REVERSIBLE REDOX TITRATIONS OF CYTOCHROME c AND CYTOCHROME c OXIDASE USING DETERGENT SOLUBILIZED ELECTROCHEMICALLY GENERATED MEDIATOR-TITRANTS

Yoko Fujihira and Theodore Kuwana
Department of Chemistry
The Ohio State University
Columbus, Ohio 43210

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

Received October 4,1974

SUMMARY: The repetitive, reversible equilibrium redox cycling of cytochrome c, cytochrome c oxidase, or mixtures thereof has been made possible by the use of the oxidant, ferricinium ion. This ion is electrochemically generated by the use of non-ionic detergent solubilized ferrocene which is apparently incorporated as micelles and readily electron transfers with an electrode. The ferricinium-ferrocene couple equilibrates rapidly with these heme proteins. Electrochemically generated benzylviologen radical cations are used as the reductant. The $E^{O'}$ values for cytochrome c oxidase at pH 7.0 are 209 ± 15 mv ($2e^-$) and 340 ± 15 mv ($2e^-$).

INTRODUCTION: In the previously reported (1) indirect coulometric titrations of mixtures of cytochrome c (cyt. c) and cytochrome c oxidase (cyt. aa_3), electrochemically generated methyl viologen radical cation served as the reductant and molecular 0_2 as the oxidant.

The charge distributions between the heme components were evaluated from the change in optical absorbance (ΔA) at 550 nm (cyt. c) and 605 nm (cyt. aa_3). The plot of the oxidative ΔA-q (change in absorbance vs charge) was not a mirror image of the reductive one, particularly for the 605 nm band. An interesting question is, indeed, should these plots indicate electrochemical equilibria when the nature of the reductant, viologen radical (n = 1), is so different from the oxidant, 0_2 (n = 4, H^+ = 4)? Also,

attainment of equilibrium during oxidative titrations of oxidase has been slow (> 10 min.) when oxygen in the presence of small amounts of ferricyanide or only ferricyanide was employed (2). This kinetic inhibition was particularly noticeable during latter stages of oxidation. These results posed interesting questions since 0_2 readily oxidized the reduced form of cyt. $\underline{aa_3}$ in the absence of cyt. $\underline{c}$.

Repetitive, reversible equilibrium titrations of cyt. $\underline{c}$, cyt. $\underline{aa_3}$, and mixtures are possible through the use of the oxidant ferricinium ion, electrochemically generated from ferrocene solubilized by detergent (non-ionic, Tween 20). Apparently, the ferrocene micelles undergo heterogeneous electron transfer with the electrode and then diffuse into the bulk solution to transfer charge with cyt. $\underline{c}$ or cyt. $\underline{aa_3}$ (3).

EXPERIMENTAL: Samples of isolated purified horse heart cyt. $\underline{c}$ and beef heart cyt. $\underline{aa_3}$ were similar to those used previously (stored at T less than $5^{o}C$) (1,2,4). Ferrocene was initially solubilized in high concentration of Tween 20, then added to the heme protein-benzylviologen-phosphate buffer solution and diluted with doubly distilled water so that final concentrations were 0.5-0.8m$\underline{M}$ ferrocene, 0.5m M benzylviologen, 20$\mu\underline{M}$ cyt. $\underline{c}$ or cyt. $\underline{aa_3}$, 5% Tween 20 and 0.1$\underline{M}$ phosphate buffer at pH = 7.0. The cell design, optical and electrochemical instrumentation, and general procedure have been previously described (5). All solutions were made anaerobic by vacuum degassing ($0_2 < 5 \times 10^{-7}\underline{M}$). After each increment of charge, spectra were obtained using a Harrick Rapid Scanning Spectrophotometer on slow sweep range (10-30 sec. per spectrum, 100-200nm scan width) and the spectra were recorded on a Houston Instruments Model 2000 X-Y recorder. The absorbance and charge were calibrated and were accurate within 2 and 5%, respectively.

