Thermodynamic Volume Cycles for Electron Transfer in the Cytochrome *c* Oxidase and for the Binding of Cytochrome *c* to Cytochrome *c* Oxidase

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ABSTRACT Dilatometry is a sensitive technique for measuring volume changes occurring during a chemical reaction. We applied it to the reduction-oxidation cycle of cytochrome *c* oxidase, and to the binding of cytochrome *c* to the oxidase. We measured the volume changes that occur during the interconversion of oxidase intermediates. The numerical values of these volume changes have allowed the construction of a thermodynamic cycle that includes many of the redox intermediates. The system volume for each of the intermediates is different. We suggest that these differences arise by two mechanisms that are not mutually exclusive: intermediates in the catalytic cycle could be hydrated to different extents, and/or small voids in the protein could open and close. Based on our experience with osmotic stress, we believe that at least a portion of the volume changes represent the obligatory movement of solvent into and out of the oxidase during the combined electron and proton transfer process. The volume changes associated with the binding of cytochrome *c* to cytochrome *c* oxidase have been studied as a function of the redox state of the two proteins. The volume changes determined by dilatometry are large and negative. The data indicate quite clearly that there are structural alterations in the two proteins that occur on complex formation.

INTRODUCTION

Cytochrome *c* oxidase is the terminal component of mitochondrial electron transport (see Wikstrom et al., 1981, for a review of the early literature; see Babcock and Wikstrom, 1992; Malmstrom, 1993; Williams, 1995; Gennis and Ferguson-Miller, 1995, for more recent views). Physiologically, the oxidase accepts electrons one at a time from cytochrome *c* (cyt *c*) and stocks them in one of four sites before reducing dioxygen to water. For electron transfer to occur, a molecule of cyt *c* must bind, electron transfer must occur, and the cyt *c* must dissociate. For the oxidase to reduce a molecule of dioxygen, this cycle of binding, transfer, and release must occur four times. Some time during these cycles, the cyt *c* oxidase pumps protons from one side of the mitochondrial membrane to the other, thereby creating an electrochemical potential of protons across the membrane.

The structure of beef heart cyt *c* oxidase has been known at the coarse level for many years (Deatherage and Henderson, 1982). As isolated and purified, the active molecule of cyt *c* oxidase contains 13 different subunits, two molecules of heme *a* (cytochrome *a* and cytochrome a_3), two atoms of copper as CuA, one atom of copper as CuB, as well as Zn(II), Mg(II), and a variable amount of phospholipid. Quite recently the x-ray structures of the 13-subunit mitochondrial (Tsukihara et al., 1995, 1996; available from the Brookhaven Data Bank: PDB1OCC) and the four-subunit *Paracoccus* (Iwata et al., 1995) enzymes have been determined at 2.8 Å. The unit cells of both crystals are dimers.

The overall structures of the core portions of the two proteins are quite similar. Subunit I, the largest of the subunits, contains a total of 12 membrane-spanning helices. The helices support the two hemes *a* and a_3, as well as one of the redox active coppers, CuB. There are water-filled cavities within the helices that coalesce to form the presumptive proton channel. The helices also form a binding site for subunit II. The latter contains the presumed tight binding site for cyt *c* as well as the other two redox active coppers.[1]

The exact nature of the binding site or sites for cyt *c* is not known, but we think that it or they contain a substantial amount of negative charge to match the positive charge on the cyt *c* (Margoliash and Bosshard, 1983). The structure of the cyt *c*/cyt *c* peroxidase complex has been determined (Pelletier and Kraut, 1992); it contains one cyt *c* per two cyt *c* peroxidases. The interface of the complex is thought to bear structural similarity to the cyt *c*/cyt *c* oxidase interface. In addition to recognizing positive charge, the latter has a requirement for sequestered water (Kornblatt et al., 1993; Nicholls et al., 1996).

Stable complexes of cyt *c* and cyt *c* oxidase will form at low ionic strength; these complexes will catalyze electron transfer from artificial donors to cyt *c* to cyt *c* oxidase to

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[1] The terminology is somewhat confusing. CuA was originally thought to be a single metallic center. More recently it was characterized as containing two coppers. CuA is, therefore, a dinuclear center in which the charge on each Cu is formally 2 when the oxidase is oxidized and 1.5 when the protein is reduced (Kroneck et al., 1988). Its structure has been elucidated in both the *Paracoccus* (Iwata et al., 1995) and the beef heart (Tsukihara et al., 1995, 1996) enzymes. More recently the structure of the CuA site of an engineered oxidase of *Escherichia coli* has been determined (Wilmanns et al., 1995). In the four crystal structures available, it is clearly a dinuclear center.

oxygen. When the salt concentration is increased, dissociation occurs on the time scale of catalysis. The ionic strength of the cytosol is not far removed from 0.15 M; because there are few permeability barriers to ions between the cytosol and intercristal space of the mitochondrion, it is likely that rapid dissociation and reassociation probably occur under most physiological conditions. At intermediate ionic strength (~30 mM) it is clear that one can detect more than one catalytically significant K_m for cyt *c* (Mochan and Nicholls, 1972; Ferguson-Miller et al., 1978). This means that there is the potential for more than one recognition site occurring during catalysis. It is not clear whether, in any single oxidase molecule, more than a single site is active during a turnover. At moderate salt and relatively high protein concentration, the oxidase can exist as a dimer, as is found in the crystal. There are data that indicate that cyt *c*, bound to only one of the monomers in the dimer, may communicate with both monomers (Kornblatt and Luu, 1986; Bisson et al., 1980). The fact that we have the possibility of at least two aggregation states of the oxidase as well as one to four binding sites per dimer makes the study of cyt *c* binding to cyt *c* oxidase rather challenging.

We have used the technique of dilatometry to assess the volume changes associated with the partial reduction and complete reduction of the oxidase. We have used the same technique to assess the volume changes associated with the binding of cyt *c* to cyt *c* oxidase when the two proteins are either oxidized or reduced. The data indicate several steps in which large volume changes occur. Volume changes of the magnitude found here can arise from two sources: 1) the intermediates can be hydrated to different extents and 2) small voids can open or close during the passage from one intermediate to another. Both probably contribute to the changes measured here.

