A Nontraditional Role for Water in the Cytochrome *c* Oxidase Reaction†

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ABSTRACT: The passage of electrons through cytochrome *c* oxidase is directly related to the activity of water. Reducing the activity in a system containing reductant, oxygen, and cytochrome oxidase blocks electron transfer between reduced cytochrome *a* and oxidized cytochrome a_3. The extent of the block is directly related to the osmotic pressure of the system, implying that the protein shell of the oxidase acts as a semipermeable membrane that excludes osmotic perturbants but not water. It appears that approximately 10 water molecules must enter and leave the oxidase in order for internal electron transfer to occur.

Cytochrome c oxidase (cytox)[1] is the terminal electron-transfer component of the mitochondrial electron-transfer chain and a site of energy coupling. The enzyme accepts electrons from the one-electron donor, cytochrome *c*, and subsequently carries out the transfer of four electrons to oxygen. During this process, energy is immediately stored in the form of an electrochemical potential for protons and ultimately converted into ATP. The mechanism by which the enzyme pumps protons is unknown, but it is felt that conformational changes are likely to be involved and are also likely to be among the early steps in the transduction process. [Information related to the role of conformations in energy transduction by the oxidase has recently been reviewed by Brunori et al. (1987), by Malmstrom (1989), and by many others.]

The catalytic cycle of the oxidase is complex; it involves many intermediates and conformations. The resting, pulsed, and oxygenated conformations have long been the subject of our study, as well as that of many other laboratories. The interconversions of these three forms, as well as some hypothetical states, during the catalytic cycle have been summarized and are repeated in Scheme I for the convenience of the reader. The scheme is not meant to be exhaustively complete. It omits all partially reduced forms except those of immediate interest here [see Hill et al. (1986) for a review]; it also omits all consideration of the monomer/dimer equilibrium (Nalecz et al., 1983) and the role it might play in proton pumping. Lastly, the scheme omits the fact that the enzyme also contains Mg, Zn, and additional Cu (Einarsdottir & Caughey, 1985; Bombelka et al., 1986); these have been omitted since roles for them have not been assigned.

The known effectors for the interconversions of the conformers are protons or hydroxyl, and reduced or oxidized cytochrome *c*. The significant aspect of the conformational changes exhibited by cytox is that at least two pathways are available to it. Low pH and a high cytochrome *c*(III)/cytochrome *c*(II) ratio promote the pathway leading to the pulsed oxidase (Antonini et al., 1977; Brunori et al., 1979) (the upper pathway); high pH and a low ratio lead to the "oxygenated" (Okunuki et al., 1958) or 428-nm form (the lower pathway). The latter two conformations are unstable[2] and convert on standing to the resting form.

This fact should be emphasized because it points out an obvious, but sometimes overlooked, aspect of work dealing with conformations. In an ensemble of molecules in solution, the molecules are never all poised in the same state. In any given solution of oxidase at equilibrium, the different conformations are continuously being sampled by the ensemble. A pure resting, pulsed, or oxygenated sample is never encountered, nor do we ever see a pure spectrum for any of the above conformations.

When a protein undergoes a conformational change the partial specific volume of the structure changes. Often this change, ΔV, is small and cannot be easily detected; sometimes the change is large, the equilibrium between the forms is in a reasonable range, and ΔV can readily be evaluated at pressures below 3 kbar. With this in mind, we embarked several years ago on a pressure analysis of the conformational changes of cytochrome *c* oxidase. The volume change between the resting and oxygenated conformations, as well as that between the resting and pulsed conformations, is small as can be seen in Scheme I. An important difference shows up when the aerobic steady state is probed; here there is a step that is sensitive to hydrostatic pressure that has a volume change of about −76 mL/mol and that introduces a block in internal electron transfer between cytochrome *a* and cytochrome a_3 (Kornblatt et al., 1988).

We reasoned that −76 mL/mol was too large to be the result of only a conformational change and that solvent fluxes into and out of the oxidase might be obligatory steps during the electron-transfer reactions and the associated conformational changes. We hypothesized that solvent entry followed the entry of the first pair of electrons and that solvent entry was favored by high hydrostatic pressures. We proposed further that solvent exit was required for the reorganization of the molecule such that internal electron transfer from the cytochrome $a\mathrm{Cu_A}$ couple to the cytochrome $a_3\mathrm{Cu_B}$ couple could occur. We recognized that we were proposing a "futile" water cycle but felt, first, that such a scheme was consistent with the data and, second, that it might be part of the transduction process whereby the oxidase pumps protons.

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[1] Abbreviations: P_o, osmotic pressure; P_h, hydrostatic pressure; ΔV_o, osmotic component of ΔV; ΔV_h, ΔV due to hydrostatic pressure; ΔV_c, conformational component of ΔV; ΔV_{ao}, all other components of ΔV, including conformation, intramolecular, and "other"; cytox, $a^3\mathrm{Cu_A(II)}$-$\mathrm{Cu_B(II)}a^3{}_3$, cytochrome *c* oxidase.

[2] A recent report has found that the pulsed oxidase can be isolated in stable form (Brandt et al., 1989).

