cytochrome c_1; [-·-·-], calculated line for Cu_A EPR signal (see text).



EPR spectra. The near infrared band, 800 minus 980 nm, was also observed during several titrations and found to correspond satisfactorally to the EPR intensity of the detectable Cu. The second electron-equivalent appears to be distributed among several centers as only small changes occur in the cytochrome *a* and cytochrome *c* signals. The third electron-equivalent nearly completes the reduction of the *a* cytochromes and is accompanied by significant reduction of the cytochrome *c* and Cu_A. The results are consistent with five-electron equivalents being able to fully reduce the enzyme, as expected from the composition.

Figure 3 shows the results of an electrochemical titration of cytochrome c_1aa_3. The *a* cytochromes are being reduced at higher potential than the *c* cytochrome. In contrast to the beef heart enzyme [16], the system can be titrated along both oxidative and reductive paths with the same result. Brief inspection of Figure 3 reveals that the midpoint potential for the *a* cytochromes is ~300 mV (NHE) while that of the *c* cytochrome is ~200 mV (NHE). As discussed below, the redox behavior of the *c* cytochrome is simple Nernstian while that of the *a* cytochromes is more complicated.

FIGURE 3. Potentiometric titration of cytochrome c_1aa_3. The solution contained 60.5 μM cytochrome c_1aa_3 in 150 mM NaCl, 0.5 mM EDTA, 0.2% lauryl maltoside, and 30 mM each of the following buffers: *N*-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid (TAPS), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), and 2-(*N*-morpholino)ethane sulfonic acid (MES), adjusted to pH 7.8. The mediators present were: 52 μM benzylviologen, 10 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 52 μM PMS, 5.2 μM 1,2 naphthaquinone; and 104 μM potassium ferricyanide. [▲] oxidative titration, cytochrome c_1 (548 nm); [X] reductive titration, cytochrome c_1; [♦] reductive titration, cytochrome aa_3 (602 nm); [△] oxidative titration, cytochrome aa_3. The curves were drawn from the theoretical calculations using the parameters shown in the Table 1 (see text).

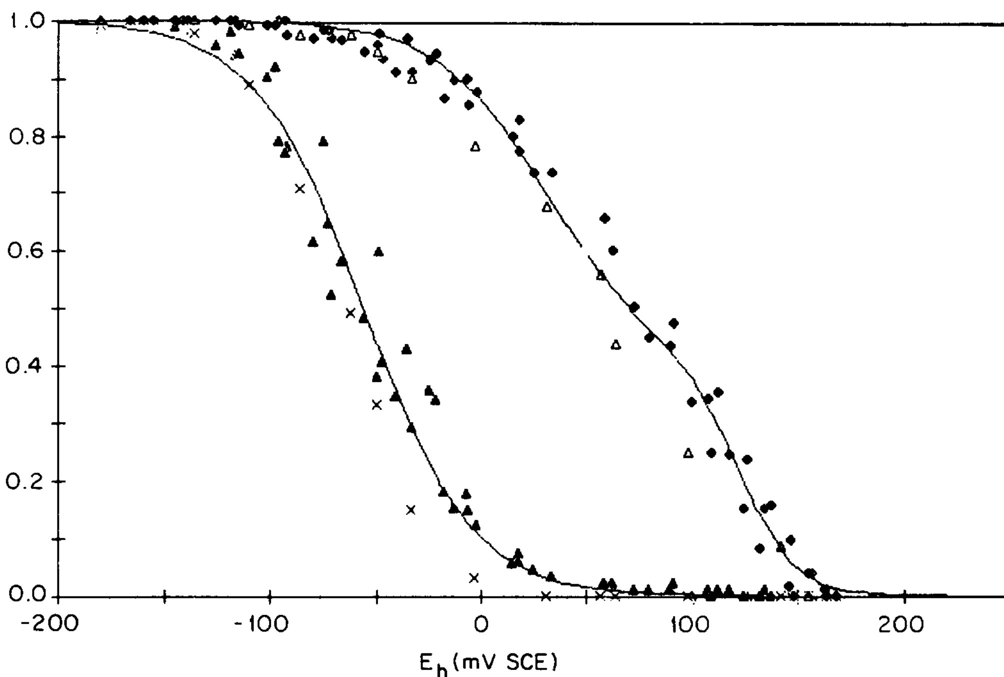


TABLE 1. Midpoint Potential of Redox Centers Associated with the 603 nm Absorption Band of Cytochrome c_1aa_3

	Center 1	Center 2
Spectral contribution[a]	0.78	0.22
n	0.71	2.28
E_m	291 mV	363 mV
Spectral contribution[b]	0.60	0.40
n	1.0 (fixed)	2.0 (fixed)
E_m	271 mV	362 mV

[a] Fit for five parameters, i.e., fraction (1), $E_m(1)$, $E_m(2)$, $n(1)$, and $n(2)$.
[b] Fit for three parameters, i.e., fraction, $E_m(1)$, and $E_m(2)$. These parameters were used to simulate the data for aa_3 in Figure 3.

