INDIRECT ELECTROCHEMICAL TITRATION OF BEEF HEART CYTOCHROME c OXIDASE*

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SUMMARY. Titration of beef heart cytochrome c oxidase with electrochemically generated reductant, methyl viologen cation radical MV^{+}, and oxidant, molecular oxygen, has enabled cycling of the oxidase repeatedly from its totally oxidized to its totally reduced forms. Spectrocoulometric results clearly show that cytochrome oxidase accepts four electrons during both reduction with MV^{+} and oxidation with O_2. Oxidation with coulometrically generated O_2 produces oxidized oxidase, with no evidence of the "oxygenated" form.

Attempts are being made to determine the mode and sequence of electron transfer in isolated and fragmented components of mitochondria. Potentiometric titrations with several redox reagents have been recently used to obtain mid-point potentials and n values for these components (1,2). We are applying electrochemical titration methods using electrogenerated titrants such as viologens for reductions (3) and ferricyanide or oxygen for oxidations in our laboratory. Results obtained for the titration of cytochrome c oxidase indicate that the value of n is four electrons per minimal functional cytochrome c oxidase molecule (four electrons per two heme a units).

A representation of the coulometric titration of oxidase is shown in

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Figure 1. The oxidase in its oxidized form was first dissolved in a solution which contained ca. 0.6 mM of methyl viologen dication MV^{2+}, 0.1% Tween 20,

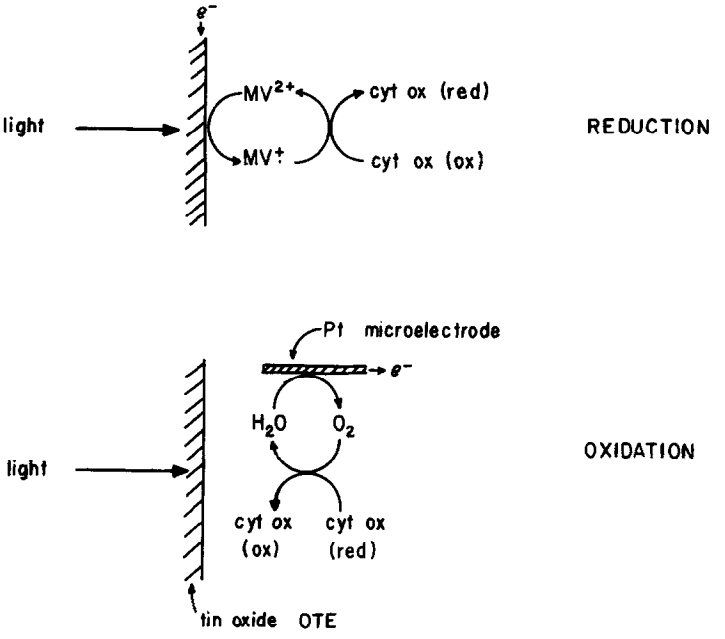

Figure 1. Pictorial representation of reduction and oxidation of cytochrome c oxidase by electrochemically generated MV^{+} and O_2.

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and 0.1M NaCl in 0.3M phosphate buffer at pH 7.0. The design of the electrochemical cell enabled spectral measurements to be made conveniently through the tin oxide OTE. The optical path length and volume of the lucite cell were 1.7 cm and 1.55 ml respectively. Vacuum degassing of solution in the cell enabled O_2 removal to ca. 10^{-7} M. Reduction of the oxidase was accomplished by stepping the potential of the tin oxide optically transparent electrode (OTE) to -0.90 V vs Ag/AgCl reference so that the MV^{2+} at the electrode surface was reduced to MV^{+}. This MV^{+} then reduced the oxidized oxidase by a homogeneous chemical reaction in solution, and MV^{2+} was thereby regenerated. The solution

was stirred during generation to ensure good mixing of MV^{+} and oxidase and to maintain MV^{2+} at the electrode surface. Reduction of oxidase directly at the electrode surface was sufficiently slow to be considered negligible. Thus, the oxidase was coulometrically pumped from its oxidized to reduced forms entirely by reaction with MV^{+} ($E^{o'} = -0.446 \pm 0.005$ V vs. NHE (3)), while maintaining essentially invarient solution conditions. After the oxidase was entirely in the reduced form, it was reoxidized by titration with molecular oxygen which was generated at +1.5 V by the oxidation of water at a platinum microelectrode.

