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Conformational changes in cytochrome oxidase as studied by sedimentation difference*

FERNANDO CABRAL and BRUCE LOVE

Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. (U.S.A.)

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SUMMARY

Sedimentation difference measurements show that a conformational change occurs in cytochrome oxidase upon reduction. The nature of the change in the sedimentation coefficient indicates an expansion of the molecule when the oxidized enzyme is reduced. A much smaller conformational change is detected when the $a^2a_3^2$CO and $a^2a_3^2$ or $a^3a_3^3$ and $a^3a_3^2$CO species are compared, indicating a relationship between conformation and the oxidation state of heme *a*.

At the present time considerable attention is being given to the conformation of cytochrome oxidase, and to the relationship between conformation and oxidation state or ligand binding. Generally speaking, the experimental approaches to this problem have fallen into two categories: analysis of spectroscopic differences observed in absorption and ORD–CD spectra, and changes in chemical properties or reactivities associated with the redox state of the enzyme. Extensive ORD–CD spectroscopic investigations by King and co-workers[1-3] have demonstrated that the optical rotatory properties of the enzyme, in both the Soret and alpha regions of the spectrum, are oxidation state dependent. King *et al.*[4] have discussed in some detail the origins of the strong rotatory power of oxidase, and have proposed that the spectroscopic differences observed between ferric and ferrous oxidase arise from an altered geometrical relationship between the heme and the protein backbone. Recently, Yamamoto and Okunuki[5] have shown that the susceptibility of cytochrome oxidase to proteolysis is greater in the ferrous than in the ferric oxidation state, suggesting a conformational change on reduction.

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In this communication we present what we believe to be the first hydrodynamic evidence for conformational changes in cytochrome oxidase. The data have been obtained from sedimentation difference measurements which determine directly the magnitude and direction of gross conformational changes in the molecule. Furthermore, interpretation of the data does not require any assumptions involving the spectroscopic properties of cytochrome *a* and a_3.

Cytochrome oxidase was prepared from beef heart mitochondria by the Yonetani[6] method and the final dialysis was against 0.05 M potassium phosphate buffer (pH 7.4) containing 0.5% Tween 80. The purified oxidase obtained in this way contained 8.5 nmoles of heme per mg of protein and had an activity of 135 electrons transferred per second per heme.

A Spinco Model E analytical ultracentrifuge and Schlieren optics was used for all experiments. To obtain the maximum precision possible with the Schlieren system, the difference sedimentation methodology described by Schumaker and Adams[7] was followed. Two cells, each containing one of the two solutions to be compared, were run in an An-D rotor with RTIC unit set to regulate at 20 °C, and thus in any one run fluctuations in temperature and rotor speed affect both samples equally. When identical samples were placed in the two cells, the sedimentation differences measured were always less than 0.03 S, and therefore differences greater than this value were taken to be significant. All sedimentation coefficients were corrected to $s_{20,\,w}$ values using relative viscosities measured with a Cannon–Ubbelohde viscometer. The rotor speed was obtained by timing the odometer during the entire run, and calculating the average speed which was usually within 100 rev./min of 51 200 rev./min. Ultracentrifuge plates were read on a Nikon two-dimensional microcomparator (Nippon Kogaku, K.K., Tokyo, Japan). Boundary positions were located by visually splitting the Schlieren peak with the vertical crosshair of the microcomparator. The average of three such readings was taken as the boundary position.

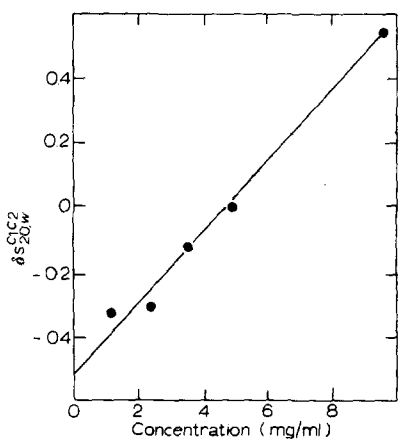
Data were processed on an IBM 360/44 computer, which calculated the sedimentation velocity from linear least squares slope and also computed the difference S from the slope of a direct difference plot for the paired cells as described by Schumaker and Adams[7].