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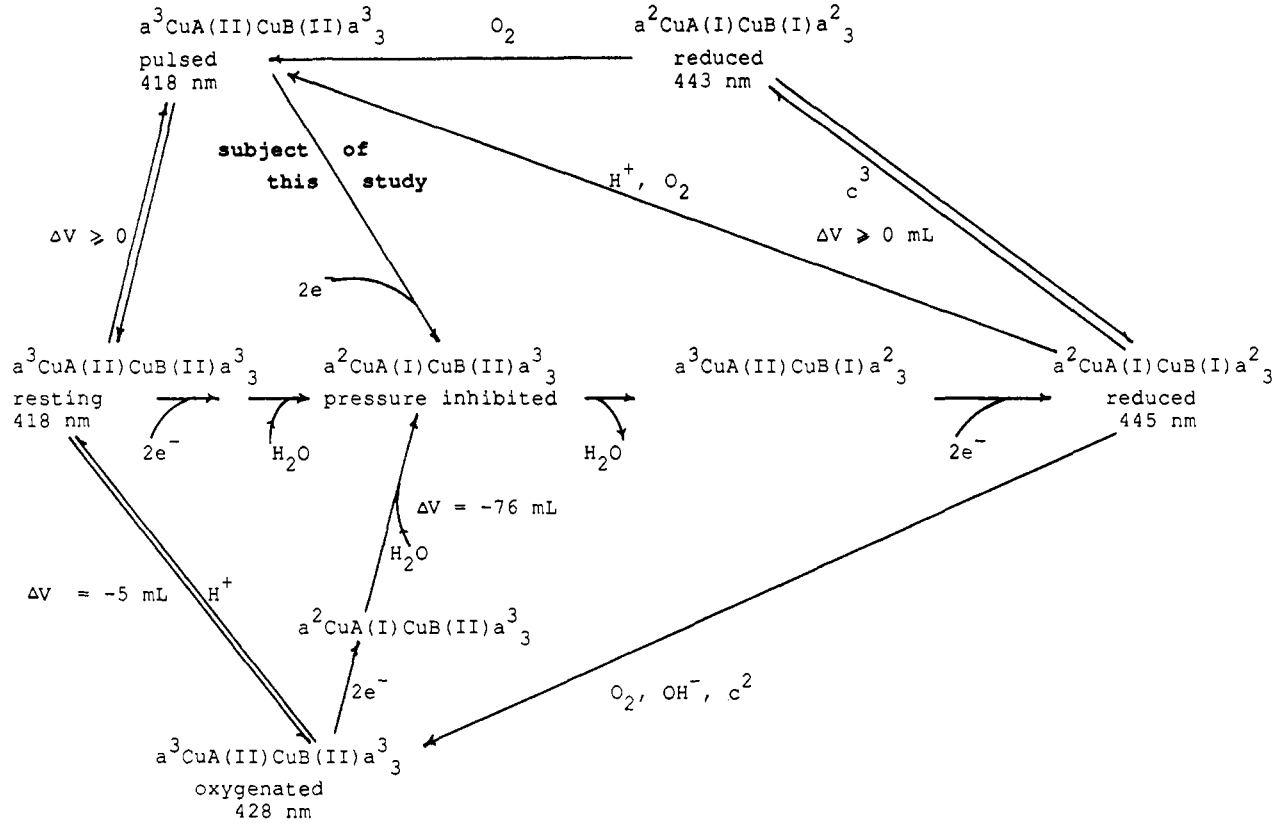
Scheme I: The Catalytic and Conformational Cycles of Cytochrome *c* Oxidase[a]



[a] The enzyme as isolated is in the resting form. During and just after turnover it exists as either the pulsed (upper path) or oxygenated (lower path) oxidase. The choice of pathway is dictated by the environmental conditions during and after turnover. The upper pathway is favored by low pH and/or a high ratio of cytochrome *c*(III)/*c*(II) as well as the presence of glycerol. The lower pathway involving the oxygenated form is favored by the opposing conditions, high pH and a low ratio. The oxygenated enzyme has been shown to be generated when the pulsed enzyme reacts with peroxide (Kumar et al., 1984; Witt & Chan, 1987); it is likely, however, that other routes can lead to the same spectral species. All three forms, pulsed, resting, and oxygenated, are totally oxidized oxidase and have been shown to be different conformations of the protein. When subjected to high hydrostatic pressure in the presence of reductant, all three forms yield the partially reduced form shown in the center of the diagram. The volume change that accompanies the reaction oxygenated to the form trapped by *hydrostatic* pressure has been well-defined; the value is −76 mL mol^{-1} (Kornblatt et al., 1988). The volume change for the transition pulsed to *hydrostatic* pressure trapped form is shown in this study to be about −60 mL mol^{-1} (Figure 5). The volume change associated with the resting to trapped form is not known with certainty. Since the four forms, resting, oxygenated, pressure inhibited, and pulsed, form a closed cycle, and since the net volume change around a cycle must total zero, we can conclude that the volume change for the resting to pulsed oxidase must be about 10 mL mol^{-1} negative in the direction of pulsed to resting.

There is an obvious test of the hypothesis. If the activity of water can be lowered sufficiently, entry of water into the oxidase might become rate limiting for internal electron transfer during the aerobic steady state. If lowering the water activity to 10% or 20% of its normal value has no effect on the distribution of oxidase redox states, we would be forced to conclude that any water bound to the oxidase was so tightly bound that it could not be taking part in the overall volume change we had seen. If lowering water activity caused the appearance of reduced cytochrome a_3, it would be difficult to draw any conclusions about entry and exit of water. Since so many things are capable of binding to the reduced, pentacoordinate cytochrome a_3, determining whether the effect was directly on the cytochrome as opposed to solvent movement would be difficult. The third possibility is that lowered water activity would result in the accumulation of reduced cytochrome aCu_A while cytochrome a_3Cu_B remained oxidized; this result would be consistent with our original hypothesis.

