EVALUATION OF THE ENERGETICS OF CYTOCHROME *C* OXIDASE IN THE ABSENCE OF CYTOCHROME *C*

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1. Introduction

As previously reported [1], the redox potentials of heme a_H and heme a_L of purified beef heart cytochrome *c* oxidase in the presence of horse heart cytochrome *c* for the indirect coulometric titration method, without potentiometric mediators present, were $E^{\circ\prime}_{\text{heme } a_H} = 340–350$ mV and $E^{\circ\prime}_{\text{heme } a_L} = 210$ mV. Spectrophotometric data alone were used to determine the potential differences between cytochrome *c* and heme a_L, and cytochrome *c* and heme a_H. The heme a_L and heme a_H potentials were then set with respect to the cytochrome *c* potential, which was determined independently [2]. This method eliminated the need for potentiometric mediators which have varied effects on the cytochrome *c* oxidase [3,4].

Muijsers et al. [5] reported the potentials of the hemes of cytochrome *c* oxidase in the presence of cytochrome *c* ($E^{\circ\prime}_{\text{heme } a_3} = 335–360$ mV and $E^{\circ\prime}_{\text{heme } a} = 200–250$ mV), and asserted that the hemes titrated as indistinguishable entities in the absence of cytochrome *c* ($E^{\circ\prime}_{\text{heme } aa_3} = 280$ mV). Potentiometric mediators were present in solution. Thus Muijsers et al. suggested that the hemes of cytochrome *c* oxidase are equivalent in purified cytochrome *c* oxidase and become split in the presence of ligands such as cytochrome *c* [5].

Wharton and Cusanovich [3] presented potentiometric data for purified cytochrome *c* oxidase in the presence of varying amounts of ferricyanide ranging from zero to 5×10^{-2} M. They concluded that the hemes of cytochrome *c* oxidase are split in potential in the presence of ferricyanide independent of cytochrome *c*. Wharton has also reported the midpoint potential of at least one copper of cytochrome oxidase, spectrally observed at 830 nm, to be 278 mV.

Tsudzuki and Wilson [6] have reported that the hemes of highly purified beef heart cytochrome oxidase, studied by potentiometric techniques, titrate as a single component with a midpoint potential of 285 mV. The heme components were resolved into high and low potential components in partially purified preparations. Both the 830 nm absorption band and EPR measurements showed the copper to have a midpoint potential of 225 mV.

Malmström et al. [7] have run kinetic experiments on cytochrome *c* – cytochrome *c* oxidase mixtures. They attribute an initial fast reaction to the reduction of cytochrome *a* by cytochrome *c*. For this to occur, the potential of cytochrome *a* must be more positive than the potential of cytochrome *c*. Therefore, they maintained that the separation of potential of heme a_L and heme a_H, even in the presence of cytochrome *c*, is not manifested in their experiments.

With these discrepancies in the states of the hemes of cytochrome *c* oxidase in mind, the energetics of purified cytochrome *c* oxidase have been examined by coulometrically titrating cytochrome *c* oxidase–ferricyanide and cytochrome *c* oxidase–tetramethyl-*p*-phenylenediamine (TMPD) mixtures and optically

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monitoring the 605 nm band of cytochrome *c* oxidase. The cytochrome *c* oxidase mixtures were initially in the completely oxidized forms, and were titrated indirectly with electrochemically generated reductant, methyl viologen cation radical (MV^+). These mixtures were reoxidized with either electrochemically generated ferricyanide or oxygen.

2. Experimental

The solutions contained 7 to 15 μM cytochrome *c* oxidase, 0.1 to 2.0 mM ferricyanide, 10–100 μM TMPD, 0.1 M potassium phosphate buffer, pH = 7.0, 0.1 M NaCl, and 0.1% Tween 20 in doubly distilled water. The cell design, optical and electrochemical equipment, and general procedure have previously been described [2]. The solutions were made anaerobic ($O_2 < 5 \times 10^{-7}$ M) by vacuum degassing.