ANALYSIS OF DATA

The results of the potentiometric titration have been analyzed under the assumptions that the system is at equilibrium and that the individual redox centers are behaving independently of one another. The optical changes associated with cytochrome c, ΔA_{548}, as the potential of the system was changed were simulated by the expression

$$\Delta A_{548}/\Delta A_{max} = (1 + 10^{n/59(E_h - E_m)})^{-1}$$

where n is the number of electrons involved in the equilibrium, E_h is the solution potential, and E_m is the midpoint potential of the cytochrome c. The solid line in Figure 3 was calculated with the parameters $n = 1.0$ and $E_m = 205$ mV (NHE). The fit is very good, and this result strongly suggests that the c cytochrome is equilibrating with the electrode independently of the other centers.

The optical changes associated with the reduction of the cytochromes a, ΔA_{603}, do not adhere to a single $n = 1$ process. However, they can be fitted using the following expression:

$$\Delta A(\%) = A_1(1 + 10^{n_1/59(E_h - E_{m1})})^{-1} + A_2(1 + 10^{n_2/59(E_h - E_{m2})})^{-1}$$

where A_i is the spectral contribution of each species simultaneously being observed at 603 nm. E_h corresponds to the potential of the system, E_{mi} to the potential of the chromophore, and n_i is the number of electrons involved in each equilibrium. The data were fitted to a least-squares minimization procedure under a number of assumptions concerning the unknown parameters. The poorest fit was obtained when the values of n were constrained to 1, while the best fit was obtained when all parameters were allowed to float. In the latter case, as shown in Table 1, the value of n_1 is close to 1 (0.71) while the value of n_2 is close to 2 (2.28). If n_1 is contrained to 1 and n_2 to 2, an acceptable fit to the data was obtained with E_{m1} = 291 mV and E_{m2} = 363 mV. The relative spectral contributions to ΔA vary substantially with the different constraints. With all parameters floating, A_1 settles

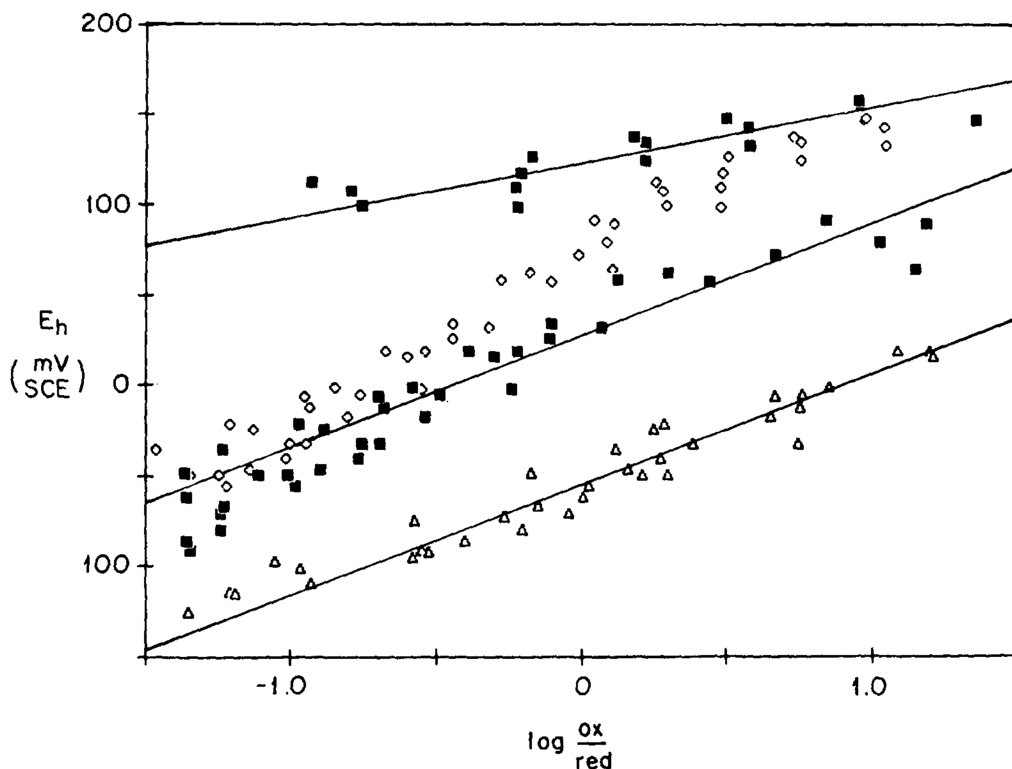


FIGURE 4. Nernst plots of the potentiometric titration of cytochrome c_1aa_3. Data are identical to those used in Figure 3. [△], cytochrome c_1 (548 nm); [◇], cytochrome aa_3 (602 nm); [■], resolved cytochrome aa_3 according to the optimal parameters shown in Table 1. The upper line corresponds to cytochrome a_3 and has a slope of 30 mV/decade. The middle line corresponds to cytochrome a and has a slope of 60 mV/decade. The bottom line indicates cytochrome c with a slope of 60 mV/decade.