MATERIALS AND METHODS

Cyt *c* oxidase was purified from beef heart as previously described (Yonetani, 1966). Cyt *c* Type VI and Tris base were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were purchased from Fluka and were of the highest purity available.

The dilatometry bath was a 120-liter fish aquarium that was insulated on five sides. The back, sides, and bottom were insulated with 10-cm-thick styrofoam; the front was a 10-cm dead air space, and the top was open. Water in the bath was stirred with two high-power motors on which were mounted shafts equipped with two propellers each. The bath was cooled continuously with a circulating water bath; water was passed through a copper coil immersed in the aquarium. The bath was heated discontinuously with a bank of five 100-W light bulbs. The power to the bank of lights was provided by a Tronac PTC-41 temperature controller (Tronac, Orem, UT). The complete apparatus, when performing under optimal conditions, provides a constant temperature within 0.0001°C over the course of several days. The LED lights on the thermal controller indicated that the bath was performing optimally. Our precision digital thermometer is accurate to 0.01°. It registered no fluctuation over the course of several days. All experiments described in this paper were performed at 15.00XX ± 0.0003°C, in which 0.0003°C represents the maximum fluctuation in the temperature over the course of the experiment. (We have verified a value of 0.0002°C by using a very large dilatometer tube filled with 50 ml of water and a 0.075 μl/cm capillary. The maximum fluctuation over the course of 1 h was 0.2 cm, indicating a fluctuation of less than 0.0002°C.) Even though we did not know the exact temperature to the fourth significant figure, the temperature did not vary by more than the cited amount, and this is the important consideration in dilatometry. The dilatometer tubes, modified versions of the original Carlsberg dilatometer (Linderstrom-Lang and Lanz, 1938), were produced in the Concordia University glass blowing shop. The dilatometer capillaries (internal diameter 0.1 mm) were purchased from Wilmad Glass; the capillary tubes were individually calibrated (Kahn and Briehl, 1982). The capillaries had internal volumes between 0.070 ± 0.001 and 0.077 ± 0.001 μl/cm. The assembled dilatometer tube is shown very schematically in Fig. 1. (Dilatometry is not a commonly used technique. Photographs of the dilatometer tubes, details of bath heating and the cooling setup, as well as indications of the "interface" between the two will gladly be provided on request.)

Dilatometry is a technique that measures the volume change that occurs on mixing two very similar solutions. The volume change is determined by the change in the level of heptane in the capillary of Fig. 1. The technique has recently been applied to the volume changes associated with native to molten globule(s) transitions of cyt *c* (Foygel et al., 1995). We have used the same basic apparatus, but have modified their protocol. The oxidase experiments are complicated by the fact that substantial amounts of detergent (Tween 80) are present in the protein solutions.

The protocol for the determining the volume changes associated with electron transfer between intermediates follows.

Oxidase (5 μmol, ~1 g) was thawed and diluted to ~20 ml with 10 mM Tris, 10 mM EDTA, 0.1 M NaCl, pH 7. One gram of Tween 80 was added. The protein solution was dialyzed to equilibrium against the same buffer solution, but omitting the Tween. Tween micelles are sufficiently large that they do not pass through the dialysis membrane. The dialyzed solution was centrifuged at 19,000 rpm for 60 min at 15°C. To this oxidase solution we now added all of the components, with the exception of the single component that would bring about the conversion between the two states: this one component was ascorbate plus tetramethylphenylenediamine (TMPD). If the final oxidase solution in the dilatometer tube was to be reduced, the solution was bubbled with argon; if the final state contained CO, it was bubbled with CO. If the final state contained cyanide, KCN was added. The solution was divided in two. The single component at low concentration was added to one half; an equivalent amount of water was added to the

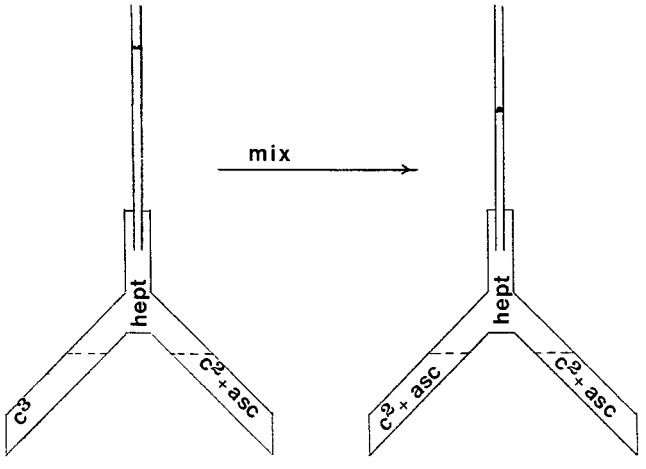


FIGURE 1 Operation of the dilatometer tube. The tube on the left contained 5 ml of 413 μM cyt c^3 in the left arm, and 5 ml of 413 μM cyt c^2 plus 5 mM ascorbate, and 50 μM TMPD in the right arm. The heptane bridge (hept) is contiguous with the capillary. When the dilatometer tube is mixed, the cyt c^3 is reduced by the ascorbate plus TMPD. The concentration of cyt *c* stays constant; the concentration of the reductant is halved. The control for this tube contained no cyt *c* on either side. The change in the level of the heptane in the capillary tube is indicative of the change in the volume of the total dilatometer tube. In the experimental tube, the heptane level in the capillary dropped by ~1 cm.

other half. They were then placed in the two arms of a dilatometer tube. Heptane that had been equilibrated with the buffer and subsequently bubbled with Ar or CO was added to the tube. Both arms and the heptane were again bubbled with the gas before the tubes were capped with the dilatometer capillary. The assembled apparatus was put into the thermostatted bath and allowed to come to thermal equilibrium.

When the two solutions are mixed, there are two volume changes that occur. The first is associated with the change in state of the protein. The second is associated with the dilution of the single added component and any chemistry that occurs because of it. The dilution and chemistry were assessed in controls run at the same time in matched dilatometers. These controls contained everything present in the experimental tubes, but the oxidase concentration was reduced by a factor of 100. Changes occurring in the control tubes were subtracted from the values obtained in the experimental tubes. Accordingly, the values that we report are representative of the change in state of the oxidase and do not contain a contribution from the dilution of the single component or from the chemistry that results from the addition. The pH of the samples was checked at the end of each experiment. In no case did it change by more than 0.1 pH units.