The 605 nm band was seen to be the composition of two separate bands, namely the two heme components of cytochrome *c* oxidase, as could be seen by a 4 nm shift of the 590 nm isosbestic during a titration. The heme a_L isosbestic remained stationary through 50% of the titration. The isosbestic then shifted 4 nm to a shorter wavelength for the remaining 50% of the titration. The two isosbestics were evaluated as being direct probes on which heme was being titrated.

3. Results and discussion

Cytochrome *c* oxidase is known to contain four titratable redox components [1]. The cytochrome *c* oxidase used in this study contained 14 μmoles of titratable heme *a*/g protein and 15 μmoles of titratable copper/g protein [1]. The amount of titrant added was monitored with nanoequivalent accuracy by measuring the amount of charge injected with an electronic integrator. The charge injected is related to the amount of reducible species in solution by the equation $Q = nFVC$ where Q is charge in millicoulombs, F is Faradays's constant, V is the cell volume and C is the concentration in moles per liter. The absorptivity per heme of cytochrome *c* oxidase at 605 nm (reduced minus oxidized) was taken as 12 $mM^{-1}cm^{-1}$.

The potentials of the four components of cytochrome oxidase were determined by curve fitting the experimental data to computer simulated absorbance (A) vs charge (Q) data. The computer simulated A vs Q curves were derived by treating the system as a five component system of two hemes, two copper atoms and a mediator-titrant, either ferricyanide or TMPD. Account was also taken for the weak charge–transfer complex between methyl viologen dication and ferrocyanide [8]. The A vs Q curves were generated by calculating the concentrations of the components from the five Nernst equations for the components. Since the copper components were not monitored optically, their potentials were obtained indirectly by their effect on the A vs Q curves of the heme components.

Potentiometric data were also taken simultaneously during these experiments. The potential vs log (oxidized heme *a*/reduced heme *a*) plots gave good agreement with the A vs Q data. However, the potentiometric data alone was not conclusive and led to the report by Muijsers et al. [5] that the potentials of the hemes of cytochrome oxidase in the absence of cytochrome *c* were not split. The potentiometric data were scattered more than the A vs Q data, owing to the poor coupling between mediator and enzyme, and mediator and platinum electrode. If the entire heme band is considered as one component, our potentiometric data alone could also indicate an $E^{\circ\prime}$ = 280 mV for the hemes of cytochrome *c* oxidase.

The A vs Q data has the distinct advantage over potentiometric data in that the potentials of both Cu atoms of cytochrome *c* oxidase can be evaluated without having an optical probe on the Cu atoms. The potentials of the Cu atoms are indirectly evaluated by noting the manner in which they affect the A vs Q curves.

Since this method depends upon an equilibrium between the mediator-titrant and the cytochrome *c* oxidase components, when ferricyanide ($E^{\circ\prime}$ = 424 mV) is used as mediator titrant, the method is most sensitive to the high potential components of cytochrome oxidase, i.e., heme a_H ($E^{\circ\prime}$ = 350 mV) and Cu_H ($E^{\circ\prime}$ = 350 mV), and less sensitive to the low potential components, heme a_L ($E^{\circ\prime}$ = 220 ± 30 mV) and Cu_L ($E^{\circ\prime}$ = 220 ± 30 mV). (fig. 1).

When TMPD ($E^{\circ\prime}$ = 300 mV) is used as mediator-titrant, the method is more sensitive to the low potential components of cytochrome oxidase, heme a_L ($E^{\circ\prime}$ =

