CHARGE DISTRIBUTION IN ELECTRON TRANSPORT COMPONENTS:

Cytochrome c and Cytochrome c Oxidase Mixtures*

Wm. R. Heineman** and T. Kuwana
Department of Chemistry, Ohio State University
Columbus, Ohio 43210

and

Charles R. Hartzell
Department of Biochemistry, Pennsylvania State University
University Park, Pennsylvania 16802

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SUMMARY. Mixtures of cytochrome c oxidase and cytochrome c have been titrated by coulometrically generated reductant, methyl viologen radical cation, and physiological oxidant, O_2. Charge distribution among the heme components in mixtures of these two redox enzymes has been evaluated by monitoring the absorbance changes at 605 and 550 nm. Differences in the pathway of the electron transfer process during a reduction cycle as compared to an oxidation cycle are indicated by variations found in the absorbance behavior of the heme components during successive reductive and oxidative titrations. It is apparent that the potential of the cytochrome a heme is dependent upon whether oxidation or reduction is occurring.

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** Present address: Department of Chemistry
University of Cincinnati
Cincinnati, Ohio 45221

A previous communication (1) described the repetitive cycling of cytochrome c oxidase between its oxidized and reduced states by indirect coulometric titration with electrogenerated reductant, MV^{+}, and oxidant, O_2. The present paper extends this study to mixtures of cytochrome c oxidase with its precursor in the respiratory chain, cytochrome c. The ability to repetitively cycle to any redox level by quantitative incremental additions of reductive or oxidative titrant with simultaneous spectral monitoring has allowed the observation of significant differences between the reductive cycle and the oxidation by O_2.

Samples of isolated purified beef heart cytochrome c oxidase and purified horse heart cytochrome c were mixed in solutions (temperature less than 5°C) containing 0.3 M phosphate buffer, pH 7.00, 0.1 M NaCl, 0.1% Tween 20 and 0.5 mM methyl viologen dication. Samples were transferred to the electrochemical cell and then made anaerobic ($O_2 < 5 \cdot X\ 10^{-7}$ M) by repeated cycling between a vacuum and prepurified nitrogen. All titrations were performed at room temperature. For reductive titrations, methyl viologen radical cation MV^{+} was electrogenerated by the one electron reduction of the dication at an optically transparent electrode. For oxidations, molecular oxygen was quantitatively generated by the oxidation of water at a small platinum electrode in the same cell compartment (1). The cell volume was 1.55 ml and its optical path length was 1.7 cm. Each reductive or oxidative titration required approximately 30 minutes. The optical and electrochemical instrumentation and procedure for these titrations have been described (2). Cytochrome c oxidase was prepared from beef heart mitochondria according to C.R. Hartzell (manuscript in preparation). The heme a and copper content were 14 μmoles/g protein and 15 μmoles/g protein respectively.

