# Redox-linked conformational changes in cytochrome *c* oxidase

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**Abstract The CD spectrum of oxidized cytochrome oxidase in the wavelength region 185–260 nm shows that the secondary structure of the protein consists of close to 60% α-helix and slightly less than 20% β-structure. CD spectra of oxidized cytochrome oxidase, of half and fully reduced carboxycytochrome oxidase as well as of fully reduced cytochrome oxidase, have been recorded in the wavelength region 200–260 nm. The results demonstrate a conformational change on going from the oxidized to the half reduced state in carboxycytochrome oxidase; no further change occurs on full reduction. A conformational change is also seen in the fully reduced enzyme without bound CO. The conformational transitions are suggested to be part of the proton pumping mechanism of cytochrome oxidase.**

*Key words:* Cytochrome oxidase; Proton pump; Protein conformation; Circular dichroism

## 1. Introduction

Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain in mitochondria and some bacteria [1,2]. It is a redox-linked proton pump, catalyzing a vectorial process in which electron transfer from cytochrome *c* to dioxygen is coupled to proton translocation across energy-transducing membranes The electrochemical gradient thus created is used to drive the synthesis of ATP according to the chemosmotic mechanism of Mitchell [3].

Cytochrome oxidase contains four redox centers bound in two different subunits. Binuclear $Cu_A$, bound in subunit II, is the primary acceptor of electrons from cytochrome *c* [4]. It is probably not involved in proton pumping, since the structurally homologous quinol oxidase lacks this component but still pumps protons (see [5] for a review). The remaining three centers, cytochrome *a* and the binuclear cytochrome $a_3$-$Cu_B$ unit, are bound in subunit I. The protons involved in the reduction of dioxygen to water as well as the pumped protons are taken up from one specific side of the membrane. Electrons are transferred from cytochrome *a* to the cytochrome $a_3$-$Cu_B$ center, which is the dioxygen-reducing site.

Redox-linked proton pumps must exist in at least two conformations, as illustrated in the reaction cycle in Fig. 1. These conformations provide an alternating access of the proton-translocating group to the two sides of the membrane. The coupling between electron transfer and proton translocation is achieved, because the electron donor can only react in the $E_1$ conformation, whereas the electron acceptor reacts in the $E_2$ state. Thus, the exergonic electron transfer from donor to acceptor can only take place if the transducer undergoes a conformational transition.

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Redox-induced conformational changes in cytochrome oxidase have so far only been deduced by indirect methods, for example, changes in the reactivity with cyanide [6,7]. Here we show by circular dichroism (CD) that reduction of the oxygen-reducing center definitely induces a change in protein conformation.

## 2. Materials and methods

### 2.1. Protein and chemicals

Bovine cytochrome oxidase was prepared by a method yielding enzyme that reacts rapidly and homogeneously with cyanide [8]. All measurements were made at 25°C in potassium phosphate buffers (0.5 mM or, in the experiment illustrated in Fig. 3A, 5 mM), pH 7.5, containing 0.05% lauryl maltoside. All chemicals were of analytical grade.

### 2.2. Sample preparations

Mixed-valence cytochrome oxidase, in which CO is bound to the reduced cytochrome $a_3$-$Cu_B$ center, whereas cytochrome *a* and $Cu_A$ are oxidized, was prepared in an anaerobic 1-cm cuvette connected to a vacuum line. The cuvette with the enzyme solution (3 ml, 0.1 μM) was first evacuated and then filled with CO; this procedure was repeated 3–4 times. The cuvette was also provided with a rubber septum port, and the fully reduced enzyme with bound CO was made by the addition of 5 μl of an anaerobic dithionite solution (approx. 10 mM) to the mixed-valence enzyme in the cuvette with a Hamilton syringe.

Samples in the absence of CO were made with 1 ml of 0.15 μM cytochrome oxidase in an anaerobic 2-mm cuvette, which was evacuated and filled with $N_2$ 4–5 times. In this case, the partially and fully reduced enzyme was made by the addition of 15 μl and 35 μl, respectively, of an anaerobic dithionite solution (approx. 2 mM).