We report here that lowering water activity results in raising the steady-state ratio of reduced to oxidized cytochrome *a* and that the changes are consistent with the oxidase behaving like a semipermeable membrane vis a vis the perturbants that lower water activity. To a first approximation, the oxidase is sensing the osmotic pressure of the medium via water molecules that should be bound in order for internal electron transfer to occur. It appears from the sensitivity that approximately 10 water molecules may be involved.

Materials and Methods

The preparation of beef heart cytochrome *c* oxidase has been described (Yonetani, 1966; Kornblatt et al., 1973). Following the final chromatography step on Sepharose 6B-CL, the protein was transferred to a dialysis sack and concentrated to about 150 μM (heme *a*) using Aquacide II (Calbiochem). The resulting oxidase solution was brought to 50% glycerol and stored at −20 °C.

The cosolvents, with the exception of glycerol, were all freshly distilled. Glycerol (Janssen) was of the highest purity available and contained no endogenous reductants when tested with cytochrome *c*. LiCl was purchased from Merck.

Ethylene glycol and glycerol were added to the oxidase at temperatures maintained between −5 and −10 °C. Adequate mixing of the viscous solutions was ensured by using an overhead stirrer turning at 300 rpm. LiCl was dried to constant weight, and solutions were prepared that contained the requisite concentration after the final addition of cytochrome *c* oxidase. The pH of the glycerol and ethylene glycol solutions was controlled as described (Douzou, 1977).

Osmolalities of the ethylene glycol and LiCl solutions were calculated by using published data (*Handbook of Chemistry*

and Physics, 46th ed.); the osmolalities of the glycerol solutions were estimated by extrapolations of the data contained in the *Handbook of Chemistry and Physics*. Osmotic pressure (P_o) was evaluated (Glasstone, 1947; Marshall, 1978) by using

$$P_o\bar{V}_{H_2O} = RT \ln X_{H_2O} \quad (1)$$

where P_o is the osmotic pressure in bars and X_{H_2O} is the mole fraction of water determined from the osmolality.

Experiments at high hydrostatic pressure were carried out by using a pressure bomb interfaced to a Cary 210 spectrophotometer. The apparatus has been described (Hui Bon Hoa et al., 1982). The experimental data were all obtained at temperatures between 4 and −4 °C. Temperatures below zero were required for the samples containing greater than 30 *m* glycerol.

The typical experiment on the aerobic steady state was carried out as follows: The oxidase/cosolvent mixture contained 2.5–7.5 μM cytox (5–15 μM heme *a*), 0.1 M Bistris, 5 mM KCl, 0.5 mM EDTA, 0.5 mM Tris, and 0.05‰ Tween 80, cosolvent, or LiCl, pH 7, 4 to −4 °C. The aerobic steady state was initiated by the addition of the reductants ascorbate and TMPD to final concentrations of 8 mM and 80 μM, respectively. The samples were transferred to cuvettes, and where necessary, the cuvettes were centrifuged in order to rid them of bubbles. Each sample was covered with a 2-cm layer of light mineral oil to ensure that it would ultimately go anaerobic. The spectrum of each sample was monitored until the steady state was reached. At the low temperatures used here this required between 20 min for the low concentrations of osmotic perturbant to 45 min for the higher concentrations. The samples were later monitored spectrally for the totally reduced protein.

When the effects of high hydrostatic pressure were studied, the samples were transferred to the high-pressure cuvette and placed into the bomb. The spectrum of the sample was monitored until the steady state was reached. The pressure on the sample was then increased, and the effects were followed spectrally. Ultimately, the samples went completely anaerobic.

The absorbances at 445 and 605 nm in the totally reduced state were used to calculate the total oxidase present. The absorbances at 600 nm during the aerobic steady state were used to calculate the ratios of reduced cytochrome *a* to pulsed oxidase; the outline of the calculation, which was used for the evaluation of the ratio of reduced cytochrome *a* to oxygenated, has been previously described (Kornblatt et al., 1988). The details of the assumptions and calculations follow: The pulsed enzyme has a Soret peak which is indistinguishable from that of the resting enzyme (Brunori et al., 1979; Kornblatt & Luu, 1986); we assume that the spectral contributions of cyt *a* and cyt a_3 to this peak are the same as in the resting enzyme as described by Vanneste (1966). Importantly, there is no hump in the Soret band of the pulsed enzyme (which would indicate reduced cyt *a* or a_3) even though the total extinction coefficient in the α band is increased from 9 to 13.6 mM^{-1} cm^{-1}. It follows therefore that the α band absorption is the result of the altered heme environment in the pulsed enzyme rather than its altered redox state. The extinction coefficient of partially reduced enzyme, cyt $a^2a^3_3$, in the α region is 18 mM^{-1} cm^{-1}. We assume that the cyt a^3_3 contribution in the pulsed and partially reduced cyt $a^2a^3_3$ form is the same and that the contributions of the cyt a^3 (pulsed) and cyt a^2 are linear functions of the difference between the 600-nm extinction coefficient of the pulsed and that of the cyt $a^2a^3_3$. For example, treating the oxidase with 46% glycerol led to a relative extinction coefficient of 14.8 mM^{-1} cm^{-1}. This value is the sum of the individual values such that