Spectra recorded during equal (0.5 millicoulomb) incremental additions

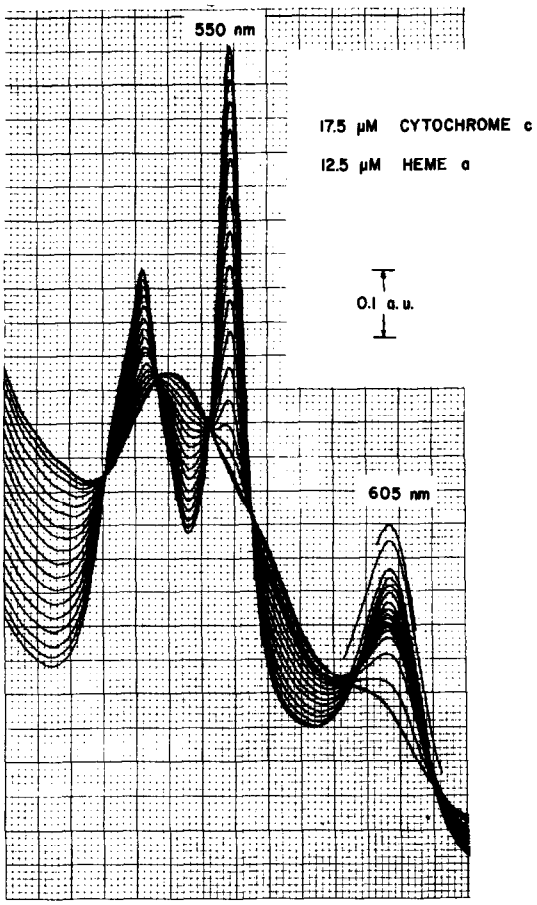

Figure 1. Spectra of cytochrome c (17.5 μM)--cytochrome c oxidase (12.5 μM heme a) mixture during reduction cycle by coulometrically generated MV^{+}. Titrant generated in increments of 0.5 mc (1.00 millicoulomb = 1.04×10^{-6} equivalents). Spectrum recorded after each addition of MV^{+}. Spectra correspond to titration from totally oxidized to totally reduced forms. Last two spectra at 605 nm were taken after excess MV^{+} was present.

of reductive titrant (MV^{+}) are shown in Figure 1. The intensity of the 605 nm α-band of the oxidase changes during the initial and final portions of the titration; the intensity of the 550 nm α-band of cytochrome c changes predominantly during the intermediate stages. It is assumed that the high potential component of the oxidase is titrated initially and the low potential one during the latter stages. The high potential component has been assigned to cytochrome a_3 and

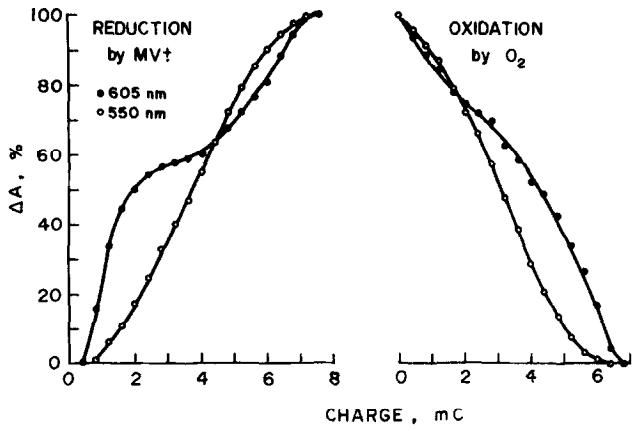

Figure 2. Typical plot of absorbance changes at 605 and 550 nm during reduction cycle by coulometrically generated MV^{+} and oxidation by coulometrically generated O_2. Absorbance plotted in terms of percentage of total absorbance change. Charge corrected for 20% background. Mixture of cytochrome c (17.5 μM) and cytochrome c oxidase (12.5 μM heme a).

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the low potential component identified as cytochrome a by Wilson and coworkers (3,4). All of the isobestic points hold rigorously throughout the titration except the one at ca. 590 nm which moves until approximately 50% of the total heme of oxidase is titrated. Whether this phenomenon is independent of the presence of cytochrome c is presently being investigated.

The absorbance change of the 605 nm band and the 550 nm band as a function of the amount of titrant added are shown in Figure 2. The amount of titrant added is expressed in terms of the charge required to electrochemically generate the reductant or oxidant (1.00 mC = 1.04×10^{-8} eq). Similar curves for the reduction of such a mixture by conventional titration with NADH have been recently reported by van Gelder (5). However, quantitative results for oxidative titrations, particularly with the physiological oxidant, O_2, have not been previously assessed. Evaluation of the relative behaviors of the 605 and 550 nm bands in Figure 2 indicates that the standard redox potential, $E^{o'}$,

of cytochrome c is intermediate of the redox potentials of the spectrally observed heme groups of cytochromes a and a_3. It is also evident that the oxidative A-Q curve of the 605 nm band of cytochrome oxidase is not a mirror image of the reductive curve, while there is little difference between the A-Q curves of cytochrome c. These curves include charge which contributes to the titration of all redox components including the two units of copper present in each molecule of cytochrome oxidase which were not monitored optically. Consequently, quantitative evaluation of the shape of the curves in Figure 2 requires consideration of 5 redox couples.