to 0.78 and A_1 to 0.22 while when the n values are fixed A_1 becomes 0.6 A_2 = 0.4.

Using values of $n_1 = 1$ and $n_2 = 2$, we have resolved ΔA_{603} into two components and plotted the resolved data in the form of Nernst diagrams [17], as shown in Figure 4. Here one sees that the low-potential portion of the data fits very nicely to an $n = 1$ process and the high-potential portion of the data fits nicely to an $n = 2$ process. Such plots show dramatic deviation from the data when other than these parameters are used in the simulation.

Combining the above information with the observation that virtually no high-spin heme or any other EPR signals develop during the electron titration, we make the following tentative conclusions: The high-potential heme A center corresponds to the $\{a_3, Cu_B\}$ pair, which behaves as a two-electron acceptor in this enzyme, and the low-potential heme A center corresponds to cytochrome a.

We now extend this analysis to the electron titration data of Figure 2. Here we make the same assumptions as above but specifically include the assumption that electrons are able to freely equilibrate within the molecule. This allows us to use the spectrally isolated cytochrome c as a reporter of the internal potential of system. We followed the method Olson et al. [18] used to describe the

thermodynamically determined distribution of electrons within xanthine oxidase, and we wrote a series of equilibria in which the reduced cytochrome *c* acts as reductant to each of the other components. These are given in Table 2 along with the relative values for each K calculated from the potentials derived from the potentiometric titration. When the observable, e^-/c_1aa_3, is calculated for ratios of $c_1{}^0/c_1{}^r$ varying from 0 to ~ 10, the solid lines in Figure 2 are generated. One can immediately observe that the model is inadequate, suggesting that the enzyme is behaving differently in the two experiments. While it is possible that internal equilibrium is not established in the electron titration, the rapidity of electron transfer observed in (unpublished) transient kinetic studies makes this unlikely. We prefer rather to believe that *intra*molecular equilibrium in the electron titration differs from *inter*molecular equilibrium in the potentiometric titration. The simplest explanation for the lack of correspondence between the two experiments then is that the relative potential of the $\{a_3, Cu_B\}$ pair is much lower in the electron titration than in the potentiometric titration. Indeed, if K_4 (Table 2) is lowered to ~ 5 in the above analysis, a much better fit is obtained, although the details are still unacceptable.

DISCUSSION

Wikström, Krab, and Saraste [19] have reviewed the extensive literature on the redox properties of bovine cytochrome oxidase. They conclude that the system is best described in terms of the "neoclassical" model of Nicholls [20]. Recently, Carithers and Palmer [16] have provided strong evidence for this model by simultaneously measuring a_3 and a during potentiometric titrations. Both cytochromes are indeed reduced over the entire range of potentials, both have intrinsic potentials of ~ 350 mV, and an anticooperative interaction of ~ -90 mV occurs between the two hemes.

There are several observations which suggest the neoclassical model may not be applicable to the c_1aa_3 complex of *Thermus*: If an allosteric interaction occurs in the aa_3 portion of the molecule during potentiometric reduction it is certainly not "sensed" by the cytochrome *c*, which behaves as an independent entity even though it appears to be tightly bound to the A-protein [2]. Previous analyses of potentiometric titration curves with beef heart oxidase have yielded two $n = 1$ processes separated by ~ 100 mV [19]. In contrast, analysis of the potentiometric titration curves of c_1aa_3 yields one $n = 1$ process with $E_m = 270$ mV and one $n = 2$ process with $E_M = 360$ mV. Moreover, unpublished work has shown that the $n = 2$ process is sensitive to changes in pH and the presence of CO while the $n = 1$ process is not. This would suggest that the $n = 2$ process is reporting an equilibrium involving a_3 and a tightly coupled Cu_B (i.e., $K_3 \ll K_4$ in Table 2). Finally, almost no high-spin heme appears during the EPR titration. This suggests a strong chemical coupling between a_3 and Cu_B and offers support for an $n = 2$ process. For these reasons, we have chosen (at this time) not to interpret our data in terms of the neoclassical model.