The modified protocol for the oxidase was necessary to avoid artifacts due to dilution of the high concentrations of protein and Tween 80. We could easily control for the former, because the concentration of protein was known to two significant figures. The uncertainty in the Tween concentration is more problematical. The volume change for diluting Tween and oxidase is positive. In all of the oxidase experiments, the mixing volumes for oxidase and Tween were zero, because their concentrations were held constant; this was verified experimentally. In addition, the salt concentration was held constant.

In the experiments in which the oxidase was reduced by (TMPD)ascorbate, there was no contribution to the overall volume change from the reaction ascorbate + 1/2 O_2 → dehydroascorbate + H_2O; this was verified in separate experiments.

All volume changes reported for the oxidase are the average of at least four determinations and four control determinations. The average estimated error in the precision of any number in Tables 1 or 2 is ~15%.

The protocol for the binding experiments was similar to that described above. Oxidase (5 μmol, ~1 g) was thawed and diluted to ~20 ml with 10 mM Tris, 10 mM EDTA, 0.1 M NaCl, pH 7. One gram of Tween 80 was added; the protein solution was dialyzed and centrifuged as above. The solution was divided in two. Solid cyt *c* was added to one half; the two solutions were then placed in the two arms of a dilatometer tube. When the two solutions are mixed, there are two volume changes that occur. The first is associated with binding of cyt *c* to oxidase; the second is the result of the dilution of the cyt *c*. The dilution was assessed in controls run at the same time in matched dilatometers. In the experiment in which we determined the volume change that occurs when reduced oxidase binds to reduced cyt *c*, TMPD (50 μM) and ascorbate (5 mM) were added to both arms of the dilatometer tube. Changes occurring in the control tubes were subtracted from the values obtained in the experimental tubes.

The binding reactions were carried out at high protein concentrations, on the order of 150 μM oxidase and 200–500 μM cyt *c*. We had little information on the aggregation state of the oxidase and its binding constants at these protein concentrations and ionic strengths. Accordingly, we measured the binding of cyt *c* oxidase and cyt *c* by the gel filtration method of Hummel and Dreyer (1962): a column of Sephadex G-50 (52 cm × 1.4 cm) was equilibrated with cyt *c* in Tris, EDTA, NaCl, 1% Tween 80. A 0.5-ml sample of cyt *c* oxidase (~50 μM) was applied to the top of the equilibrated column and allowed to percolate through the cyt *c*. The oxidase was excluded from the Sephadex, whereas the cyt *c* was not. As the oxidase passes through the cyt *c* it binds, and the extent of binding can be estimated from absorption measurements at 604 nm (ϵ = 40 mM^{-1} cm^{-1}, the α band of the reduced oxidase) and 550 nm (ϵ = 27.7 mM^{-1} cm^{-1}, the α band of reduced cyt *c*). If the experiment is performed over a range of cyt *c* concentrations, the stoichiometry of binding and the binding constants can be evaluated by Scatchard analysis. A series of nine experiments allowed us to establish the stoichiometry and binding affinities.

It will be noted that all of the volume changes that we report here are negative values, a fortuitous result. We have measured the volume changes that occur when enolase dimers dissociate and when enolase monomers reassociate. The volume change for the association reaction is the same as that for dissociation, except that the signs are opposite (data not shown).

TABLE 1 Reaction volumes for the partial or complete reduction of the oxidase

Overall reaction	$\triangle V$ (ml/mol protein)
$c^3 + 1e^- \rightarrow c^2$*	−22 ± 2.3
$CuA^2a^3CuB^1a^2{}_3CO + 2e^- \rightarrow CuA^1a^2CuB^1a^2{}_3CO$#	−155 ± 9.2
$CuA^2a^3CuB^2a^3{}_3CN + 3e^- \rightarrow CuA^1a^2CuB^1a^3{}_3CN$§	−117 ± 22
$CuA^2a^3CuB^2a^3{}_3 + 4e^- \rightarrow CuA^1a^2CuB^1a^2{}_3$¶	−95 ± 19

The dilatometer operation is detailed in Materials and Methods. The reactions were all carried out in the buffer listed in Materials and Methods. The numerical value shown is the mean ± standard deviation.

*See Fig. 1. Cytochrome c_3 was reduced by mixing 413 μM cyt *c* in buffer containing 5 mM ascorbate plus 50 μM TMPD with the same solution that contained neither ascorbate nor TMPD.

#193 μM oxidase that had been bubbled and dialyzed against CO was mixed with the same solution that contained 5 mM ascorbate plus 50 μM TMPD. We verified that the partially reduced CO complex had been formed before mixing with TMPD/ascorbate and that the totally reduced CO complex had formed after mixing (Greenwood et al., 1974; Nicholls, 1979a).

§Oxidase (88 μM) that had been exhaustively dialyzed versus the standard buffer containing 1 mM cyanide was mixed with the same solution containing 5 mM ascorbate plus 50 μM TMPD. The spectrum before mixing indicated complete conversion to the oxidized cyanide complex; after mixing the spectrum was indicative of the three-electron-reduced species (Nicholls, 1979b; Johnson et al., 1981).

¶Oxidase (182 μM) in deoxygenated standard buffer was mixed with the same solution containing 5 mM ascorbate plus 50 μM TMPD plus 10 μM cytochrome *c*. The identities of the oxidized and reduced oxidase were verified spectroscopically, in the dilatometer tubes, before and after mixing.