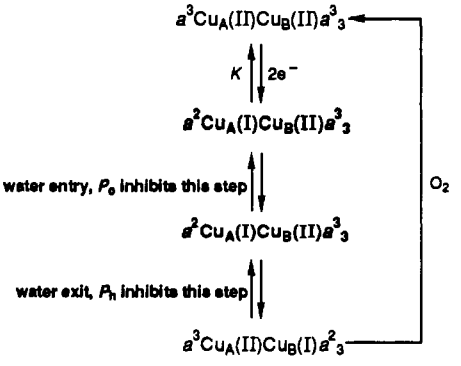
14.8 mM^{-1} cm^{-1} = (100 − % reduced cyt *a*) × (13.6 mM^{-1} cm^{-1}) + (% reduced cyt *a*)(18.0 mM^{-1} cm^{-1})

This represents 27% reduced cyt *a* and 73% pulsed cyt *a* (100–27); the ratio of reduced cyt *a* to pulsed would be 0.37 [0.27/(1 − 0.27)]. The assumptions leading to this number are almost certainly valid when the steady state is perturbed by hydrostatic pressure. In the latter case one is able to increase the pressure above 1700 bar, which allows one to obtain spectra which are indistinguishable from cyt $a^2a^3_3$ (Vanneste, 1966). In the experiments reported here it has not been possible to increase the cosolvent to concentrations high enough such as to trap a final and stable form; the oxidase denatures at the required concentrations. Nonetheless, in experiments in which both osmotic and hydrostatic pressure were varied there was no evidence for any discontinuity in the growth of the partially reduced spectra as hydrostatic pressure was varied nor was there accumulation of reduced cytochrome a_3 in the final combined hydrostatic/osmotic pressure trapped form.

The extinction coefficients at about 600 nm used for the totally reduced oxidase, $a^2Cu_A(I)Cu_B(II)a^3_3$, and totally pulsed oxidase were 20.0 mM^{-1} cm^{-1} (Yonetani, 1966), 18.0 mM^{-1} cm^{-1} (Vanneste, 1966), and 13.6–13.9 mM^{-1} cm^{-1} (Brunori et al., 1979), respectively.

The ratios, reduced cytochrome *a* to pulsed oxidase, were taken to be an indication of the extent of inhibition of electron transfer between the cyt aCu_A pair and the cyt a_3Cu_B pair of the oxidase. The value of the ratio was treated as an equilibrium constant, recognizing that the data were obtained under nonequilibrium conditions. We previously discussed the problems to which this gives rise (Kornblatt et al., 1988). In order to apply a thermodynamic analysis to steady-state data, it is neccessary that the enzymatic step in question be at or close to equilibrium for the extracted thermodynamic parameters to have signifiance (Dixon & Webb, 1979). In the work presented here we cannot prove but we do have data that would at least indicate that our experimental conditions are probably not far from equilibrium.

The apparent equilibrium constant, *K*, that we calculate is *representative* of the hypothesized reaction shown below in bold type (Kornblatt et al., 1988); as inhibition due to osmotic pressure becomes greater, there is a commensurate increase in the level of $\mathbf{a^2Cu_A(I)Cu_B(II)a^3_3}$, resulting in an increased value of *K*.



If the hypothesized reaction is truly at equilibrium, the ratio of the pulsed species to $a^2Cu_A(I)Cu_B(II)a^3_3$ should be independent of electron flux through the oxidase. We have found that we could vary the levels of both TMPD and ascorbate, the electron donors ($2e^-$) for the steady state, over a minimum 5-fold range of concentrations without effecting the levels of pulsed and partially reduced oxidase and thereby the value of *K*. In such experiments, the samples attain steady state and

go anaerobic faster when the TMPD and ascorbate are increased; conversely, lowered TMPD and ascorbate cause the samples to attain steady state and go anaerobic more slowly. It must be emphasized however that the value of K does not appear to be influenced by the overall electron flux through the enzyme. We take this to mean that our steady-state estimate of equilibrium data is valid to a first approximation.

The variation of K as a function of pressure was treated in the standard manner in order to extract a value ΔV (eq 2).

$$\Delta V = RT\,(\partial \ln K)/(\partial P) \qquad (2)$$

While the equation is routinely applied to systems at high hydrostatic pressure where the density of the system is being perturbed, its use is also valid for a system probed with osmotic pressure (Franks, 1985; Parsegian et al., 1986; Rand, 1981; Zimmerberg & Parsegian, 1986) where the solute inaccessible space is perturbed. This value, as previously explained (Kornblatt et al., 1988), does not represent a standard change in volume, $\Delta V°$, but rather is indicative of the susceptibility of the sensitive step to pressure.

For the case where the pressure term is hydrostatic

$$\Delta V_h = \Delta V_o + \Delta V_{ao} \qquad (3)$$

where

$$\Delta V_{ao} = \Delta V_c + \Delta V(\text{intramolecular}) + \Delta V(\text{other})$$

In eq 3 all pressure-sensitive steps are probed by hydrostatic pressure (Balny et al., 1989).

For the case where the pressure term is osmotic, we make two assumptions: (1) to a first approximation there is no contribution from the other terms, and (2) there is no interaction between the osmotic and other terms. The above assumptions allow us to assign specific contributions to ΔV_o and $[\Delta V_c + \Delta V(\text{other}) + \Delta V(\text{intramolecular})]$.

