THE OXIDATION-REDUCTION POTENTIAL OF COPPER IN CYTOCHROME OXIDASE

David C. Wharton and Michael A. Cusanovich
Section of
Biochemistry and Molecular Biology
Cornell University
Ithaca, New York 14850

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SUMMARY

The oxidation-reduction potential of copper in cytochrome oxidase from beef heart has been determined by titrations with ascorbate + cytochrome c and with sodium dithionite. The mid-point potentials using these two methods are 284 mvolts and 274 mvolts, respectively. Thermodynamically, the copper is located between cytochrome a and cytochrome a_3.

The position of copper in the reaction sequence of cytochrome oxidase has been determined by means of rapid reaction techniques to lie between cytochrome a and cytochrome a_3 (1,2). Thus, an electron from cytochrome c would be transferred sequentially to cytochrome a to copper to cytochrome a_3, and finally to oxygen. Recently, we have conducted experiments to measure the oxidation-reduction potential of copper in cytochrome oxidase in order to determine thermodynamically its position in the electron transport chain. The results of these experiments are reported here.

EXPERIMENTAL PROCEDURE

Cytochrome oxidase was prepared from beef heart according to the procedure of Yonetani (3). This preparation was chosen because it did not become turbid during the period of the oxidation-reduction titrations. Its concentration was determined spectrophotometrically using the extinction coefficient $\Delta\varepsilon$(605 mμ - 630 mμ reduced) = 16.5 $mM^{-1}cm^{-1}$ (4). Cytochrome c was horse heart (Type III), from Sigma Chemical Co., and was further purified by the method of Margoliash and Lustgarten (5). Ascorbic acid was purchased from Merck and Co.; N, N, N', N',-tetramethyl-p-phenylenediamine from Eastman; disodium 2,6-

dibromobenzenoneindo-3'-carboxyphenol and sodium benzenoneindophenol from Matheson, Coleman, and Bell, Inc.; $K_3Fe(CN)_6$, KH_2PO_4, and K_2HPO_4 from Mallinckrodt Chemical Works. Sodium dithionite was a product of Hardman and Holdman, Miles Platting, Manchester, England.

Titrations were carried out in two ways - 1) by the addition of aliquots of potassium ascorbate and 2) by direct potentiometric titrations with sodium dithionite and oxidation-reduction buffers. In the former method the potassium ascorbate solution (degassed) was added by means of a Hamilton microliter syringe through a rubber serum cap to an anaerobic solution of cytochrome oxidase, cytochrome c (approximately equimolar to total heme a), and potassium phosphate (0.1 M, pH7.4) contained in an anaerobic cuvette. The titrants were permitted to reach equilibrium after each addition of ascorbate and the difference spectrum (reduced minus oxidized) from 900 mμ to 530 mμ was recorded in a Beckman model DK-2A recording spectrophotometer equipped with a thermo-jacketed cell compartment maintained at 20°C. A lead sulfide detector was used for the range 900-650 mμ and a 1P28 photomultiplier for the range 650-530 mμ. The proportion of copper reduced was based on the appearance of a trough near 830 mμ which has been attributed to copper by several authors (6-8). The proportions of cytochrome c and cytochromes a + a_3 reduced were determined by the appearance of maxima at 550 mμ and 605 mμ, respectively. All proportions were based on values obtained after total reduction by solid dithionite. Corrections were applied for the contribution of cytochrome c to the absorbance at 605 mμ and for the contribution of the a cytochromes at 550 mμ.

The second method, which permits titrations over a redox range of 500 to 180 mvolts, was carried out in a buffer consisting of 0.1 M potassium phosphate, pH 7.4, 5×10^{-3}M $K_3Fe(CN)_6$, 1×10^{-7}M N, N, N', N'-tetramethyl-p-phenylenediamine, 1×10^{-6}M disodium 2,6-dibromobenzenoneindo-3'-carboxyphenol, and 1×10^{-6}M sodium benzenoneindophenol. Cytochrome oxidase was present at a concentration of 100 μM. The mixture was deaerated by bubbling with nitrogen

that has been deoxygenated by passing through heated copper turnings. The oxidase was then reduced by the stepwise addition of sodium dithionite (1% w/v). The potential was determined directly with a platinum-AgCl combination electrode (IL-15020). The apparatus for these titrations has been described previously (9). Spectrophotometric techniques and calculations were the same as those described for the first method except that corrections for the contributions of the cytochromes to absorbance were not required. The dyes at the concentrations used in the system do not absorb light significantly in the regions of interest. Reverse titrations were performed in the case of both methods by the addition of aliquots of a degassed solution of 0.01 M $K_3Fe(CN)_6$ to the reduced complex.