RESULTS

Electron transfer into and through cytochrome *c* oxidase

The reactions studied and the volume changes that accompany them are outlined in Table 1 and Scheme 1.[2]

Two representative sets of data on which the data of Table 1 are based are shown in Fig. 2. The data of Fig. 2 *A*

[2]To put the reactions into perspective, it is useful to recall the overall catalytic cycle and some of the inhibited intermediates of the oxidase. Cyt *c*, the natural donor, transfers electrons one at a time to CuA (Hill, 1994a). There is rapid equilibration between CuA and cytochrome *a* (cyt *a*), leading to the two-electron-reduced oxidase (*A* in Scheme 1). The latter is the electron donor for the binuclear center CuB and cyt a_3 (*B* in Scheme 1). To form the completely reduced oxidase, there must be a subsequent two-electron transfer into the CuA/cyt a site (*C* in Scheme 1). A two-electron transfer out of the oxidase to oxygen produces peroxide as the first "metastable" product (part of *D*, but not shown in Scheme 1). There is then an internal electron transfer from CuA and cyt *a* to rereduce the oxidized CuB/cyt a_3 centers followed by a two-electron transfer to peroxide. thereby reforming the totally oxidized oxidase (*D* in Scheme 1). The inhibitors carbon monoxide and cyanide inhibit the overall electron transfer process (reviewed in Hill, 1994b). Cyanide binds to oxidized cyt a_3; when there is a source of electrons present, such as from cyt *c*, cyanide traps the

TABLE 2 Reaction volumes for the binding of cyt *c* to cyt *c* oxidase

Overall reaction	$\triangle V$ (ml/mol protein)
$c_3 + 1e^- \rightarrow c_2$*	-22 ± 2.3
$(CuA^2a^3CuB^2a^3{}_3)_2 + 8e^- \rightarrow (CuA^1a^2CuB^1a^2{}_3)_2$[#]	-190 ± 38
$(CuA^2a^3CuB^2a^3{}_3)_2 + c_3 \rightarrow (CuA^2a^3CuB^2a^3{}_3)_2 \cdot c_3$[§]	-322 ± 43
$(CuA^1a^2CuB^1a^2{}_3)_2 + c_2 \rightarrow (CuA^1a^2CuB^1a^2{}_3)_2 \cdot c_2$[¶]	-491 ± 70
$(CuA^2a^3CuB^2a^3{}_3)_2 \cdot c_3 + 9e^- \rightarrow (CuA^1a^2CuB^1a^2{}_3)_2 \cdot c_2$[‖]	-404 ± 41

The dilatometer operation is detailed in Materials and Methods. The reactions were all carried out in the buffer listed in Materials and Methods. The numerical value shown is the mean ± standard deviation.

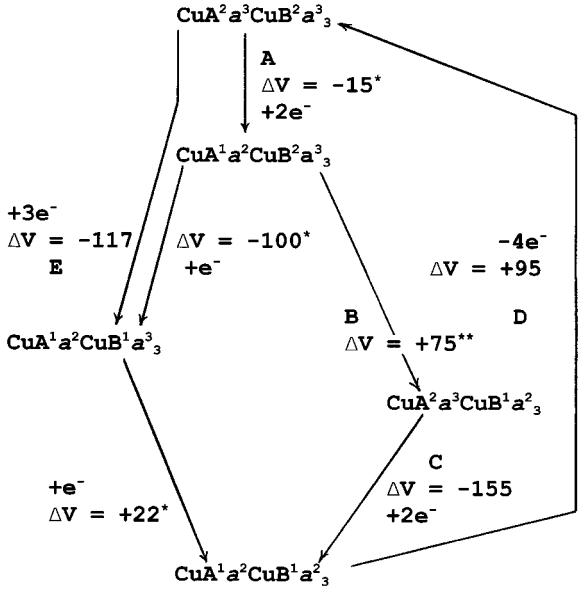
*Cyt c_3 was reduced by mixing 413 μM cyt *c* in buffer containing 5 mM ascorbate plus 50 μM TMPD with the same solution that contained neither ascorbate nor TMPD.

[#]Oxidase (91 μM) dimer in deoxygenated standard buffer was mixed with the same solution containing 5 mM ascorbate plus 50 μM TMPD plus 10 μM cyt *c*. The identities of the oxidized and reduced oxidase were verified spectroscopically before and after mixing.

[§]Oxidase (77 μM) dimer in the standard buffer was mixed with the same solution containing 415 μM cytochrome *c*. The matched controls mixed buffer with buffer containing 415 μM cytochrome *c*. At 77 μM oxidase dimer (153 μM oxidase monomer) and 208 μM cytochrome *c*, ~71% of the oxidase is in the form of the 1 *c* to 1 oxidase-dimer complex if the value of the dissociation constant is 60 μM. The value of $\triangle V$ was calculated as ($\triangle\mu$l in the dilatometer capillary)/(μmol of the 1 *c*/2 oxidase complex formed).

[¶]As in previous footnote, except that the deoxygenated buffer contained 5 mM ascorbate and 50 μM TMPD.

[‖]Oxidase (77 μM) dimer and 200 μM cytochrome *c* were mixed with the same solution containing 5 mM ascorbate plus 50 μM TMPD. The matched controls contained the same solutions, but the oxidase and cytochrome were at 3 μM.



Scheme 1. A thermodynamic volume cycle for the electron transfer reactions of cyt *c* oxidase. The value of $\Delta V = 75$** ml/mol is taken from Kornblatt et al. (1988) and is based on hydrostatic pressure measurements. The other values of ΔV that do not contain an asterisk have been taken directly from Table 1. Those values of ΔV that contain a single asterisk were obtained under the assumption that the total volume change is independent of path; this assumes that the cycle as written is a complete thermodynamic cycle. Therefore $\Delta V = -15$* is obtained from the volume change for complete reduction of the oxidized oxidase (−95 ml/mol), the volume change for the two-electron transfer to $CuB^2a_3^3$ (75 ml/mol), and the volume change for the transfer of two electrons to CuA^2a^3 (−155 ml/mol) (−15 = −95 + 155 − 75). The value of $\Delta V = -100* = -117$ ml/mol for the three-electron transfer and −15 ml/mol for the initial two-electron transfer. The value of $\Delta V = 22* = -95$ ml/mol for the complete reduction and −117 ml/mol for the three-electron reduction.

represent the volume changes associated with the reduction of cyt c^3 by ascorbate and TMPD. Fig. 1 shows the approach; oxidized cyt *c* was mixed with the same solution containing TMPD and ascorbate. There is a small dilution volume for the TMPD and ascorbate, but none for the cyt *c*. The data of Fig. 2 *B* represent those for the complete reduction of the partially reduced CO complex of the oxidase by ascorbate and TMPD. In one arm of the dilatometer is the partially reduced CO complex. In the other arm is the fully reduced CO complex plus TMPD and ascorbate. The reduction is carried out at constant oxidase concentration. Fig. 2 has one other important aspect: it indicates the relative noise level versus the magnitude of the volume change.