### 2.3. Spectral measurements

CD measurements were made in a Jasco J720 spectropolarimeter. The spectrum of the oxidized oxidase (0.2 μM) was measured in a 1-mm cuvette. In the anaerobic 1-cm cuvette, measurements could not be made on the fully reduced enzyme without CO, because of the strong absorption by dithionite in the spectral region used. To minimize the absorption, the enzyme concentration was low. This had the effect that the reduction by CO was very slow, so that the complete formation of the mixed-valence enzyme required $>6$ h. The slow reaction had the distinct advantage that the fully oxidized enzyme could be measured directly after the last addition of CO to the anaerobic cuvette. Thus, all measurements could be made on the same solution, so that no corrections for changes in concentration had to be made. In the 2-mm cell, both oxidized and fully reduced enzyme could be measured in the absence of CO.

Optical spectra were recorded in a Cary 4 UV-visible spectrophotometer to monitor the formation of the mixed-valence and fully reduced enzyme; this could only be done in the 1-cm cuvette.

The secondary structure of the oxidized enzyme was estimated from the far-UV CD spectrum (185–260 nm), as described earlier [9].

## 3. Results and discussion

The far-UV CD spectrum of oxidized cytochrome oxidase is shown in Fig. 2. This agrees with spectra recorded earlier [10–12] and is dominated by the presence of close to 60% α-helix and about 20% β-structure (cf. [12]). This structure prediction


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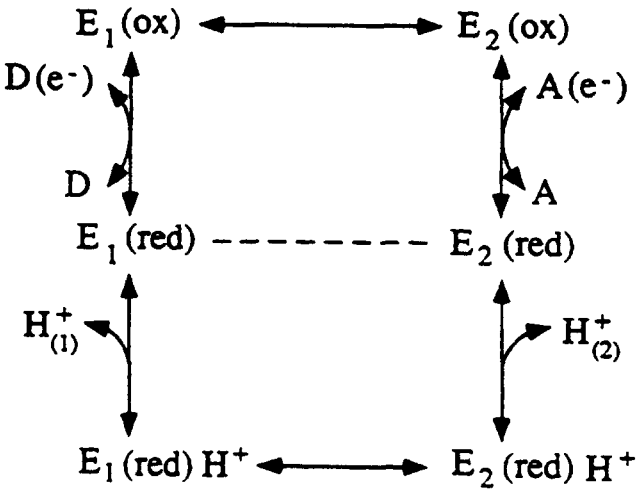


Fig. 1. Reaction scheme for a redox-linked proton pump. The dashed line represents a forbidden transition. For further explanations, see the text.

is confirmed by the recently determined crystal structure of bovine cytochrome oxidase [13].

The CD spectra in the region 200–260 nm of the oxidized, mixed-valence and fully reduced cytochrome oxidase in the presence of CO are shown in Fig. 3A; the spectra could not be recorded below 200 nm because of the strong absorption from dithionite. Even if a long time was required to form the mixed-valence enzyme at the low enzyme concentration used, the small change observed cannot be due solely to instrument instability. This could be demonstrated by generating the difference spectrum (not shown). In the case of baseline drift, the two spectra should be congruent, so that the difference should look like the original spectrum. Instead, it has a larger negative dichroism at 210 nm than at 225 nm, whereas the opposite is true for the oxidized enzyme. The fact that the two spectra are not congruent was also confirmed by plotting them against each other, which in the congruent case should give a straight line. In this plot some deviation from a straight line was, however, observed at about −40 mdegree. The spectrum of the fully reduced enzyme (Fig. 3A) was recorded only a few minutes after that of the mixed-valence enzyme, which excludes instrument drift, so it is clear that no further change occurs on going from the half reduced to the fully reduced state. The formation of the mixed-valence and fully reduced oxidase was demonstrated by the optical spectra (not shown).

The spectra of the oxidized and fully reduced oxidase in the absence of CO are given in Fig. 3B. In this case a higher dithionite concentration had to be used, as the anaerobic technique does not remove all the oxygen, whereas reduction by CO only occurs when all oxygen is consumed; this is the reason why the process takes >6 h at the low enzyme concentration employed. Because of the strong absorption from dithionite, a 2-mm cell had to be used for the experiment in the absence of CO. The observed change in the CD spectrum on reduction is larger than in the presence of CO, and, for reasons given earlier, this difference could only to a very limited extent be due to instrument instability during the long time of the CO experiment. Thus, the binding of CO to the reduced cytochrome $a_3$-$Cu_B$ site stabilizes a conformation closer to that of the oxidized enzyme.