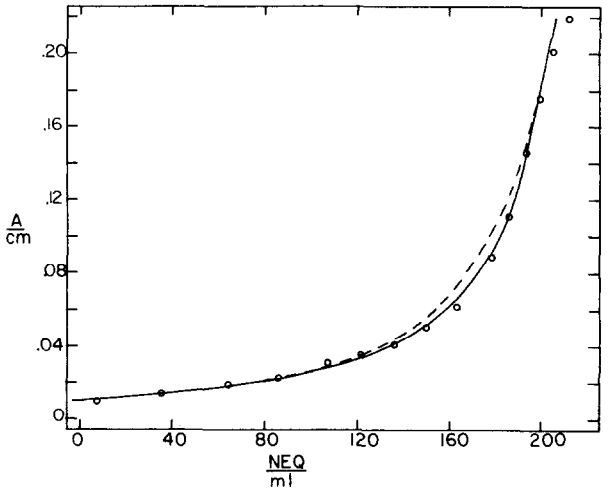

Fig. 1. Plot of absorbance (A/cm) vs charge (nanoequivalents/ml) for first reduction titration of cytochrome c oxidase–ferricyanide mixture. Solution contained 0.5 mM $Fe(CN)_6^{3-}$, 0.5 mM MV^{2+}, and 9.2 μM cytochrome c oxidase. (o—o—o) Data points for reduction of cytochrome c oxidase with electrochemically generated MV^{+}. (- - -) Computer simulated for $E^{\circ\prime}_{\text{heme } a_H}$ = 350 mV: $E^{\circ\prime}_{Cu_H}$ = 350 mV; $E^{\circ\prime}_{\text{heme } a_L}$ = 220 mV; $E^{\circ\prime}_{Cu_L}$ = 220 mV; $E^{\circ\prime}$ ferricyanide = 424 mV. (- - - -) Computer simulation for $E^{\circ\prime}_{\text{heme } a_H}$ = 350 mV; $E^{\circ\prime}_{Cu_H}$ = 280 mV; $E^{\circ\prime}_{\text{heme } a_L}$ = 220 mV; $E^{\circ\prime}_{Cu_L}$ = 220 mV; 220 mV; $E^{\circ\prime}_{\text{ferricyanide}}$ = 424 mV.

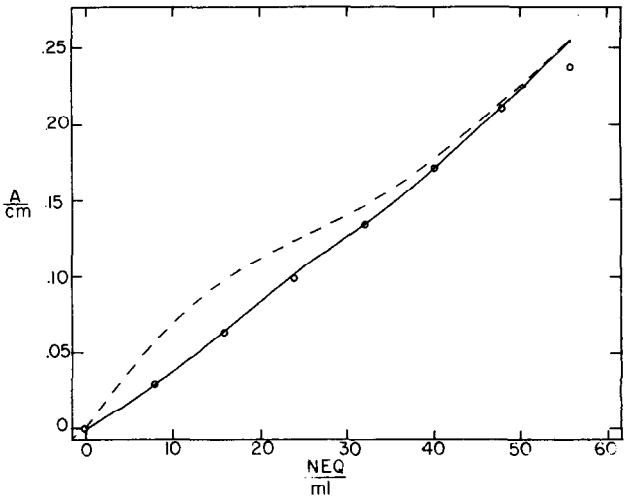

Fig. 2. Plot of absorbance (A/cm) vs charge (nanoequivalents/ml) for first reduction titration of cytochrome c oxidase–TMPD mixture. Solution contained 10^{-5} M TMPD, 0.5 mM MV^{2+}, 10.6 μM cytochrome c oxidase. (o—o—o) Data points for reduction of cytochrome c oxidase with electrochemically generated MN^{+}; (- - - -) computer simulation for $E^{\circ\prime}_{\text{heme } a_H}$ = 340 mV; $E^{\circ\prime}_{Cu_H}$ = 350 mV; $E^{\circ\prime}_{\text{heme } a_L}$ = 215 mV; $E^{\circ\prime}_{Cu_L}$ = 215 mV; $E^{\circ\prime}_{TMPD}$ = 300 mV. (- - - -) Computer simulation for $E^{\circ\prime}_{\text{heme } a_H}$ = 340 mV; $E^{\circ\prime}_{Cu_H}$ = 280 mV; $E^{\circ\prime}_{\text{heme } a_L}$ = 215 mV; $E^{\circ\prime}_{Cu_L}$ = 215 mV; $E^{\circ\prime}_{TMPD}$ = 300 mV.

215 ± 10 mV) and Cu_L ($E^{\circ\prime}$ = 215 ± 10 mV). The high potential components were again determined to be heme a_H ($E^{\circ\prime}$ = 340 ± 10 mV) and Cu_H ($E^{\circ\prime}$ = 350 ± 10 mV) (fig. 2).