RESULTS AND DISCUSSION: The quantitative aspects of the electrochemical generation of benzylviologin radical cation and ferricinium ion were established by linearity of the plots of ΔA_{594} vs q, as performed previously for methyl viologen-oxygen (see Fig. 4, ref. 5). After correction for non-faradaic charge, the slopes gave an n value of unity for generation of both oxidant and reductant.

In titrations of cyt. c, the results were quite identical to those previously reported (5) with the exception that the ΔA_{550} - q plots (see Fig. 7, ref. 5) are essentially linear for most of the titration. This linearity results from the $E^{O'}$ of the ferrocene couple being more positive in value (3) than iron cyanide.

For cyt. aa_3, (no cyto c present) the ΔA_{605} - q plots were curved initially during the reductive titration compared to the linearity of the previous (4) methyl viologen-oxygen titrations because the high potential components of oxidase were in equilibrium with the ferrocene couple. During the latter part of the reductive or the initial part of the oxidative titrations, the ΔA - q plots were linear. Extrapolation of these linear portions to the charge axis gave an intercept value which corresponds to one half of the total equivalents of charge required for complete titration, assuming n=4 for oxidase (4). Thus, the distribution of charge was consistent with 2 electrons being involved in the high and low potential portions. From analysis of the curved portions, it was possible to calculate the difference in the $E^{O'}$ value of the high potential components relative to the ferrocene couple as 103 $\pm$ 5 mv.

Cyt. aa_3 had been previously titrated in the presence of cyt. c (1), ferricyanide and TMPD (6). Analysis of the reductive portions of the ΔA - q curves yielded values of $E^{O'}$ consistent with the assignment: $E^{O'}_{\text{heme } a_H}$ = 345 $\pm$ 15 mv and $E^{O'}_{\text{heme } a_L}$ = 215 $\pm$ 15 mv. The use of ferricinium ion as an oxidant removed the aforementioned hysteresis between the reductive and oxidative $(O_2)\Delta A$ - q curves, and the problems associated with ferricyanide.

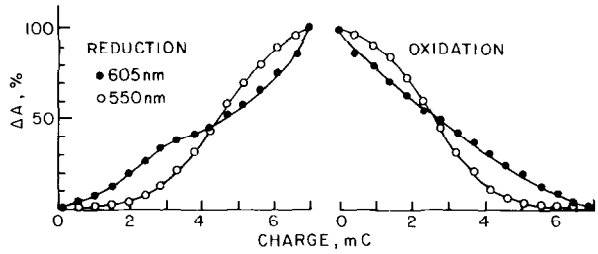

Figure 1. Plot of absorbance changes at 605 and 550 nm during reduction by electrochemically generated benzylviologen radical cation and during oxidation by ferricinium ion. Solution concentrations: cyt. c: 20μM, cyt. aa_3: 17μM, benzylviologen: 0.5mM, and ferrocene: 0.8mM

Fig. 1 shows the absorbance changes of the 605 and the 550nm bands as a function of amounts of charge (titrant) added.

About 20-40 seconds were required for each incremental addition of 0.5 mC of charge (corresponds to 1.04×10^{-6} eq). Equilibrium in the solution was attained within three minutes depending somewhat on the stirring rate. Assuming the validity of previous computer simulation of the Nernstian behavior of the optical components (6), the relative potentials of the two hemes with respect to cyt. c can be evaluated. These potential differences are summarized in Table 1. The $E^{O'}$ values of heme a_H and heme a_L are separated by ca. 130 mv in agreement with previous results.