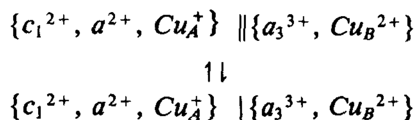
The Analysis shows that if the redox sites of c_1aa_3 are independent in the potentiometric titration, where *inter*molecular equilibria are established by the mediators, they are definitely dependent on one another in the electron titration in which there are no mediators. Such behavior can be rationalized, with some

TABLE 2. Model of Equilibrium Distribution

Equilibrium	K_{rel}	Relative potential[a]
$c_1{}^r + a^0 = c_1{}^0 + a^r$	$K_1 = 29.8$	65
$c_1{}^r + Cu_A{}^{2+} = c_1{}^0 + Cu_A{}^{1+}$	$K_2 = 0.9$	~ −3
$c_1{}^r + \{a_3, Cu_B\}^{5+} = c_1{}^r + \{a_3, Cu_B\}^{4+}$	$K_3 = 0.01$[b]	
$c_1{}^r + \{a_3, Cu_B\}^{4+} = c_1{}^r + \{a_3, Cu_B\}^{3+}$	$K_4 = 1080$	157

[a] Cytochrome c_1 was assumed to have a relative potential of 0 mV. Other values were calculated from the data in Table 1.
[b] This value was assigned arbitrarily to account for the apparent $n = 2$ process described in the text and to preclude formation of high-spin heme.

success, by assuming an allosteric equilibrium between two partially reduced forms of the enzyme, only one of which is able to transfer electrons from a to a_3. This barrier is indicated by the vertical bars in the following scheme:



This equilibrium should have the quality of an *electron gate* within the molecule, but it can be circumvented by the mediators present in the potentiometric titration. A similar situation evidently occurs in the resting state of the bovine enzyme (cf. Ref. 21 and references therein).

We had hoped that a cytochrome oxidase with a simpler subunit structure would exhibit a correspondingly simpler electrochemical behavior. This is unfortunately not the case with the enzyme from *Thermus*.

REFERENCES

1. J. A. Fee, M. G. Choc, K. L. Findling, R. M. Lorence, and T. Yoshida, *Proc. Natl. Acad. Sci. USA* **77**, 147 (1980).
2. T. Yoshida, R. M. Lorence, M. G. Choc, G. E. Tarr, K. L. Findling, and J. A. Fee, *J. Biol. Chem.* **259**, 112 (1984).
3. T. Yoshida and J. A. Fee, *J. Biol. Chem.* **259**, 1031 (1984).
4. L. Powers, B. Chance, Y. Ching, and P. Angiolillo, *Biophys. J* **34**, 465 (1981).
5. G. T. Babcock, P. M. Callahan, M. R. Ondrias, and I. Salmeen, *Biochemistry,* **20**, 952 (1981).
6. T. A. Kent, L. J. Young, G. Palmer, J. A. Fee, and E. Munck, *J. Biol. Chem.*, **258**, 8543 (1983).
7. N. R. Orme-Johnson, R. E. Hansen, and H. Beinert, *Biochem. Biophys. Res. Commun.* **45**, 871 (1971).
8. K. Honnami and T. Oshima, *Biochemistry* **23**, 454 (1984).
9. B. Kadenbach and P. Merle, *FEBS Lett.* **135**, 1 (1981).
10. B. Ludwig, *Biochim. Biophys. Acta* **594**, 177 (1980).
11. N. Sone, Y. Yanagita, K. Honnami, Y. Fukumori, and T. Yamanaka, *FEBS Lett.* **155**, 150 (1983).
12. B. F. van Gelder and A. O. Muijsers, *Biochim. Biophys. Acta* **81**, 405 (1964).
13. C. Bull, D. P. Ballou, and S. Ostuka, *J. Biol. Chem.* **256**, 12681 (1981).
14. R. C. Prince, S. J. G. Linkletter, and P. L. Dutton, *Biochim. Biophys. Acta* **635**, 132 (1981).

15. M. T. Stankovich, *Anal. Biochem.* **109**, 295 (1980).
16. R. P. Carithers and G. Palmer, *J. Biol. Chem.* **256**, 7967 (1981).
17. D. F. Wilson and P. L. Dutton, *Arch. Biochim. Biophys.* **136**, 583 (1970).
18. J. S. Olson, D. P. Ballou, G. Palmer, and V. Massey, *J. Biol. Chem.* **249**, 4363 (1974).
19. M. Wikström, K. Krab, and M. Saraste, *Cytochrome Oxidase: A Synthesis,* Academic Press, London, 1981.
20. P. Nicholls and L. C. Petersen, *Biochim. Biophys. Acta* **357**, 462 (1974).
21. T. M. Antalis and G. Palmer, *J. Biol. Chem.* **257**, 6194 (1982).

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