RESULTS AND DISCUSSION

Titration curves for the reduction of the near-infrared band of cytochrome oxidase by dithionite and by ascorbate + cytochrome c are shown in Figs. 1A and 1B. respectively. The mid-point potential for the former titration has been calculated to be 274 mvolts with a standard deviation of 4 mvolts for

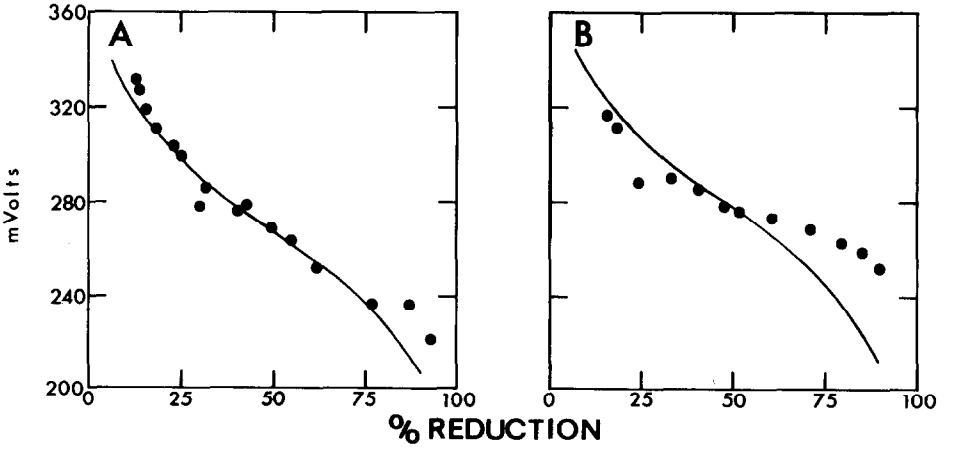

Fig. 1. Potentiometric titration of cytochrome oxidase at 830 mμ. A: titration carried out anaerobically by the addition of sodium dithionite as described in the text. B: titration carried out anaerobically in the presence of cytochrome c by the addition of ascorbate as described in the text. The solid line represents the theoretical curve for a 1-electron transfer. The dots represent experimental values for a single titration.

seven experiments. That calculated for the latter is 284 mvolts with a standard deviation of 8 mvolts for fourteen experiments. Reverse titrations of the reduced complex with $K_3Fe(CN)_6$ give similar results.

It should be noted in Figs. 1A and 1B that, while the titration curve of the oxidase reduced by dithionite closely fits a theoretical curve for a 1-electron reduction, the titration curve obtained from reduction of the enzyme with ascorbate + cytochrome c shows some deviation from that ideal. In fact, if these data are treated after the manner of Minnaert (10), by plotting the log of ferrocytochrome c/ferricytochrome c as the ordinate versus the log of cuprous/cupric as the abscissa, the resulting slope is equal to about 1.4. The reason for this deviation is not clear although Minnaert (10) observed deviations from unity in his titrations of the α-band and suggested the presence of additional oxidation-reduction groups in the enzyme as one possible explanation. However, the difference between titrations made with dithionite and with ascorbate + cytochrome c and with their respective reverse titrations using $K_3Fe(CN)_6$ is puzzling. Perhaps an interaction of cytochrome oxidase with cytochrome c results in an alteration in the oxidation-reduction behavior of Cu in the enzyme.

The mid-point potential for the α-band of cytochrome oxidase at 605 mμ has been calculated to be 292 ± 1 mvolts with ascorbate + cytochrome c as the reductant and 284 ± 3 mvolts with dithionite. These potentials, as well as the slopes of the curves, agree quite favorably with those previously reported (10-12). It must be remembered, however, that the α-band is composed of two cytochromes, a and a_3 (13,14). The former has a mid-point potential more negative than 250 mvolts (11,12) while the mid-point potential of the latter is more positive than 300 mvolts (12,15).

These results are consistent with the conclusion of Gibson and his associates (1,2) who, by rapid reaction studies, found that the following sequence of reactions occurs in cytochrome oxidase:

$$\text{cytochrome } a \longrightarrow \text{copper} \longrightarrow \text{cytochrome } a_3 \longrightarrow O_2.$$

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