In reductions by viologen radicals, both cyt. c and cyt. aa_3 were rapidly reduced. In O_2 oxidations, native cyt. c was oxidized slowly while the cyt. aa_3 was oxidized relatively rapidly (4). Therefore, in mixtures, the cyt. c must be oxidized by O_2 via the cyt. aa_3. However, ferricinium ion readily oxidizes both cyt. c and cyt. aa_3 so, equilibrium between redox components can be attained through homogeneous electron exchange involving the ferricinium-ferrocene. In the case of ferricyanide, there is considerable evidence that either ferricyanide/ferrocyanide or some dissociated cyanide kinetically

TABLE 1

Summary of $E^{O'}$ Values (mv)

Redox Component[a]	Present Results	Previous Results
cyt. c	254 ± 10	257 ± 17
cyt. aa_3 (heme a_L)	209 ± 15	215 ± 15
cyt. aa_3 (heme a_H)	340 ± 15	345 ± 15
Ferrocene[3]	437 ± 5	
Benzylviologen[12]		352 ± 8

a. phosphate buffer, pH 7.0.

inhibits electron transfer from oxidase when four equivalents per oxidase oxidations are attempted, even when molecular 0_2 is employed. These results suggest that the 0_2 transfer "site" is kinetically modified or blocked.

In the summary of potential differences given in Table 1, if the potential of $E^{O'}$ = 437 is assigned to the ferricinium/ferrocene couple (3), then it is possible to assign potentials to heme a_H = 340 mv; cyt. c = 254 mv; and heme a_L = 209 mv. These values are close to those found previously (1,6,7), (previous values listed also in Table 1). It should be emphasized that the separation of $E^{O'}$ values for heme a_H and heme a_L does not necessarily justify any conclusions about the similarities or differences between the hemes in the fully oxidized or fully reduced states of the oxidase (7).

The use of detergent to solubilize species able to undergo facile heterogeneous and homogeneous electron-transfer has interesting mechanistic implications to the field of biological electron transfer processes involving

redox centers with peripheral structures, such as lipid-protein interactions in membranes. Use of surfactants for formation of micellular structures incorporating reactants which are accelerated or inhibited in their reactions with enzymes have been considered and discussed previously (6,9). Perhaps more significantly, this solubilization approach, demonstrated previously by others (10,11) for electron transfer species, but not applied to bio-electron transport problems, will greatly enlarge the available number of redox reactants for mechanistic studies of a wide variety of bio-redox components.

Acknowledgement

The assistance of M. Fujihira and L. Mackey in these titrations is greatly appreciated. We thank P. Yeh for electrochemical data on solubilized ferrocene oxidation. The cyt. c and cyt. aa_3 samples were from the same preparation used previously (CRH). We gratefully acknowledge financial support from PHS-NIH Grant CM 19181 and National Science Foundation Grant No. GP-40622X. C.R.H. is an Established Investigator of the American Heart Association.

References

1. W.R. Heineman, T. Kuwana, and C.R. Hartzell, Biochem. Biophys. Res. Commun., 50, 892 (1973).
2. L.N. Mackey and T. Kuwana, unpublished results.
3. P. Yeh and T. Kuwana, unpublished results.
4. W.R. Heineman, T. Kuwana, and C.R. Hartzell, Biochem. Biophys. Res. Commun., 49, 1 (1972).
5. F.M. Hawkridge and T. Kuwana, Anal. Chem., 45, 1021 (1973).
6. L.N. Mackey, T. Kuwana, and C.R. Hartzell, FEBS Letters, 36, 326 (1973).
7. W.R. Heineman and T. Kuwana, 2nd International Symposium on Bioelectrochemistry, Pont a Mousson, France, Oct. 1973, J. Bioelectrochem. and Bioenergetics, Ed. G. Milazzo, in press.
8. M.B. Lowe and J.N. Phillips, Nature, 190, 262 (1961); ibid., 194, 1058 (1962)
9. F.M. Menger and C.E. Portnoy, J. Am. Chem. Soc., 89, 4698 (1967).
10. S. Hayano and N. Shinozuka, Bull. Chem. Soc. Japan, 42, 1469 (1969); 43, 2083 (1970); 44, 1503 (1971).
11. P.G. Westmoreland, R.A. Day, Jr., and A.L. Underwood, Anal. Chem., 44, 737 (1972).
12. E. Steckhan and T. Kuwana, Ber. Bunsenges. Physik. Chem., 78, 253 (1974).