A change in the CD spectrum on reduction of the oxidase had earlier been reported by Myer [10]. His observation does not agree with our results, however, since he found an increase in the negative dichroism, whereas we saw a decrease (Fig. 3A,B). The discrepancy is probably due to the fact that Myer did not use an anaerobic cell, since we found that a high dithionite concentration shifts the baseline towards a negative value.

The experiments described here provide this first direct evidence for a redox-linked conformational changes in cytochrome oxidase. They furthermore demonstrate that one such change occurs on a two-electron reduction of the enzyme, since no change is seen on going from the mixed-valence to the fully reduced state (Fig. 3A). This agrees with the earlier finding [14] that two-electron reduction triggers the transition from the closed to the open conformation [6,7]. A change in conformation on two-electron reduction is also evidenced by the fact that internal electron transfer to the cytochrome $a_3$-$Cu_B$ site does not occur in the singly reduced oxidase [15,16].

To some extent it is surprising that such a large changes in the CD spectra are observed, since protein conformational transitions often do not involve changes in secondary structure. This suggests that the interaction between cytochrome *a* and cytochrome $a_3$ is mediated by transmembrane helices in subunit I, as proposed earlier [17]. In this context, it should be noted that the two cytochromes are bound on opposite sides of the same helix [13].

It is tempting to assume that the conformational changes observed here correspond to the transition from the $E_1$ to the $E_2$ state in the scheme of Fig. 1. Wikström [18] has, however, reported that the initial two-electron reduction of the oxygen-reducing site is not coupled to proton pumping, which only occurs in the subsequent steps in the presence of oxygen. It is, on the other hand, possible to formulate a reaction cycle in which the enzyme switches from the input to the output state

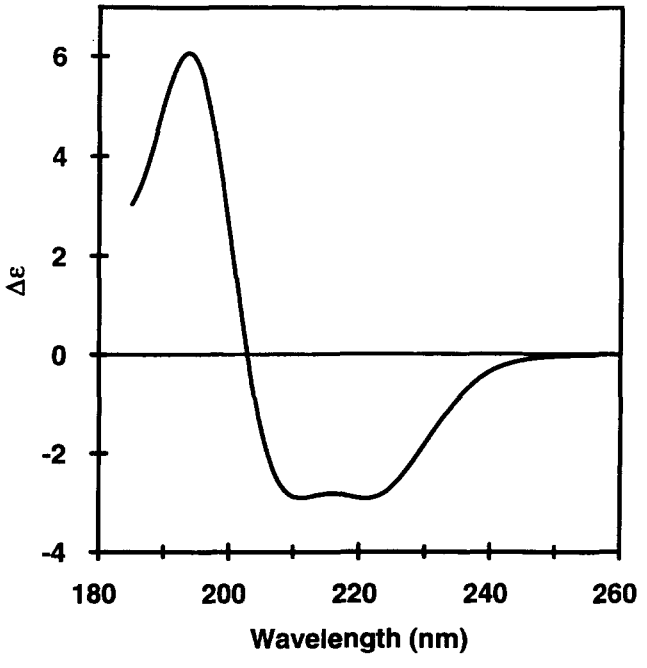


Fig. 2. The CD spectrum of oxidized cytochrome oxidase. The CD data are here expressed as differential molar absorption coefficients ($\Delta\varepsilon = \varepsilon_l - \varepsilon_r$) per amino acid residue.