Dilutions of the enzyme stock were made with the potassium phosphate buffer used for dialysis, and the samples were then made 0.2 M in KCl. Aliquots of 0.6 ml were degassed with N_2 and syringed into centrifuge cells which had been purged with N_2. 0.2 mg of sodium dithionite, used as the reductant, was placed in the centerpiece of the cell before purging. In all cases viscosities were exactly matched in the two cells being compared by the addition of sodium thiosulfate to balance the reducing agent. Carbon monoxide complexes were prepared by slow bubbling of reduced enzyme solutions with CO after waiting 30 min for full reduction. In all experiments the absorption spectrum and pH of the cell contents were measured at the conclusion of the run to ensure that the samples had not changed. Reoxidation of reduced and reduced–CO species was carried out by the addition of 0.08 ml of a stock solution of potassium

ferricyanide (25 mg/ml) to 0.5-ml samples of the enzyme. The increase in ionic strength due to the addition of ferricyanide was compensated by lowering the KCl concentration.

The concentration dependence of the sedimentation coefficient for ferrous and ferric oxidase was determined from direct difference data as described by Schumaker and Adams[7], using 0.4% cytochrome oxidase as the reference. All dilutions were made from a single enzyme stock.

The concentration dependence for ferric and ferrous oxidase is shown in Figs 1 and 2. A linear concentration dependence of the form $s^{c}_{20,w} = s^{o}_{20,w}(1-kc)$ was assumed where c is the concentration in mg/ml and k is the concentration-dependence parameter.



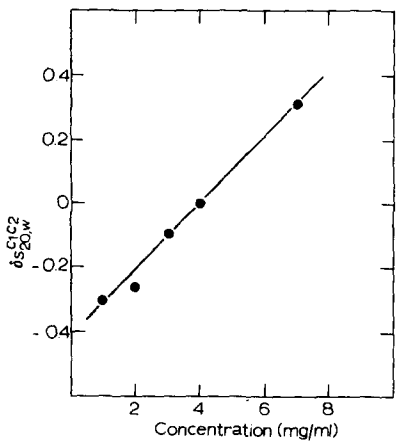


Fig.1. Concentration dependence of ferric oxidase. For each point the reference protein, cytochrome oxidase (4.8 mg/ml), was placed in Cell 1, which had a 1° positive wedge window. Cell 2 contained oxidase at concentrations varying from 1.2 to 9.6 mg/ml. The difference, $\delta s^{c_1 c_2}_{20,w}$, for each paired run was calculated as described in the text and plotted against the variable concentration, c_2. The slope of the least squares line yields $k = 0.135 \pm 0.008$ S/mg per ml.

Fig.2. Concentration dependence of ferrous cytochrome oxidase. Again Cell 1 held the reference protein, this time ferrous oxidase at a concentration of 4.0 mg/ml. The results were obtained as described in the legend for Fig.1. The value of k obtained for the reduced enzyme was $k = 0.133 \pm 0.010$ S/mg per ml.

To obtain the best value for k, a trial value was first determined by plotting $\ln r_1 - \ln r_2$ *vs* time, where r_1 and r_2 are the boundary positions in Cells 1 and 2. The slope provides a difference sedimentation coefficient, $\delta s^{c_1 c_2}_{t,s}$, for the concentration pair c_1 and c_2 at temperature, t, in the solvent used. This value was corrected to water at 20 °C, by using the expression given below[8].

$$\delta s^{c_1 c_2}_{20,w} = \delta s^{c_1 c_2}_{t,s} \frac{\eta_{t,s}(1-\bar{v}\rho)_{20,w}}{\eta_{20,w}(1-\bar{v}\rho)_{t,s}}$$