Results

Effects of Cosolvents on the Totally Oxidized and Totally Reduced Cytochrome Oxidase—The Controls. We have tested several cosolvents for their effects on the structure of cytochrome oxidase. All, if brought to sufficiently high concentrations, were capable of denaturing the protein as judged by spectral measurements or by precipitation. The cosolvents tested included methanol, ethanol, propanol, ethylene glycol, propanediol, glycerol, dimethylformamide, dimethyl sulfoxide, and LiCl. All but glycerol, ethylene glycol, and LiCl denatured the oxidase when the cosolvents were at relatively low concentrations. Glycerol (Figure 1) could be added to a final concentration of 60% (vol/vol) before there was any alteration in either the Soret or visible spectra of the protein. At 90% glycerol the oxidase still maintained the majority of its spectral character, but there were noticeable shifts of the protein toward low-spin forms (Phillips, 1963) as evidenced by the red shift in the oxidized and blue shift in the reduced Soret spectra (Figure 1). The most important aspect of Figure 1 is the fact that both oxidized and reduced proteins show an invariant spectrum up to 60% glycerol after which there is some modification of the reduced spectrum at 75% glycerol. All further experiments using glycerol as perturbant were carried out at concentrations at or below 63% in order to ensure that we were dealing with the same phenomena throughout.

Ethylene glycol could be added to concentrations somewhat above 60% before there were noticeable changes in the spectrum. If the protein/cosolvent mixture was allowed to warm above 10 °C, the solutions ceased to be optically clear and the protein could be sedimented. Ethylene glycol that was not freshly distilled from Mg metal also had a tendency to alter the spectral character of the oxidized protein. The experiments

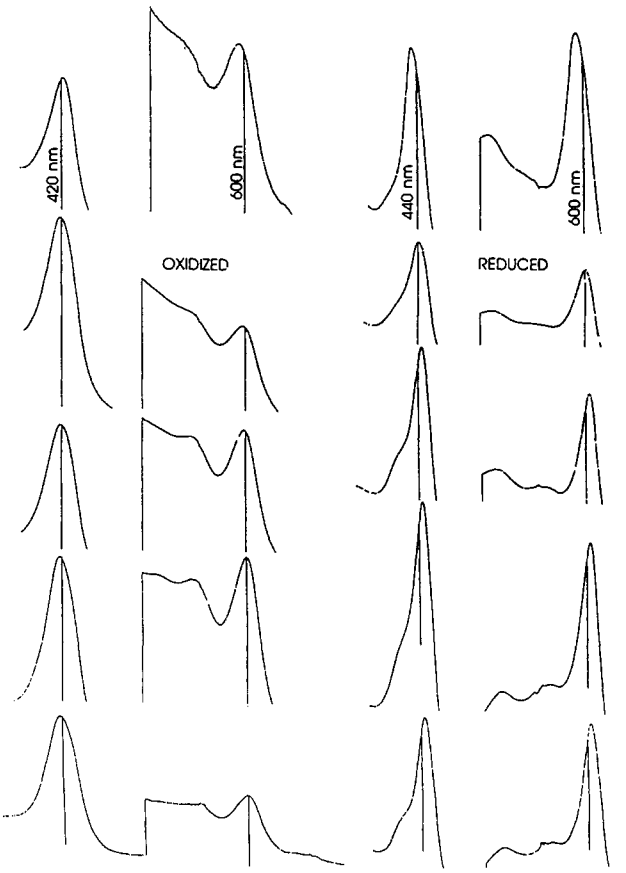


FIGURE 1: Glycerol effects on the spectra of cytochrome *c* oxidase. The oxidized enzyme is shown on the left, the dithionite reduced enzyme on the right. The enzyme was prepared as detailed under Materials and Methods; the glycerol, expressed as vol/vol, and the oxidase concentrations, expressed as heme *a*, were as follows: bottom row, 0%, 7.6 μM oxidized, 6.3 μM reduced; second row from bottom, 40%, 23 μM oxidized, 20 μM reduced; middle row, 60%, 20 μM oxidized, 17.4 μM reduced; second row from top, 75%, 14 μM oxidized, 10 μM reduced; top row, 90%, 5 μM oxidized, 4.5 μM reduced. The scales for all the spectra were chosen so as to maximize the definition of the spectra; the bars at 420, 600, 440, and 600 nm are wavelength markers.

reported here used ethylene glycol at concentrations at or below 60% at temperatures at or below 4 °C.

LiCl could be added at concentrations up to 10 *m* without inducing major changes in the protein. An oxidase solution containing 10 *m* LiCl had the same spectral character as an oxidase solution in 80% glycerol.

The glycerol solutions containing either oxidized or reduced cytochrome oxidase (Figure 1) were subjected to hydrostatic pressures up to 3500 bars (data not shown). In no instance did the hydrostatic pressure cause more than a 2-nm shift in the absorption bands. The absorbances remained constant when corrected for solvent compression. This indicates that the predominant forms of the oxidized and reduced oxidase, which are in equilibrium with alternate forms, do not have partial molar volumes which are substantially different.

In that which follows we present the data for glycerol since the data set is more complete. The glycerol data are representative of the other cosolvents.