The potentials of heme a_H and heme a_L of cytochrome oxidase obtained from A vs Q data were substantiated by Minnaert [9] plots of log (TMPD(ox)/TMPD(red)) vs log ((heme a_H + heme a_L)ox/(heme a_H + heme a_L)red). Experimental data were fitted with a computer simulated curve. The potentials of hemes a_H and a_L agreed with the potentials determined by the A vs Q method (fig. 3).

These values for the potentials of heme a_H and heme a_L in the presence of ferricyanide and TMPD agree with the values previously reported in the presence of cytochrome c [1].

The experimentally observed split in potential of the hemes and coppers of cytochrome c oxidase is not proof that they are distinguishable in the fully oxidized or fully reduced states. A reasonable explanation for the difference in potentials observed experimentally is that each electron transferred alters the charge and electron density distribution within the oxidase molecule which consequently affects to $E^{\circ\prime}$ for the next electron transferred. Since the potentials of the four redox components of cytochrome c oxidase are reproducible and independent of the mediators used in this study, it is considered unlikely that ligand effects are causing the splittings.

The 830 nm band of cytochrome c oxidase which usually has been attributed to copper was monitored simultaneously with the 605 nm band in order to compare the potentials of the copper atoms with those determined by the A vs Q curves. A close parallelism was observed for the A vs Q plots at both wavelengths. This suggests that the 830 nm band is not due solely to one copper, or due to one copper and one heme unless the molar absorptivities are very similar.

The assigned $E^{\circ\prime}$ values are summarized in table 1 along with selected literature values for comparison purposes.

Table 1
$E^{\circ\prime}$ Values for purified cytochrome *c* oxidase $E^{\circ\prime}$ values, mV (n values).

Source	Heme *a*		Copper		Conditions
	Low*	High**	Low	High	
This paper	220 ± 30 (1.0)	350 ± 10 (1.0)	220 ± 30 (1.0)	350 ± 10 (1.0)	Ferricyanide present
	215 ± 10 (1.0)	340 ± 10 (1.0)	215 ± 10 (1.0)	350 ± 10 (1.0)	TMPD present
Heineman[1]	210 (1.0)	340–350 (1.0)			Cyt. *c* present
van Gelder[2]	280 (1.0)***				Cyt. *c* absent
	200–250 (1.0)	335–360 (1.2)			Cyt. *c* present
Wilson[5]	285 (0.5)***				Highly purified
	225 (1.0)	375 (1.0)	225 (1.0)†		Partially purified

* Frequently designated cyt. *a*.
** Frequently designated cyt. a_3.
*** Titrated as single component.
† Based on 830 nm band and EPR.

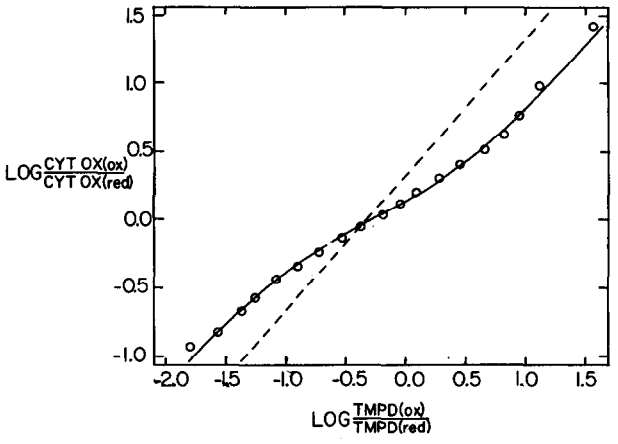

Fig. 3. Plot of log [heme a_H + heme a_L)ox/(heme a_H + heme a_L)red] vs log [TMPD(ox)/TMPD(red)]. Solution conditions the same as in fig. 2. (o–o–o) Data points for reduction of cytochrome *c* oxidase with electrochemically generated MV^+. (- - - -) Computer simulation for $E^{\circ\prime}_{\text{heme } a_H}$ = 335 mV and $E^{\circ\prime}_{\text{heme } a_L}$ = 220 mV. (- - - -) Computer simulation for $E^{\circ\prime}_{\text{heme } a_H} = E^{\circ\prime}_{\text{heme } a_L}$ = 280 mV for comparison.

Acknowledgements

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