In order to separate the behavior of the optically monitored redox components, logarithms of the ratios of the reduced to oxidized forms were plotted in a manner following Minnaert (6). Data for the reductive cycle in Figure 2 are plotted as log [c(red)/c(ox)] versus log [aa_3(red)/aa_3(ox)] in Figure 3 where c(red)/c(ox) = $\Delta A_{550}/(100\% - \Delta A_{550})$ and aa_3(red)/aa_3(ox) = $\Delta A_{605}/(100\% - \Delta A_{605})$ where ΔA represents the percentage of the total absorbance change. The result is a sigmoid curve which is characteristic of a multi-redox situation. The standard redox potentials of the three redox species involved can be found by computer simulating the behavior of the following Nernst equations as the potential E of the system is varied:

$$E = E^{o'}_{cyt\ c} - 59 \log (c(red)/c(ox))$$

$$E = E^{o'}_{cyt\ a} - 59 \log (a(red)/a(ox))$$

$$E = E^{o'}_{cyt\ a_3} - 59 \log (a_3(red)/a_3(ox))$$

The solid curve in Figure 3 represents the curve generated for $E^{o'}_{cyt\ c}$ = 250 mV, $E^{o'}_{cyt\ a}$ = 210 mV and $E^{o'}_{cyt\ a_3}$ = 350 mV. The fit is quite good. Since the method gives the relative potentials of the three components, the above potentials are based on a value of 250 mV for cytochrome c which was deter-

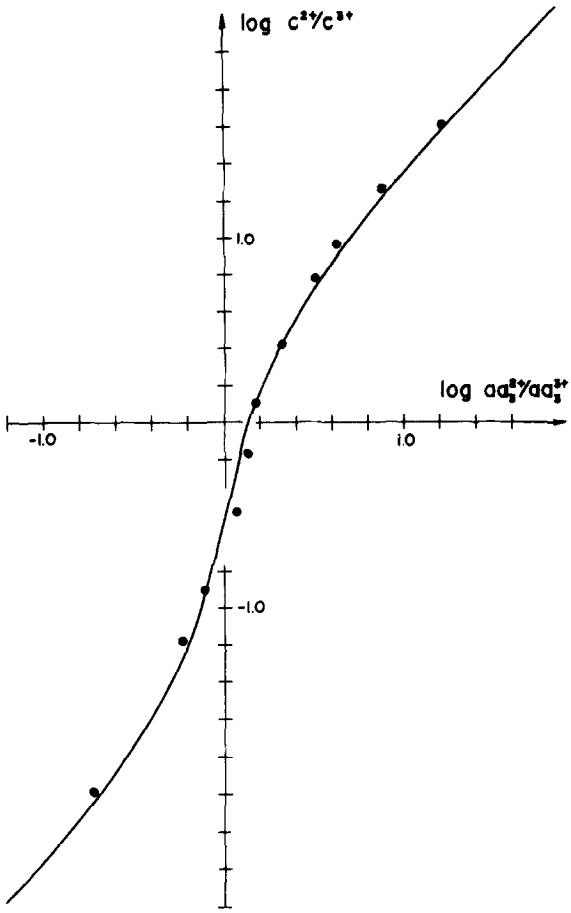

Figure 3. Plot of log [c(red)/c(ox)] versus log [aa_3(red)/aa_3(ox)] for first reduction titration of cytochrome c - cytochrome c oxidase mixture. (●) - data points obtained from data for reduction by MV^+ in Figure 2. (——) - computer simulation for $E^{o'}_{cyt\ c}$ = 250 mV, $E^{o'}_{cyt\ a}$ = 210 mV and $E^{o'}_{cyt\ a_3}$ = 350 mV.

mined independently by comparison with the potential for ferro-ferricyanide (2). The measurement of standard potentials of redox enzymes by this method as compared to the potentiometric method offers the significant advantage of not requiring coupling of the solution potential with a platinum indicator electrode. This eliminates the necessity of adding numerous coupling agents (mediators) to the solution as is necessary for the potentiometric study of enzymes which do not themselves couple with the platinum electrode (3,4). The possibility