Effects of Cosolvents on the Aerobic Steady State. The behavior of cytochrome oxidase during the steady state has already been described (Kornblatt et al., 1988; Kornblatt & Luu, 1986); there is a striking dependence on the history of the protein and on environmental conditions as indicated in Scheme I. At 1 bar, in the absence of cytochrome *c* and cosolvents and at neutral pH, the steady-state form that is

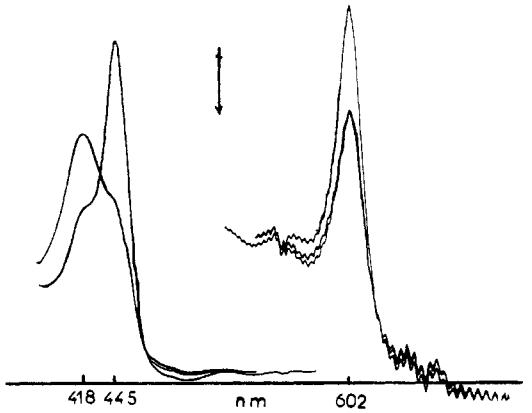


FIGURE 2: Effects of 47% glycerol on the spectra of cytochrome oxidase during the aerobic steady state and when totally reduced. The oxidase in glycerol was prepared as described under Materials and Methods. The steady state (lower trace) was initiated with TMPD and ascorbate, and the spectra were monitored until constant, about 30 min. The sample went reduced after another hour (upper trace). The arrow indicates the scale: 0.1 A for the Soret region and 0.02 A for the visible. The significant aspects of the steady-state spectrum are the extinction coefficient of the visible peak, 14.8 mM^{-1} cm^{-1}, and the pronounced hump at 440 nm indicating partial reduction of the oxidase.

routinely found is predominantly "oxygenated" (Kornblatt et al., 1975, 1988; Kornblatt & Luu, 1986); under the same conditions, in the presence of low concentrations of glycerol (0.2 M), the form of the oxidase found during the aerobic steady state was predominantly pulsed as indicated by peak positions and extinction coefficients in the Soret and α regions (Kornblatt & Luu, 1986). We have referenced all the steady-state spectra to the pulsed state since we find it to be the predominant form whenever cosolvent is present; the extinction coefficient at 600 nm of this form is 13.6–13.9 mM^{-1} cm^{-1}.

The addition of higher concentrations of glycerol gave rise to steady-state spectra that were displaced from the pulsed as can be seen in Figure 2. Here the glycerol concentration is 11.7 *m* (46%), and in the presence of TMPD and ascorbate, the extinction coefficient of the visible peak is 14.8 mM^{-1} cm^{-1} (lower curve) instead of 13.6–13.9 mM^{-1} cm^{-1}. We have used the spectral data from this experiment to calculate an "equilibrium constant", (reduced cytochrome *a*)/(pulsed oxidase), as described under Materials and Methods; the value is about 0.37.

At lower concentrations of glycerol the same block is present, but it is not as obvious as at the high concentrations; it becomes increasingly difficult to determine the ratios, and thereby the K_e, of the two forms, as the absorbance of the α band approaches that of the "pure" pulsed form. The errors in the estimation become quite large.

We have obviated this problem in the following manner. Hydrostatic pressure also introduces a block in internal electron transport between cytochrome *a* and cytochrome a_3 (Kornblatt et al., 1988). We have therefore subjected a series of samples containing varying concentrations of glycerol and constant concentrations of cytochrome oxidase and TMPD/ascorbate to high hydrostatic pressures; the plots of ln *K* vs pressure were linear, as they are in the absence of glycerol, thereby allowing us to extrapolate back to K_e at 1 bar. This ruse allowed us to accurately determine K_e values, at 1 bar, which were as low as 0.05; this would not have been possible in the absence of hydrostatic pressure. The complete data set ln $(K_e)_{1bar}$ was plotted against glycerol concentration and the osmotic pressure of the solution; the results are shown in Figure 3. What is most striking about this plot is that ln (K_e) scales as a linear

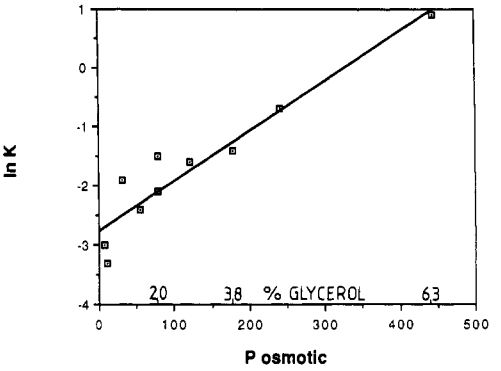


FIGURE 3: ln *K* (reduced cytochrome *a*/pulsed oxidase) scales as a linear function of osmotic pressure at a hydrostatic pressure of 1 bar. The equilibrium constants were calculated as detailed under Materials and Methods. The volume change calculated on the basis of the slope was −190 mL mol^{-1}.

function of osmotic pressure. ΔV, calculated by using eq 2, evaluates to 190 ± 22 mL mol^{-1}. The assumption that we are making here is obvious: We ascribe the perturbation of the oxidase to water and its reduced activity and not to a direct interaction of glycerol with the oxidase. Further, we postulate that cytochrome *c* oxidase contains an osmotically active compartment when the protein is turning over which is not detectable when the protein is either in the fully oxidized or fully reduced static states.