In Table 1, the reduction of cyt *c* is included for the sake of comparison; it indicates that electron transfer itself does not contribute greatly to the overall volume changes seen in the other three lines of the table. The absolute value of the change, −22 ml/mol, agrees reasonably well with the literature value of −24 ml/mol (Cruañes et al., 1992). The values of ΔV for all four reactions shown in the table represent changes associated only with oxidation state.

The stoichiometry and affinity of cytochrome *c* binding to cytochrome *c* oxidase

Fig. 3 shows a typical Hummel and Dreyer (1962) type experiment in which cyt *c* oxidase is percolated through a column equilibrated with cyt *c*. Nine experiments were performed at varying concentrations of cyt *c*, and these data gave rise to the Scatchard plot shown in the inset of the figure. At pH 7.0 and ionic strength 120 mM, the oxidase binds 1/2 (0.56 ± 26) cyt *c* per monomeric unit or one cyt *c* per oxidase dimer. This is the same stoichiometry as is found in the crystal structure of the cyt *c*/cyt *c* peroxidase

three-electron-reduced oxidase (*E* in Scheme 1). Carbon monoxide binds to the reduced cyt a_3. When there is a source of electrons present, it traps the totally reduced oxidase. In the absence of external reductants, as in the experiments reported here, the oxidase can oxidize carbon monoxide. In the presence of saturating CO, this results in trapping of the two-electron-reduced oxidase in which CuB and cyt a_3 are reduced, whereas CuA and cyt *a* are oxidized. Subsequent reduction of the latter has allowed us to measure the volume change in step C of Scheme 1.

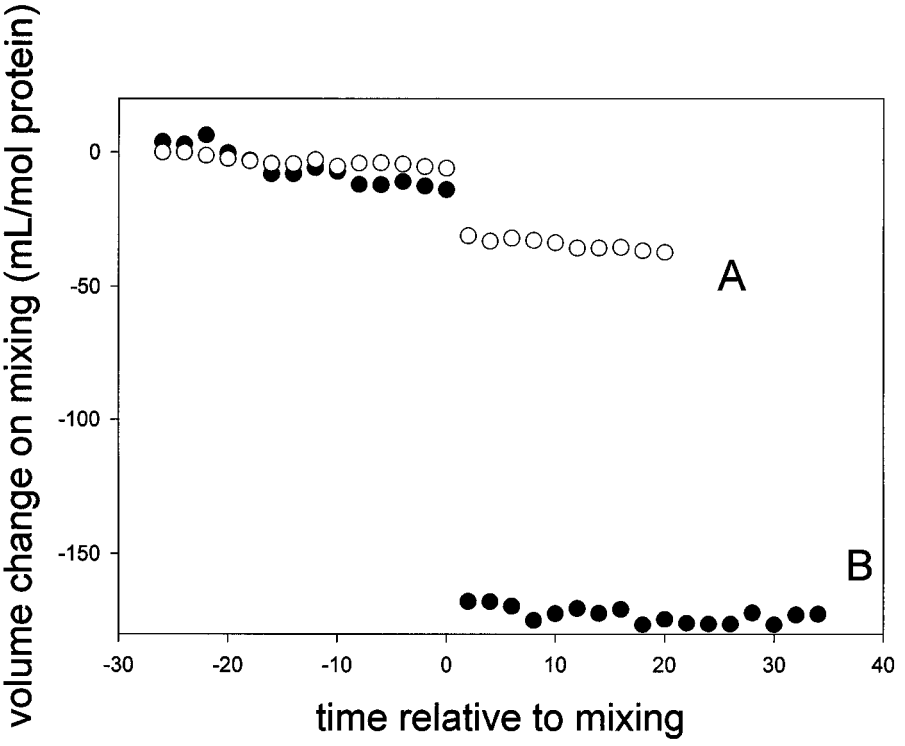


FIGURE 2 Volume changes resulting from electron transfer. (*A*) ○, The reduction of cyt c^3 by TMPD (50 μM) and ascorbate (5 mM); these are the raw data on which the volume change of Table 1, row 1 are based. (*B*) ●, The reduction of $CuA^2a^3CuB^1a^2{}_3CO$ by TMPD (50 μM) and ascorbate (5 mM). These are the raw data on which the volume change of Table 1, row 2 are based.

complex (Pelletier and Kraut, 1992). It indicates that the minimum unit in our experiments is not the monomeric oxidase but the dimeric, the form present in the oxidase crystal structure. It is important to recognize that this does not mean that the oxidase in our solutions is exclusively dimeric. It means that regardless of whether the aggregation state of the oxidase is dimer, tetramer, or nondefined multimer, the functional unit is the dimer. The aggregation state of the oxidase is not likely to change during the course of the experiment. Writing the expression for the dissociation constant as

$$K_d = [\text{oxidase dimer}]^*[\text{cyt } c]/[\text{complex}]$$

the dissociation constant of the complex is ~60 μM (61 ± 25). There is scatter in the data of the Scatchard plot. It is the result of viscous solutions and a traditional Sephadex column. High-performance liquid chromatography with silica-based resins would give far better quality data but cannot be used with cyt *c*; the latter binds, even at ionic strengths of 20 mM. The scatter is not such as to mask the fact that one cyt *c* binds per two monomeric units of oxidase. It is also not such as to substantially change the value of K_d.

Volume changes associated with the binding of the electron transfer partners

The reactions studied and the volume changes that accompany them are outlined in Table 2. In the first and second rows we repeat the molar volume changes that occur on reduction of monomeric cyt *c* or dimeric cyt *c* oxidase (this appears as −95 ml/mol, the value for the monomer, in Table 1). Rows 3 and 4 indicate the volume changes found when a single cyt *c* binds to the dimeric oxidized or reduced oxidase. Row 5 represents the change that occurs when the oxidized complex is reduced. In the three latter cases, the data are expressed in terms of the single monomeric cyt *c* binding to dimeric cytochrome *c* oxidase.