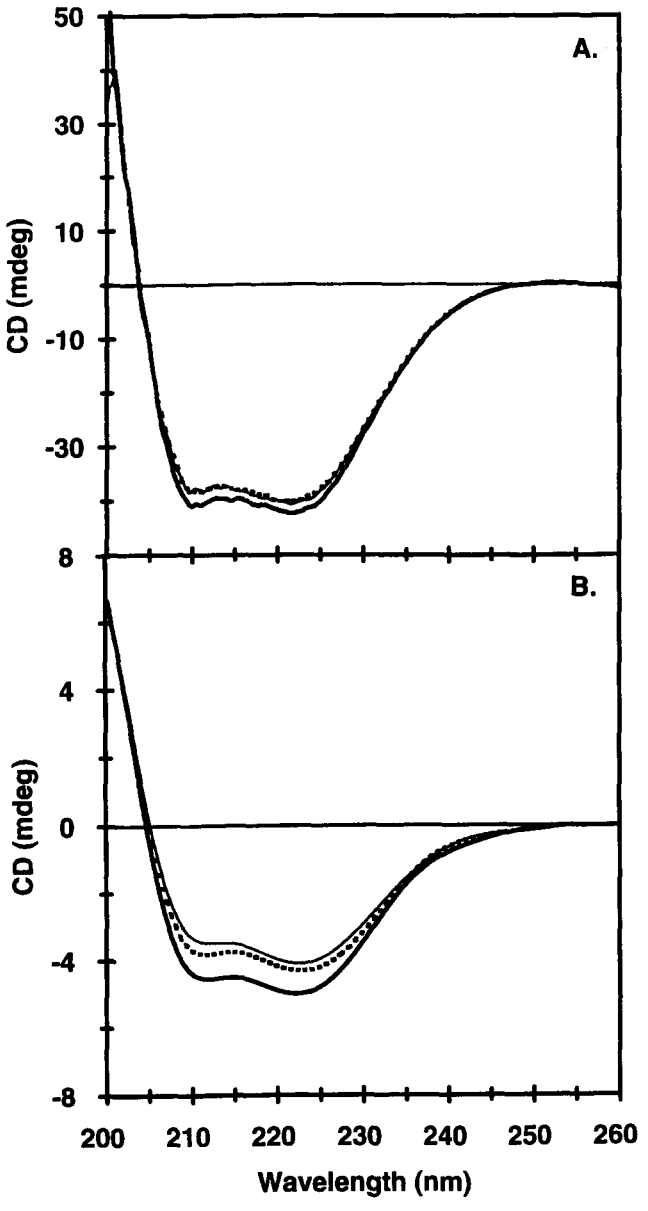


Fig. 3. CD spectra of oxidized, half reduced and fully reduced cytochrome oxidase in the presence and absence of CO. A: With CO present; the bottom spectrum is of the oxidized enzyme and the dotted spectrum of the fully reduced protein. B: In the absence of CO; the heavy line is the spectrum of the oxidized protein and the other two spectra of a partially and fully reduced protein.

also in the initial two-electron transfer [19], and the results reported here support such a mechanism.

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## References

[1] Malmström, B.G. (1990) Chem. Rev. 90, 1247–1260.
[2] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–309.
[3] Mitchell, P. (1961) Nature 191, 144–148.
[4] Brzezinski, P., Sundahl, M., Ädelroth, P., Wilson, M.T., El-Agez, B., Wittung, P. and Malmström, B.G. (1995) Biophys. Chem. 54, 191–197.
[5] Hosler, J.P., Ferguson-Miller, S., Calhoun, M.W., Thomas, J.W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M.M.J., Babcock, G.T. and Gennis, R.B. (1993) J. Bioenerg. Biomembr. 25, 121–136.
[6] Jones, M.G., Bickar, D., Wilson, M.T., Brunori, M., Colosimo, A. and Sarti, P. (1984) Biochem. J. 220, 57–66.
[7] Jensen, P., Wilson, M.T., Aasa, R. and Malmström, B.G. (1984) Biochem. J. 224, 829–837.
[8] Brandt, U., Schägger, H. and von Jagow, G. (1989) Eur. J. Biochem. 182, 705–711.
[9] Wittung, P., Källebring, B. and Malmström, B.G. (1994) FEBS Lett. 349, 286–288.
[10] Myer, Y.P. (1971) J. Biol. Chem. 246, 1241–1248.
[11] Bazzi, M.D. and Woody, R.W. (1985) Biophys. J. 48, 957–966.
[12] Park, K., Perczel, A. and Fasman, G.D. (1992) Protein Sci. 1, 1032–1049.
[13] Tsukihara, T., Aoyama, H., Yamshita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Ito, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) Science (in press).
[14] Scholes, C.P. and Malmström, B.G. (1986) FEBS Lett. 198, 125–129.
[15] Antonini, E., Brunori, M., Greenwood, C. and Malmström, B.G. (1970) Nature 228, 936–937.
[16] Brzezinski, P., Thörnström, P.-E. and Malmström, B.G. (1986) FEBS Lett. 194, 1–5.
[17] Malmström, B.G. (1989) FEBS Lett. 250, 9–21.
[18] Wikström, M. (1989) Nature 338, 776–778.
[19] Malmström, B.G. (1993) Acc. Chem. Res. 26, 332–338.