We have also perturbed the aerobic steady state of the oxidase with ethylene glycol and with LiCl. With these two products we obtained the equilibrium constants directly, i.e., they were not obtained from pressure extrapolations. This has the advantage of increasing the rate at which data can be obtained with the disadvantage that the data set is necessarily far smaller. K_e that lie between 0.2 and 2 are reliable whereas below 0.2 the accuracy of the determination is dubious and above 2 the concentration of ethylene glycol denatures the protein. The values obtained for ΔV were 233 ± 38 mL mol^{-1} for ethylene glycol and 248 ± 9 mL mol^{-1} for LiCl. In neither case are the data shown.

The ΔV values for the three perturbants are virtually the same and cannot be distinguished experimentally.

In the case of glycerol the data indicate that K_e should be about unity when the osmotic pressure is about 330 bar (Figure 3) or about 55% glycerol. Qualitatively this agrees well with the spectrum shown in Figure 2. When ethylene glycol was the perturbant, K_e = 1 was obtained at 340 bar osmotic pressure; when LiCl was the perturbant, the corresponding value was 270 bar. These three values are reasonably close to one another and indicate that all three perturbants are probing the same aspect of the protein.

The data relating K_e to the osmotic pressure of the glycerol solutions were obtained via extrapolations from ln K_e vs P_h plots. We have used the same plots to extract the value of ΔV_o at 1000 bar. For a rigorous analysis of the combined effects of hydrostatic and osmotic pressure, it is necessary that ΔV_o be independent of hydrostatic pressure. The variation of ln $(K_e)_{1000bar}$ vs P_o is shown in Figure 4. Once again, the two are linearly related; ΔV at 1000 bar extrapolates to 165 ± 20 mL mol^{-1}, indicating little interaction between the osmotic term and other processes occurring after the block.

However, ΔV_h is not a constant function of osmotic pressure but varies as shown in Figure 5; this indicates that ΔV_o and ΔV_{ao} are not totally independent of one another.

Discussion

In Peter Mitchell's original formulation of the chemiosmotic hypothesis, he assigned a passive role to cytochrome *c* oxidase;

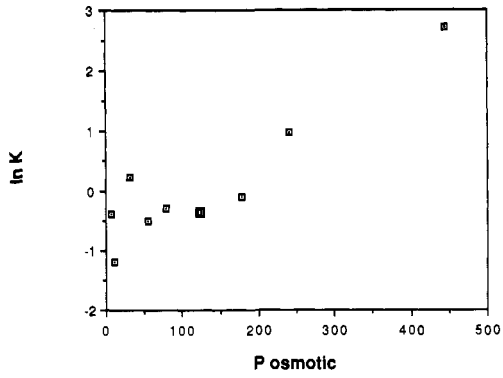


FIGURE 4: ln K (reduced cytochrome a/pulsed oxidase) scales as a linear function of osmotic pressure at a hydrostatic pressure of 1000 bar. The equilibrium constants were calculated as detailed under Materials and Methods. The volume change calculated on the basis of the slope was −165 mL mol^{-1}.

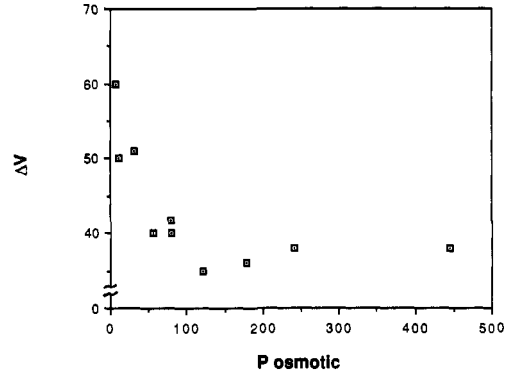


FIGURE 5: ΔV (hydrostatic) as a function of osmotic pressure. Oxidase samples, in the aerobic steady state, at various glycerol concentrations were subjected to hydrostatic pressure and the equilibrium constants determined. ΔV's were calculated from the slopes of the ln K vs P_h curves.

the protein established a proton gradient by reducing oxygen on the mitochondrial side of the inner membrane, thereby generating hydroxyl ions (Mitchell, 1966). With Wikstrom's subsequent discovery that the oxidase acted as a proton pump (Wikstrom, 1976), the question arose as to how to couple electron transport to proton translocation. We still have little idea as to the molecular mechanism by which the two processes are coupled, but it is safe to say that the purely osmotic aspects of chemiosmosis have been largely ignored as being potentially of interest in the establishment of the proton gradient.

The osmotic stress approach to the study of the interactions between membranes and between macromolecules was pioneered by Parsegian and Rand and their associates. The approach has been extended to an electrophysiological study of the voltage-dependent anion channel (VDAC) of the mitochondrion (Zimmerberg & Parsegian, 1986). The data from their study indicate that the VDAC undergoes major structural changes. The osmotically active compartment of the VDAC, the channel, occupies a volume of 30 000 Å^3 mol^{-1} (20 000 mL mol^{-1}), which agrees reasonably well with values from other studies. The analysis used in this study is similar to that used with the VDAC.