DISCUSSION

The data presented in this paper are based, for the most part, on the technique of dilatometry (Linderstrom-Lang and Lanz, 1938). In principle it yields the same sort of information as that obtained from hydrostatic pressure perturbations, but it makes fewer assumptions. In particular, there are no compressibility factors (β-factors or pressure-dependent terms) that must be taken into account in the analysis. This means that the compression of small voids does not contribute to the measured volume change. Perhaps more importantly, it is model independent. In the analysis of hydrostatic pressure derived data, the variation of K_{eq} with pressure is assumed to involve no measurable concentration of intermediates. If these are present, but not detectable, the calculated value of $\Delta V°$ is lower than the true value. (The difference between the enthalpies calculated by van't Hoff analysis and that estimated by calorimetry is well established. The van't Hoff analysis is model dependent and presumes no measurable concentration of intermediates. Direct calorimetry is model independent. When the two do not agree, and the van't Hoff value is less than the calorimetry, it sometimes indicates the presence of intermediates

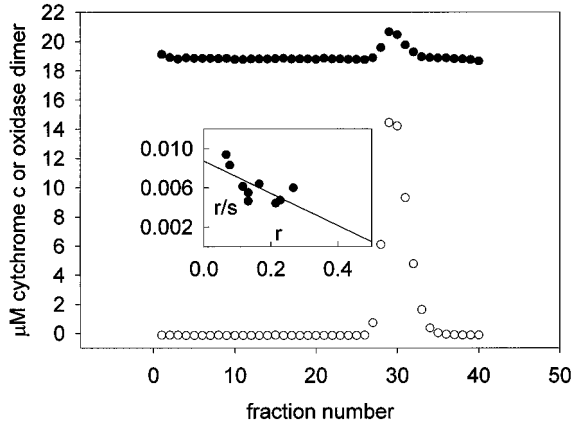


FIGURE 3 Gel filtration of cyt *c* oxidase through a column preequilibrated with ~18.8 μM cyt *c*. The column buffer was 10 mM Tris, 10 mM EDTA, 100 mM NaCl, 1% Tween 80, pH 7.1. The closed circles represent the concentration of cyt *c*, and the open circles are the concentration of cyt *c* oxidase monomer. (*Inset*) Scatchard analysis of the number of cyt *c* binding sites on cyt *c* oxidase and their affinity. *r* is the number of moles of cyt *c* bound per mole of cyt *c* oxidase; *s* is the concentration of free cyt *c*. At the peak of the oxidase profile in the main figure, *r* is ~0.12 mole of cyt *c* bound per mole of oxidase (1.66 μM excess cyt *c*)/(14.2 μM cyt *c* oxidase monomer). *r*/*s* in the inset is ~0.0062. A series of nine such experiments yielded the data in the inset. The least-squares regression line for the nine experimental points yields an average of 0.5 cyt *c*/cyt *c* oxidase or one *c* per oxidase dimer. The average dissociation constant obtained from the slope of the line is 60 μM. It is the dissociation constant for the reaction

$$c(aa_3)_2 \rightarrow c + (aa_3)_2$$

(Privalov, 1979). $\Delta H°$ and $\Delta V°$ are analogous in this respect.) The comparison of dilatometry-derived and hydrostatic pressure-derived data is complicated still further: it is rare that one can make measurements using both techniques without radically changing conditions. Dilatometry has allowed us to measure volume changes for reactions that were not accessible with the high-pressure technique, such as the full reduction of the oxidase and the reduction of the heme a_3 and CuB.

What contributes to the measured volume changes during either the catalytic cycle of cytochrome *c* oxidase or the binding of cytochrome *c* to cytochrome *c* oxidase? How can the density of a protein-containing solution change as the protein cycles or binds substrates?

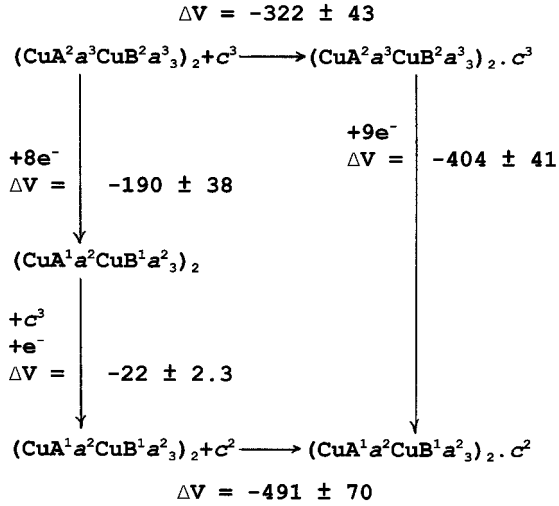
There are two factors that contribute to the volume changes that we have measured in this paper. The first is opening and closing of small voids in the proteins. The three-dimensional structure of the oxidase reveals many small voids. It would be surprising if these did not change as the protein cycled or bound its substrate. Differential hydration also contributes to the measured volume changes. During the catalytic cycle there are water movements into and out of the oxidase. During binding of the cyt *c*, water is sequestered at the interface. Our osmotic stress studies, based on the approach of Parsegian and Rand and their colleagues (1995), clearly indicate that water movements, independent of void formation and collapse, are critical aspects of cyt *c* oxidase and its reactions (Kornblatt et al., 1990, 1993). In what follows, we have interpreted the data in terms of water movements, but we fully recognize that there must be contributions of void formation and collapse to the overall volume change.

A volume of water must be sequestered from bulk solution into the structure of the protein or protein complex when the sign of the volume change is negative. Conversely, when the sign is positive, water is expelled from the protein or protein complex. The volume of water sequestered or expelled could be associated with either the external surface of the protein(s) or with internal surfaces or cavities. Regardless of whether the surface is internal or external, the net result of water binding will be the same: it will lead to a decrease in the system volume (Kauzmann, 1959; Zhang et al., 1996). We can state unequivocally the volume change that is associated with the transition from one redox state to another, but this cannot be translated into a fixed number of water molecules. We have no way of dissecting out the contributions from void formation/collapse and hydration.

We have used the data of Tables 1 and 2 to construct two thermodynamic cycles (Schemes 1 and 2); Scheme 1 details the interrelations between the different redox intermediates of the oxidase. Scheme 2 deals with the binding of cyt *c*.

The catalytic cycle of cytochrome *c* oxidase

In Scheme 1 our major assumption is that the volume changes detailed in Table 1 are additive, that they behave as state properties from the point of view of thermodynamics.