when $\eta_{t,s}$ is the viscosity of solvent at the temperature of the experiment, $\eta_{20,w}$ is the viscosity of water at 20 °C, $\bar{v}$ is the partial specific volume of the protein, and ρ is the solution density. These $\delta s^{c_1 c_2}_{20,w}$ values were plotted against c_2, where c_2 is the variable concentration. The slope of this line gave a trial value for k which was used to obtain the $\delta s^{c_1 c_2}_{20,w}$ values plotted in Figs 1 and 2. The new slopes obtained from Figs 1 and 2 yield the best values for k, which are 0.135 ± 0.008 S/mg per ml for ferric oxidase and 0.133 ± 0.010 S/mg per ml for ferrous oxidase. These values of k were used to obtain the $\delta s^{\circ}_{20,w}$ values for the difference experiments reported below. Since the k values are equal, no change in the state of association occurs when the enzyme is reduced. This result is of some significance since all our enzyme preparations contain a small amount of monomer in addition to the predominant dimer species. Therefore, the concentration-dependence data indicate that the change in the sedimentation coefficient which accompanies a change in the redox state of the enzyme as reported in this communication cannot be due to a different state of association of the enzyme between the oxidized and reduced forms.

The results summarized in Table I and Fig. 3 show that there is a 1.08% decrease in S when cytochrome oxidase is reduced (Fig. 3, A). A decrease of this magnitude corresponds to an increase in molecular volume of about 3%, assuming that oxidase is a globular protein. This is a relatively small change in volume when compared to results reported for a variety of allosteric proteins. For example, X-ray diffraction data show that the molecular volume of hemoglobin decreases by about 8% upon oxygenation[9]. Gerhart

TABLE I

SUMMARY OF THE SEDIMENTATION DIFFERENCE EXPERIMENTS

*Species observed**	$\delta s^{\circ}_{20,w}$**	*% Change in S****
$a^3a_3^3 - a^2a_3^2$	0.141 ± 0.021	1.08
$a^2a_3^2CO - a^2a_3^2$	0.045 ± 0.006	0.34
$a^3a_3^2CO - a^3a_3^3$	0.048 ± 0.006	0.37

* Species with the higher $s^{\circ}_{20,w}$ listed first.

** Each value represents the mean of three successive experiments with the average deviation given.

*** Calculated as $(\delta s^{\circ}_{20,w}/s^{\circ}_{20,w}) \times 100\%$, where $s^{\circ}_{20,w} = 13.1$.

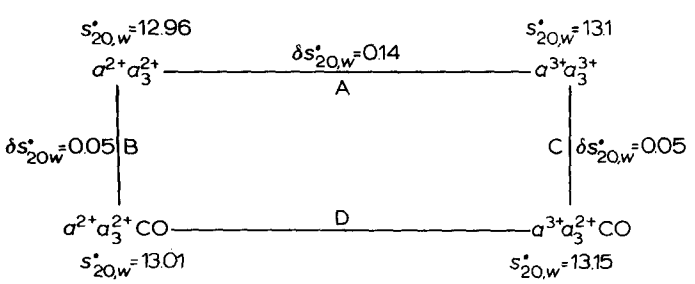


Fig.3. Summary of sedimentation difference experiments on cytochrome oxidase.

and Schachman[10] found a decrease of 3.6% in the sedimentation coefficient of aspartate transcarbamylase in the presence of carbamyl phosphate and succinate, indicating an expansion of about 13% in the molecule when the effectors were bound. Recently, Poillon and Feigelson[11] reported about an 18% contraction in ferrous tryptophan oxygenase when the allosteric effectors tryptophan or α-methyltryptophan were bound. Kirschner and Schachman[12], however, measure an increase in S of only 1.05% when succinate and carbamyl phosphate bind to the catalytic subunit of aspartate transcarbamylase. The small change we are measuring could arise either from subtle reorganization of the quaternary structure of cytochrome oxidase, or from a large change in the tertiary structure. Van Buuren *et al.*[13] have recently speculated, on the basis of cyanide binding data, that the heme cavity may be more closed in the oxidized than in the reduced form of the enzyme. The question of whether the observed sedimentation difference involves a change in tertiary or quaternary structure should be clarified by studies we now have in progress on monomeric cytochrome oxidase, which we have previously shown to retain many of the properties of cytochrome a_3 [14–16].

When the reduced and the reduced–CO enzyme are compared (Fig. 3, B), we find that addition of CO does not greatly alter the conformation of reduced cytochrome oxidase, shifting it only 0.045 S back toward the oxidized value. This is in agreement with the conclusion reached by Yong and King[3] on the basis of CD measurements.