The finding that cytochrome c oxidase was especially susceptible to hydrostatic pressure during turnover of the enzyme led us to postulate that there was an obligatory solvent cycle associated with the molecular mechanism of the oxidase (Kornblatt et al., 1988). Since the oxidase was not pressure sensitive in the totally oxidized state, it followed that reduction of the cytochrome aCu$_A$ center was necessary for the entry of solvent into the molecule. This step, solvent entry and sequestration in the protein, has a negative volume change and is favored by hydrostatic pressure. The block in electron transport from the aCu$_A$ couple to the a_3Cu$_B$ couple indicated that solvent had to leave the molecule in order for electron transport to resume. Solvent exit into the bulk solution has a positive volume change and is thereby disfavored by hydrostatic pressure. By modulating the activity of water during electron transport by the oxidase, it should be possible to disfavor the entry of water. If the major water entry step occurs just after the reduction of the aCu$_A$ couple, the net result of lowered water activity should take the same form as the application of high hydrostatic pressure; cytochrome a should go reduced at the expense of the pulsed oxidase.[3] If, on the other hand, the major entry of water occurs at some other stage of the mechanism, the interpretation becomes more difficult. We were fortunate, in terms of the analysis, that the step in the mechanism that was osmotically sensitive was that which follows the original entry of electrons into the oxidase.

We have presented data which suggest that there is a step in the mechanism of the cytochrome c oxidase that is sensitive to osmotic perturbants. In order to do so, it was necessary to choose a range of compounds that were highly water soluble such that the activity of water could be significantly lowered. Of the compounds tested, glycerol, ethylene glycol, and LiCl did not denature the oxidase at mole fractions of osmotically active components as high as 0.25. In addition, it was necessary that the perturbants not interact directly with the oxidase; their major effect had to be on the activity of water. Glycerol and ethylene glycol are sufficiently similar such that a study consisting only of the two would not have been able to detect subtle interactions with the oxidase; such a study would necessarily have been incomplete; the addition of LiCl to the list allows us to generalize our results.

There are two critical, interrelated results which indicate that the effects of perturbants are on the activity of water rather than on the oxidase itself. The first is that ΔV_o for the transition pulsed to reduced aCu$_A$ is the same for all three compounds. This is a quantitative and statistically significant result obtained from many data points. The second is that all three perturbants yield $K_e = 1$ when they are present at about the same osmotic strength. This indicates that we are working in a range of perturbant concentrations where only nonspecific effects are being sensed by the protein. The significance of these two results cannot be overstated; it indicates that even though there is some coupling between the osmotic term and all other terms in eq 3, the 1 bar results as a function of osmotic pressure imply an osmotically active compartment in the oxidase during turnover.

The water of interest in this study is detectable only during the catalytic cycle. It is not necessary that this water be directly involved in the catalytic cycle, but it is necessary that the component of the protein with which it equilibrates be linked to the catalytic cycle. We have interpreted the data in terms of blocking electron transport between the cyt aCu$_A$ couple and cyt a_3Cu$_B$; this aspect of the analysis, however, is not critical for the final conclusion. The catalytic cycle of the oxidase is sufficiently complex that it is undoubtedly naive to ascribe the block to electron transfer between two well-defined states. What is important is that overall electron transport

[3] It must be emphasized that the form would be the same, cytochrome a would go reduced, and there would be a loss of the pulsed spectrum, but the actual step being affected would be different (see Scheme I and Materials and Methods). Increasing osmotic pressure has opposite effects to increased hydrostatic pressure.

is inhibited and that the inhibition is associated with the differential hydration state of the enzyme forms present during turnover.

The water discussed here is clearly different from that recently discovered by Rousseau's group. Their work has uncovered at least two water molecules coordinated to the heme of cytochrome oxidase (Sassaroli et al., 1989); these are present in both the reduced enzyme and during turnover. The work reported here is closely related to studies by Wrigglesworth's group and by Escamilla et al. The former (John Wrigglesworth, personal communication) has studied the oxidase in solutions containing high glycerol and has concluded that the cosolvent inhibits the oxidase activity. The nature of the inhibition, they feel, is consistent with a proton or water mechanism. More recently, Escamilla et al. (1989) have studied the enzyme in reversed micelles; they concluded that low water activity inhibits the oxidase at the level of electron transfer from the aCu_A center to the a_3Cu_B center; in their study, mechanism and quantitation were not an issue.

Osmotic analysis as applied to the oxidase during turnover conditions has yielded interesting results. In this study we found that the volume change associated with the first step after the entry of electrons into the oxidase is about 200 mL mol^{-1}. Taking the partial molar volume of water as 18 mL mol^{-1} and assuming that the volume of the sequestered water is not sensed, this translates into about 10 molecules of water that must enter and exit the protein during every turnover; it must be emphasized that this number is a very rough approximation. The question of why these molecules must move is intriguing. For enzymes that accept carbon-based substrates into their active sites, enzymes such as the bacterial cytochrome P-450(cam), it is understandable that water can move into and out of the active site during a catalytic cycle. The hydrophobic cavity of this enzyme contains structured water when substrate is not present, and the water is replaced when camphor enters the pocket (Fisher & Sligar, 1987; Poulos et al., 1987). This situation does not apply to the oxidase. There are no large substrates, and no large cavities are needed to accommodate them. We speculate that the water movements that we are witnessing are part of the channel gating process of the oxidase. This channel is activated by electron transport and pumps protons across the mitochondrial membrane. At the same time, water passes through the channel. We have shown that the coupling between the electron-transfer capacity and solvent movement is rigorous; in other words, there is no slippage. It remains to be determined if there is similar obligatory coupling of the proton-transfer capacity of the oxidase to this osmotically sensitive step.

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