Typical spectral results for the cytochrome oxidase during a reductive and an oxidative titration are shown in Figure 2. Spectra obtained for the reduced and oxidized forms of the oxidase agreed with those reported by other workers (4). In the oxidized form the wavelengths for maximum absorbance for the Soret-band and the α-band were 422 nm and 597 nm respectively; in the reduced form the wavelengths were 445 nm and 605 nm respectively. We obtained a ratio of 1.28 for red/ox of the $Soret_{maximum}$ compared with the reported value of 1.25 (4).

We have no evidence that titration of reduced oxidase with the electrochemically generated oxygen leads to an "oxygenated" form. Spectral bands for such an "oxygenated" form have been reported to be $Soret_{max}$ 426 - 428 nm and α_{max} 603 nm. (4,5) However, $Soret_{max}$ and α_{max} remained at 422 nm and 597 nm for the oxidized form in these experiments even after several cycles of reduction-oxidation and in cases where an excess of O_2 was purposely generated.

In order to determine the number of electrons involved per heme a unit during reduction and oxidation of the oxidase, the change in absorbance at a particular wavelength was plotted as a function of titrant added in terms of charge. Figure 3 shows a plot at 605 nm as obtained from spectra such as those in Figure 2. The cytochrome oxidase, which existed initially in the oxidized

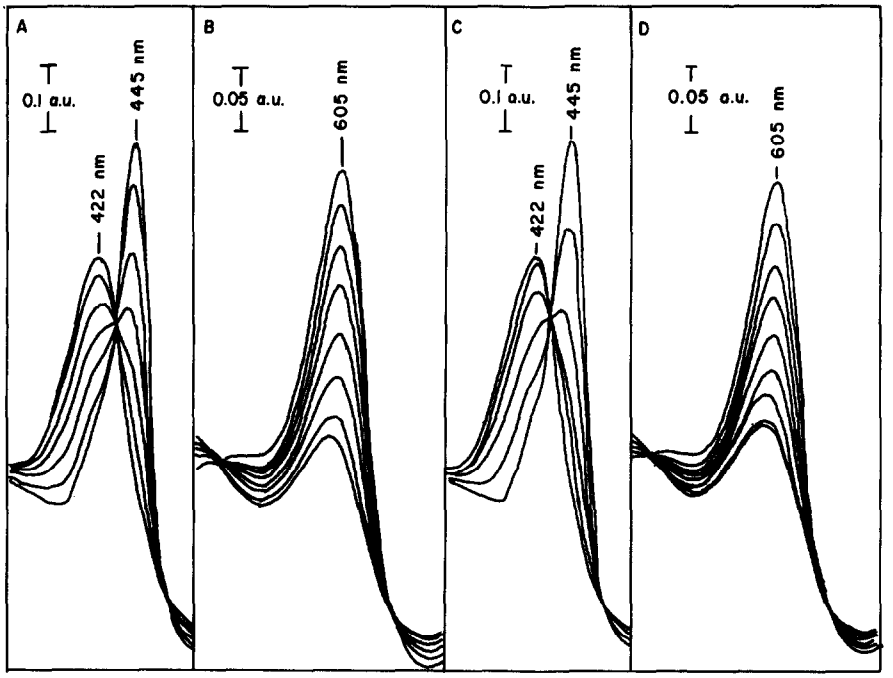

Figure 2. Spectra of cytochrome oxidase during titrations involving incremental addition of titrant. Solutions contained cytochrome c oxidase, 0.6 mM MV^{2+}, 0.1 M NaCl and 0.1% Tween 20 in 0.3 M phosphate buffer at pH 7.0. Cytochrome c oxidase was prepared from beef heart mitochondria according to C.R. Hartzell (manuscript in preparation). The heme a and copper content were 14 μmoles/g protein and 15 μmoles/g protein respectively. A. Reductive titration of 1.6 μM cytochrome oxidase by MV^{+}, MV^{+} generated in 0.25 mC increments. B. Reductive titration of 5.5 μM oxidase by MV^{+}, 0.5 mC increments. C. Oxidative titration of 1.6 μM oxidase by O_2, 0.25 mC increments. D. Oxidative titration of 5.5 μM oxidase by O_2, 0.50 mC increments.