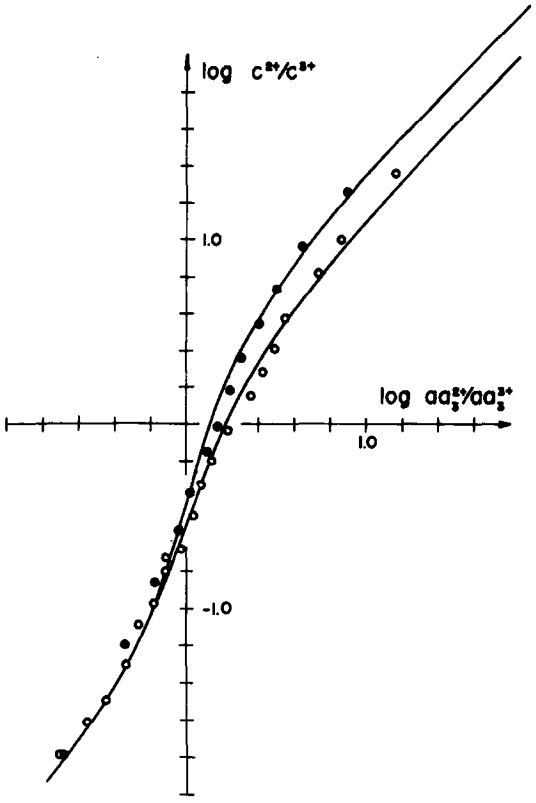

Figure 4. Plot of log [c(red)/c(ox)] versus log [aa_3(red)/aa_3(ox)] for mixture of cytochrome c (17.5 μM) and cytochrome c oxidase (12.5 μM heme a). (●) - oxidation titration by O_2 and (——) - computer simulation for $E^{o'}_{cyt\ c}$ = 250 mV, $E^{o'}_{cyt\ a}$ = 225 mV and $E^{o'}_{cyt\ a_3}$ = 340 mV. (●) - subsequent reduction titration by MV^{+} and (——) - computer simulation for $E^{o'}_{cyt\ c}$ = 250 mV, $E^{o'}_{cyt\ a}$ = 210 mV and $E^{o'}_{cyt\ a_3}$ = 340 mV.

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of poor electrode coupling with the accompanying drift of the electrode potential is eliminated.

Figure 4 shows a logarithmic plot for an oxidative titration with O_2 as compared to the subsequent reductive titration with MV^{+}. Once again the solid lines represent the best-fit computer simulation. The comparison shows a shift in the redox potential of cytochrome a from 225 mV during the oxidation cycle to 210 mV during the reduction cycle. It appears that the low potential heme of cytochrome a has a more positive $E^{o'}$ during oxidation than during

reduction. This shift in potential may be, in part, arising from differences in the pathway of the electron transfer process. In reductions by methyl viologen radical cation, both cytochrome c and cytochrome c oxidase are rapidly reduced. In oxidations by molecular oxygen, however, only reduced cytochrome c oxidase can be rapidly oxidized. Natured cytochrome c reacts with O_2 at a negligible rate. Thus, in these mixtures, cytochrome c must be oxidized by O_2 via cytochrome c oxidase. Differences in pathway of electron transfer has been previously suggested by Beinert (7) based on kinetic measurements by EPR.

In addition to the possible differences in the pathway for reductive or oxidative electron transfer to and from cytochrome c oxidase, there is the possibility that the interactions between cytochrome c and cytochrome c oxidase require a given redox state to be restored to the complex form found in mitochondria or electron transport particles. This is evidenced by the greater similarity between the A - Q curves and the Minnaert-type plots after the initial reductive titration. Thus, it can be assumed that the successive titrations after the initial one are closer to the required cytochrome c - cytochrome c oxidase equilibrium interaction than when these two entities are initially mixed or reduced. This complex is not the same as that described by Kuboyama et al (8) since the components formed here are separable by gel filtration.

Approximately 15% more titrant is required for the first reduction cycle as compared to subsequent reductions and oxidations. The exact reason has not been determined. The reproducibility of each A - Q curve from sample to sample suggests that there may be an extraneous, non-reversible redox component which is observed only on the first reductive titration.

No evidence for the formation of "oxygenated" cytochrome oxidase has been detected during oxidations by the electrogenerated O_2. Excess O_2 is

never present in these mixtures until the end of the titration at which point controlled amounts of O_2 up to the amount equivalent to total heme have been generated. There was still no evidence of oxygenated oxidase.

Preliminary indirect coulometric titrations of cytochrome c oxidase in the presence of ferricyanide/ferrocyanide indicates that the two heme a potentials are separated by approximately the same amount as in the presence of cytochrome c. Further studies are under way to accurately evaluate these potentials.

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