These authors also found that partial reoxidation of the ferrous–CO complex with ferricyanide results in a large decrease in the positive ellipticity at 600 nm, showing a conformational change in the enzyme associated with the oxidation state of cytochrome *a*. When we compare the products of ferricyanide reoxidation of the ferrous and ferrous–CO species (Table I and Fig. 3, C) the $\delta s^{\circ}_{20,w}$ observed is 0.048, which is the same value obtained when reduced and reduced–CO forms are compared. This implies that the oxidation state of heme a_3 has little or no effect on the sedimentation rate, and that it is the oxidation state of heme *a* which controls the conformation of the enzyme. Van Buuren *et al.*[13] have also recently shown that the reduction of cytochrome *a* enhances the dissociation of cyanide, and believe that this implies the conformation of oxidase is primarily determined by the oxidation state of cytochrome *a*.

Fig. 3 suggests that comparison of the $a^2a_3^2$CO and $a^3a_3^2$CO species (Fig. 3, D) by the sedimentation difference technique should give a δs of about 0.14 S. To perform the experiment, however, it is necessary to add to the $a^2a_3^2$CO cell, a substance which matches the viscosity and density effects exhibited by the potassium ferricyanide used to produce the $a^3a_3^2$ species. The natural choice to balance the effects of potassium ferricyanide would appear to be potassium ferrocyanide. It was found, however, that these two substances do not balance sufficiently for use in the difference experiment. This was evident both from the observation that the δs values obtained depend upon the absolute amounts of potassium ferricyanide and potassium ferrocyanide used in the experiment, and from independent measurements of the viscosity of solutions of the two substances using a Cannon–Ubbelohde viscometer. Thus it has not been possible to confirm experimentally the prediction that the δs value associated with Fig. 3, D should be $\delta s = 0.14$ S.

Finally, in calculating the change in volume between the oxidized and reduced enzymes, we have assumed a globular shape. The magnitude of the concentration dependence, and intrinsic viscosity data (Cabral, F., unpublished), however, indicate that cytochrome oxidase may be more asymmetric than was previously thought. Work on this problem is currently in progress.

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REFERENCES

1 Meyer, Y.P. and King, T.E. (1969) *Biochem. Biophys. Res. Commun.* 34, 170–175
2 Yong, F.C. and King, T.E. (1970) *Biochem. Biophys. Res. Commun.* 38, 940–946
3 Yong, F.C. and King, T.E. (1970) *Biochem. Biophys. Res. Commun.* 40, 1445–1451
4 King, T.E., Bayley, P.M., and Yong, F.C. (1971) *Eur. J. Biochem.* 20, 103–110
5 Yamamoto, T. and Okunuki, K. (1970) *J. Biochem. Tokyo* 67, 505–506
6 Yonetani, T. (1966) in *Biochemical Preparations* (Maehly, A., ed.), Vol. 11, pp. 14–20, John Wiley and Sons, New York
7 Schumaker, V. and Adams, P. (1968) *Biochemistry* 7, 3422–3427
8 Schachman, H.K. (1959) *Ultracentrifugation in Biochemistry*, p. 82, Academic Press, New York
9 Muirhead, H. and Perutz, M.F. (1963) *Nature* 199, 633–638
10 Gerhart, J.C. and Schachman, H.K. (1968) *Biochemistry* 7, 538–552
11 Poillon, W. and Feigelson, P. (1971) *Biochemistry* 10, 753–760
12 Kirschner, M. and Schachman, H.K. (1971) *Biochemistry* 10, 1919–1926
13 Van Buuren, K., Zuurendonk, P., Van Gelder, B. and Muijsers, A. (1972) *Biochim. Biophys. Acta* 256, 243–257
14 Love, B., Chan, S. and Stotz, E. (1970) *J. Biol. Chem.* 245, 6664–6668
15 Chan, S., Love, B. and Stotz, E. (1970) *J. Biol. Chem.* 245, 6669–6674
16 Love, B. and Auer, H. (1970) *Biochem. Biophys. Res. Commun.* 41, 1437–1442