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form, was first converted to the reduced form by titration with MV^{+}. The reduced form was then titrated with O_2 to convert it back to the oxidized state. The behavior of the 605 nm band during this sequence is shown in Figure 3. This titration could easily be repeated several times on the same solution.

The slope of the A - Q line is proportional to the number of electrons involved according to equation (1)

$$\text{slope} = \frac{\Delta A}{\Delta Q} = \frac{\Delta \epsilon \, b\underline{C}}{n \, 9.65 \times 10^{7} \, V\underline{C}} \qquad (1)$$

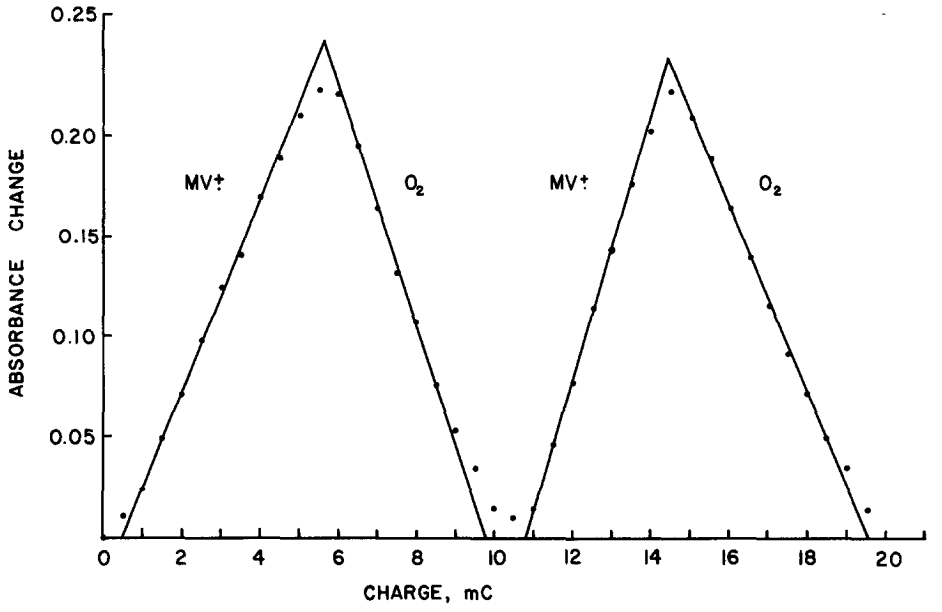

Figure 3. Typical plots of change in absorbance of cytochrome oxidase at 605 nm as a function of titrant added in mC. Cytochrome oxidase concentration 5.5 μM.

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where ΔA is the change in absorbance, $\Delta \epsilon$ is a difference in molar absorptivity, (reduced-oxidized), b is the optical path length in cm of the electrochemical cell, C is the concentration in moles/liter of species being titrated, Q is the amount of titrant added in terms of charge in millicoulombs (mC), n is the number of electrons added per molecule, 9.65×10^7 mC/equivalent is Faraday's number and V is the cell volume in liters. It is unnecessary to know the concentration of oxidase since the two concentration terms cancel. Since 12×10^3 l/mol/cm (6) was used as the $\Delta \epsilon$ for the 605 nm band of heme a, the value of n is also in terms of heme a.

The calculated slopes for the increase of the 605 nm oxidase band during reduction as a function of titrant added are 0.136 a.u./mC for $n = 1$ and 0.068 a.u./mC for $n = 2$ where n is the number of electrons added per

unit of heme a. These values are valid only for the dimensions of the particular cell which was used and are based on assumptions of 100% current efficiency for $MV^{+\cdot}$ generation and 100% efficiency of reaction of the $MV^{+\cdot}$ with oxidized oxidase. It was determined that approximately 10% of the current during each increment of $MV^{+\cdot}$ generation went to extraneous processes such as charging the double layer of the electrode. Application of this correction to the above values gave slopes of 0.122 a.u./mC and 0.061 a.u./mC for $n = 1$ and $n = 2$ respectively. These corrected slopes can now be compared with the experimental values.