Scheme 2. A thermodynamic cycle for the binding of cyt *c* to cyt *c* oxidase. The scheme represents a true thermodynamic cycle. All of the volume changes were determined by dilatometry; within the limits of the experimental error, they sum to zero, as is expected for a cycle. There are three points of substantial interest. The volume changes are large and represent water uptake during complex formation. The volume change that occurs on reduction of the components of the complex is only 52% of that occurring when the preformed complex is reduced. The volume change on formation of the oxidized complex is only 65% that found on formation of the reduced complex.

The second assumption is that the addition of a ligand, cyanide or CO, to cyt a_3 does not substantially change the measured volume for the electron transfer step. We think that this is reasonable; Ogunmola et al. (1976) found that the binding of both high-spin and low-spin ligands to methaemoglobin caused an absolute volume change of only 10 ml/mol at most. The third assumption is that the volume change obtained from one series of hydrostatic pressure measurements (75 ml/mol) can be treated as equivalent to those from dilatometry; we assume that there are no intermediates in the internal electron transfer on the right-hand side of Scheme 1 (step B). We do not have values of ΔV for the reactions of Scheme 1 marked with an asterisk. They were obtained by assuming that the four-electron reduction and four-electron oxidation do in fact form a thermodynamic cycle that sums to zero.

Inspection of Scheme 1 indicates that there are at least four important reactions:

The first (step B), +75 ml/mol, accompanies an internal electron transfer from $CuA^1a^2CuB^2a_3^3$ to $CuA^2a^3CuB^1a_3^2$. It is based on the sensitivity of the oxidase to hydrostatic pressure (Kornblatt et al., 1988). This volume change indicates that there is water that is transferred from the protein into bulk solution during the internal transfer.

The second (steps E − A), about −100 ml/mol, occurs during the transfer of one electron to the CuB site. It is based on the measured value for the three-electron reduction of the oxidase (−115 ml/mol) and on the assumption that the reaction volume changes represent a true thermodynamic cycle.

The third important step (C) occurs during the transfer of the last two electrons into the oxidase, thereby rereducing the CuAcyt*a* site. This step is accompanied by a volume change of −155 ml/mol and is based on dilatometry. Water once again moves to a privileged site within the oxidase.

The fourth is the exit of the four electrons from the oxidase. It is accompanied by a volume change of 95 ml/mol and is based on dilatometry. As seen in Table 1, it was actually measured in the opposite direction. It represents water that must leave sites within the oxidase during the transfer of the four electrons to oxygen. Here it is critical to note that this represents the global figure for the oxidation step. It is possible, it is even likely, that this 95 ml might consist of three volume changes. The first would be associated with the transfer of two electrons from the CuBcyta_3 site to oxygen. The second would accompany the transfer of two electrons from the CuAcyt*a* to the CuBcyta_3 site and would be the +75 ml/mol (B) mentioned above. The third would be the transfer of the two remaining electrons out of the CuBcyta_3 site to peroxide.

The binding of cyt *c* to cyt *c* oxidase

We previously studied the binding of the oxidase to cyt *c*, using hydrostatic pressure to perturb the equilibrium. This was performed under conditions of low protein (~1 μM) and low salt (~10 mM). $\Delta V°$ for the association reaction is about −16 ml/mol. Unfortunately, the dilatometric measurements could not be performed under the same conditions as the hydrostatic pressure measurements. The oxidase and cyt *c* concentrations for the dilatometry measurements had to be close to 100 μM, whereas the salt concentration was ~120 mM. The volume changes measured by these (hydrostatic pressure and dilatometry) techniques are quite different. By dilatometry we find a system volume for oxidized cyt *c* oxidase binding to oxidized cyt *c* of −322 ± 43 ml/(mol protein), ~20 times larger than that found with hydrostatic pressure. The discrepancies between the dilatometry and hydrostatic pressure measurements may be due to the presence of intermediates in the pressure measurements. We feel it more likely that we are looking at different binding sites on the oxidase in the two sets of experiments. In the hydrostatic pressure experiments we are looking at tight binding in which there is a stoichiometry of one oxidase to one cyt *c*. In the dilatometry experiments we are looking at weak binding with a stoichiometry of two oxidase to one cyt *c*. It is essential to stress that although the numbers are different, both the hydrostatic pressure and the dilatometry measurements show that water is sequestered in the complex when cyt *c* binds to cytochrome oxidase.[3]

We have also carried out osmotic perturbations of the oxidase/cyt *c* equilibrium at low protein and low salt. In contrast to hydrostatic pressure and dilatometry, which both measure density changes when A reacts with B, osmotic pressure measures the number of water molecules involved in the reaction. Like the hydrostatic measurements, it is model dependent; the analysis requires that there be no measurable concentration of invisible intermediates. Osmotic stress indicated volume changes of about −200 mL/mol, or perhaps as many as 10 waters were sequestered when binding occurred (Kornblatt et al., 1993). Zhang et al. (1996) have studied the response of albumin to highly excluded and included compounds. They have determined, in this system, that the average density of protein-bound water is ~25% greater than that of bulk. If the density of the water bound to the oxidase/cyt *c* complex was similar to that found by Zhang et al. (1996), it would mean that the complex would be slightly sensitive to hydrostatic pressure. The value of −16 ml/mol mentioned above is perhaps somewhat low, but is consistent with the osmotic pressure results.[4] The osmotic results and those from dilatometry yield approximately the same numbers in terms of volume changes, but this is fortuitous. The dilatometry values rela-

[3]Cyt *c* binds to the oxidase via a ring of positive charge. Presumably there is a complementary region of negativity on the oxidase. This has been "noticed" in the x-ray structure. If positive and negative charges are electrostricted, then binding should lead to loss of water from the site. This is the opposite of what we find (Kornblatt et al., 1984, 1993).

[4]About 10 water molecules are sequestered per mole of complex formed. Assuming a global volume of 18 ml/mol for bulk water, (10 mol/mol of complex) * (18 ml/mol) * 0.25 yields a value of ΔV of ~45 ml/mol of complex. This value differs from that found by a factor of 3.

tive to the osmotic stress values are too high to be explained by anything other than intermediates in the osmotic stress studies or by invoking altered stoichiometry and binding to a different site on the oxidase.