In order to calculate the expected slopes for the decrease of the 605 nm oxidase band during oxidative titration with O_2, a measure of the efficiency of the electrochemical generation of O_2 was necessary. This was accomplished by generating $MV^{+\cdot}$ in the absence of cytochrome oxidase and then titrating the accumulated $MV^{+\cdot}$ with O_2. The ratio of the slope for $MV^{+\cdot}$ generation to the slope for disappearance of $MV^{+\cdot}$ during O_2 titration was found to be 1.1. Consequently, the expected slopes for the decrease in the 605 nm oxidase band during oxidative titration with O_2 are -0.111 ($n=1$) and -0.055 ($n=2$).

Table 1 shows the slopes which were obtained for several experimental determinations. The average slope of the first titration with $MV^{+\cdot}$ was lower than subsequent reductions on the same solution, probably due to the scavenging of some residual oxygen in the solution. The average slope for the second reductive titration was 0.063 ± 0.006 which corresponds to $n = 1.94 \pm 0.18$ per heme a or $n = 4$ per molecule of cytochrome c oxidase. The average slope for all of the titrations with oxygen was -0.053 ± 0.004 which corresponds to $n = 2.08 \pm 0.16$ for heme a, also $n = 4$ per molecule of oxidase.

Although during most titrations the absorbance change at 605 nm was

Table 1. Slopes of absorbance-charge plots at 605 nm for the titration of cytochrome c oxidase.

	Slope[a]			
Experiment No.[b]	MV^{+}	O_2	MV^{+}	O_2
1	0.053	-0.052	0.052	-0.056
2	0.071	------	0.071	-0.043
3	0.071	-0.052	0.075	-0.057
4	0.048	-0.059	0.054	-0.062
5	0.049	-0.053	0.065	-0.044
6	-----	------	0.060	------
7	-----	------	0.063	-0.052
average slope	0.058		0.063	-0.053[c]
average deviation	±0.010		±0.006	±0.004

[a]slope expressed in a.u./mC where mC is amount of titrant added in terms of charge (1.00 mC = 1.04×10^{-8} eq.).

[b]For each experiment the titration sequence is reduction by MV^{+}, oxidation by O_2. Sequence is repeated once. Each experiment represents a fresh solution.

[c]Average is for all O_2 titrations.

monitored, the same results were obtained at 445 nm using a $\Delta\epsilon$ (reduced-oxidized) of 82×10^3 l/mole/cm (6). The actual slope recorded at 445 nm corresponded to this increased $\Delta\epsilon$ compared to that at 605 nm. The ratio of 445 nm slope/605 nm slope was 7.0 which compares with the reported ΔSoret/$\Delta\alpha$ of 7 (4).

The coulometric generation of reductant or oxidant is a facile means of repetitively pumping an enzyme through its oxidation states. The charge consumed by the enzyme can be accurately measured electronically and spectra recorded simultaneously through the transparent generating electrode. The results reported here substantiate earlier reports (6) that cytochrome c oxidase consumes 4 electrons per molecule during conversion from the totally oxidized to the reduced form. We have also demonstrated that the subsequent oxidation of this reduced form with coulometrically generated oxygen also involves 4 electrons per molecule. There is no evidence that the so-called "oxygenated"

form (4) is produced during this electrochemical oxidation. Possibly, this is because oxygen is generated at a slow rate and rapidly consumed during the titration. The 4 electron change is consistent with the evidence for four one-electron redox species in each molecule of cytochrome oxidase -- two units of heme a and two units of copper.

This coulometric method appears to be a valuable tool for the study of electron transport in redox enzyme systems because of the ease and precision with which reductant and oxidant can be incrementally added under strictly anaerobic conditions.

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