In the mitochondrion, reduced oxidase normally interacts with oxidized cyt *c*, and oxidized oxidase normally interacts with reduced cyt *c*. We have yet to devise a method to monitor these reactions without electron transfer occurring at the same time. The reactions that we can monitor are those occurring between the totally oxidized partners in the one case, and the totally reduced partners in the other. The reactions shown in Table 2 permit us to say that even though the values of K_d are the same for the two complexes (data not shown), there are significant differences in the amount of water that must be incorporated into the complexes.

This is in contrast to the majority of binding reactions for which we have data; in most instances there is a net expulsion of water from the interfacial region. Hydrostatic pressure favors dissociation of the cyt b_5/cyt *c* complex, (Rodgers et al., 1988), and of oligomeric proteins such as enolase (Paladini and Weber, 1981; Kornblatt et al., 1995) or lactate dehydrogenase (reviewed in Gross and Jaenicke, 1994); hydrostatic pressure depolymerizes tubulin (Salmon, 1975). All of the above examples exclude water from the interfacial regions of the complexes. There are only two systems that we know that sequester net amounts of bulk water when one protein binds to another. The two are the binding of cyt *c* by the cyt *c* oxidase (Kornblatt et al., 1993), and the binding of the antibody D1.3 by hen egg white lysozyme (Bhat et al., 1994).

The data of Table 2 were used to construct the thermodynamic cycle of Scheme II. Here, too, our major assumption is that the volume changes detailed in the table are additive; this is borne out by the data. The sum of the volume changes for a cycle should be zero; for Scheme II, it is. The most striking feature of Scheme II is that large volume changes occur upon binding of cyt *c* to the oxidase and upon reduction of the oxidase, with or without cyt *c* bound. The total volume change that occurs when cyt *c* and cyt *c* oxidase are reduced individually is less than that which occurs when the complex is reduced. This can only mean that the structure of the two proteins in the complex differs from that of the free components.

What do we mean by different? At a minimum, the reduced complex must be more hydrated than the components. Most likely it means that substantial conformational changes occur in the oxidase when the complex forms and is subsequently reduced.

We have previously shown (Kornblatt et al., 1988; Kornblatt and Hui Bon Hoa, 1990) that changes in hydration are linked to electron transfer. A percentage of the volume changes shown in Tables 1 and 2 must be associated with solvent movements during the catalytic cycle. Blocking the movement of water, by either osmotic or hydrostatic pressure, blocks electron transfer within the complex. Electron transfer is linked to proton pumping. We speculate that at least some of the changes in hydration that we are measuring represent water moving into and out of the channel through which protons are pumped.

The whereabouts of this channel remain a subject of speculation. Everyone who tries is able to trace a path in the oxidized protein from the matrix face of the protein into the region just before the hemes. From the cytosolic face one can similarly trace a cavity into the region near the hemes. Between the two open spaces, there is packed protein. It is clear that during catalysis the protein moves; it opens. It rearranges the amino acid side chains so that space can be made for the transported proton and its solvent shell. In so doing it creates new spaces and collapses some of the old.

The volume changes that we have measured are the sum of these two processes. Clearly, both are extremely important in the process of moving protons from one side of the membrane to the other. At a minimum, the process probably involves opening a privileged compartment from the matrix side in response to electron transfer. A water-borne proton would then enter this space, which would then be closed. Release of the hydrated proton would only occur when a pore opened on the cytosolic side; the pore would have to close for the cycle to start again. This opening, closing, opening, and closing has to involve solvent flux and it has to involve rearrangement of the protein. Dilatometry has allowed us to measure the combined change. We speculate that steps in which large volume changes occur correspond to those openings and closings. The cyt *c* oxidase is behaving as though it were processing protons from one side of the mitochondrion to the other. We obviously take water movements and the associated conformational changes seriously. Is there reason for anyone else to do so? Are they just another interesting sideline in the overall reaction scheme of the oxidase?

There is one cogent reason for thinking that they should be taken seriously: there are four energy-transducing proteins described to date that seem to have water chains or water cavities required for transduction.

1. The crystal structures of the *Paracoccus* and beef heart oxidases both reveal water pockets; these pockets contain ~10 water molecules that are sufficiently rigid such that they appear in the crystal structure. Some of the waters are seen in detail in the latest beef heart structure (Tsukihara et al., 1996). (The waters are seen in the original journal article, but are not present in the PDB file (PDB1OCC).) The pockets are close to the hemes and near the proposed proton transfer path. In previous work we proposed that there should be at least one protected compartment that contained ~10 waters; this compartment should open and close on opposing sides of the membrane, thereby resulting in water flow along with the pumped protons (Kornblatt and Hui Bon Hoa, 1990). We showed that four water molecules could be trapped within the oxidase such that they did not exchange on an hour time scale (Kornblatt and Kornblatt, 1992). Most importantly, we have shown in the osmotic stress work and in the high hydrostatic pressure work that the oxidase will not catalyze electron transfer unless water

is free to move in and out of the protein (Kornblatt et al., 1988; Kornblatt and Hui Bon Hoa, 1990).

2. Bacteriorhodopsin is the major proton pump of the Halobacteria. The photocycle of bacteriorhodopsin involves protonation steps as well as hydration linked to the protonation. Water molecules have been identified in the presumed proton transfer path (Cao et al., 1991; Yamazaki et al., 1995). The photocycle has been shown to occur with volume changes on the order of 30 ml/mol for the protonation of the Schiff base from the extracellular side (Varo and Lanyi, 1995). The imposition of pressure blocks the cycle at the level of proton uptake (Varo et al., 1995).

3. The bacterial photoreaction center was shown to contain a continuous chain of water molecules that stretch from one side of the membrane to the other (Ermler et al., 1994). Mutations that lead to disruption of this chain lead to a reduction in the rate of proton pumping by the center (Baciou and Michel (1995).

4. The cyt b_6f has a chain of five waters that pass through much of the molecule but form a privileged site, well insulated from the aqueous surroundings. The residues that form the tunnel for this chain are highly conserved throughout the 13 known cyt b_6f sequences (Martinez et al., 1996).

In conclusion, we have shown that there are several steps in the catalytic cycle that are associated with large movements of solvent. We believe that these movements are part of the proton pumping machinery of the oxidase. To date, everything that disrupts the movement of water between bulk and the protected environment of the protein disrupts electron transfer. This appears to be a characteristic of the cyt *c* oxidase as well as that of other energy-transducing proteins.

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