# Responses of Two Protein-Protein Complexes to Solvent Stress: Does Water Play a Role at the Interface?

Jack A. Kornblatt,*‡ M. Judith Kornblatt,*‡ Gaston Hui Bon Hoa,§ and A. Grant Mauk¶

‡Enzyme Research Group, Departments of Biology, Chemistry and Biochemistry, Concordia University, 1455 de Maisonneuve, Montreal, Quebec, Canada H3G 1M8; §INSERM-Unite 310, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France; and ¶Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

ABSTRACT We have analyzed the stability of the cytochrome *c*-cytochrome $b_5$ and cytochrome *c*-cytochrome *c* oxidase complexes as a function of solvent stress. High concentrations of glycerol were used to displace the two equilibria. Glycerol promotes complex formation between cytochrome *c* and cytochrome $b_5$ but inhibits that between cytochrome *c* and cytochrome *c* oxidase. The results with cytochrome $b_5$ and cytochrome *c* were expected; the association of this complex is largely entropy driven. Our interpretation is that the cytochrome *c*-cytochrome $b_5$ complex excludes water. The results with the cytochrome *c* oxidase and cytochrome *c* couple were not expected. We interpret them to mean that either glycerol is binding to the oxidase, thereby displacing the cytochrome *c*, or that water is required at this protein-protein interface. A requirement for substantial quantities of water at the interface of some protein complexes is logical but has been reported only once.

## INTRODUCTION

Brownian dynamics analysis has shown that the assembly of simple 1:1 protein-protein complexes can involve more than one docking trajectory; the initial encounter complex(es) formed upon protein-protein contact may not be identical to the final, thermodynamically stable complex (Northrup et al., 1988; Northrup and Erickson, 1992). For complexes of moderate stability ($K_d$ ca. $10^{-6}$ to $10^{-7}$ M) involving proteins of opposite electrostatic charge, more than one binding geometry may occur at equilibrium (Mauk et al., 1986). The relative stability of the alternative complexes will be a function of ionic strength, pH, and temperature. During formation of such 1:1 complexes, two limiting possibilities can be envisaged: 1) The point of closest approach between the two proteins includes the water shell encompassing one or both proteins. In this case, the shortest point of contact between the two proteins cannot be less than about 8 Å (Smith, 1991). 2) Alternatively, the hydration shell of one or both proteins may be removed upon complex formation such that the proteins approach each other within van der Waals radii of their surface amino acids.

Crystal structures of several 1:1 macromolecular complexes have been established. In most antibody/antigen structures, water appears to be excluded almost completely from the interface between the interacting molecules (Amit et al., 1986; Davies et al., 1988). There is one recently discovered exception. In the 1.8-Å resolution structure of the chicken lysozyme (HEL)-Fv D1.3 complex, it has been possible to detect 25 extra water molecules trapped at the interface of the two proteins (Bhat et al., 1993). These waters occupy the space that would otherwise constitute a void at the interface. The absence of trapped water appears to be the rule rather than the exception; this is true for the intersubunit interfaces of most, but not all, oligomeric proteins (Matsumura et al., 1989; Schlunegger and Gruetter, 1992; Daopin et al., 1992). On the other hand, the specificity of protein/nucleic acid complex formation appears to involve hydrogen bonds mediated by water molecules at the protein/base interface (Otwinowski et al., 1988; De Vlieg et al., 1989). With the exception of the bacterial photoreaction centers (Deisenhofer et al., 1985; Allen et al., 1987), crystal structures of cytochromes interacting with physiologically relevant partners were not available until very recently. The hypothetical model proposed by Salemme (Salemme, 1976; Wendoloski et al., 1987) for the complex formed by cytochrome $b_5$ and cytochrome *c* did not attempt to account for solvation changes that occur with complex formation, though the author did note that the desolvation of the interacting protein surfaces was likely to make a major entropic contribution to the stability of the complex formed. Similar qualitative arguments have been made in related proposals for hypothetical structures proposed for the cytochrome *c*-cytochrome *c* peroxidase complex and for the cytochrome $b_5$-hemoglobin subunit complexes (Poulos and Kraut, 1980; Poulos and Mauk, 1983).

The recently published structures of the complexes of cytochrome *c* peroxidase with both yeast and horse cytochrome *c* have added immeasurably to our understanding of the nature of the interactions between redox partners (Pelletier and Kraut, 1992). From the point of view of electron transfer reactions, the crystal structures have clearly established possible electron transfer paths between the two metal centers which are separated by a distance of 26 Å; the probable electron transfer pathway follows from the cytochrome *c* peroxidase iron via the porphyrin to $Try^{191}$ and then to $Gly^{192}$ and $Ala^{193}$ and $Ala^{194}$. At the surface of the cytochrome *c* peroxidase the two alanines are in close proximity to ring C of the heme *c* tetrapyrrole. From the point of view of the

*Received for publication 17 February 1993 and in final form 27 May 1993.*

Address reprint requests to Jack A. Kornblatt and M. Judith Kornblatt.

*Abbreviations used:* HEL, hen egg white lysozyme; Fv D1.3, the variable domain of the D1.3 monoclonal antibody.



0006-3495/93/09/1059/07 $2.00

details of the atoms at the interface, the 2.3-Å resolution of the yeast cytochrome *c* complex allows one to draw the following conclusions: 1) Compared to the Fv D1.3-HEL complex, there are relatively few intimate contacts between the two proteins that are visible at this resolution. 2) A very qualitative picture of the interacting group of positive charges on the cytochrome *c* "would not look very different from that published 15 years ago" by Ferguson-Miller, Brautigan, and Margoliash (1978) for the cytochrome *c*-cytochrome *c* oxidase couple. 3) It is clear that some solvent has been removed from the interfacial region but there remains space in other portions of the interface that will accommodate some solvent.

Hydrostatic pressure studies should be capable of quantifying the changes in solvation that accompany protein-protein complex formation (Kundrot and Richards, 1988; Rodgers and Sligar, 1991; Kitchen et al., 1992). Previous analyses of the cytochrome $b_5$-cytochrome *c* (Rodgers et al., 1988; Rodgers and Sligar, 1991) and cytochrome $b_5$-porphyrin-cytochrome *c* complexes (Kornblatt et al., 1988) suggested that a net loss of surface bound water occurs when these proteins interact. This conclusion is consistent with the previous observation that the formation of the cytochrome $b_5$-cytochrome *c* complex is largely entropy driven (33 e.u.) (Mauk et al., 1982); ordered surface water is transferred to the bulk solution on binding of the cytochrome partners. Similar studies of the cytochrome *c*-cytochrome *c* peroxidase and porphyrin-cytochrome *c*-cytochrome *c* oxidase complexes indicated that there is either no major loss of water upon formation of these complexes or that compensatory changes occur in other aspects of interaction between these proteins (Kornblatt et al., 1984; Kornblatt et al., 1986; Kornblatt et al., 1992).

As loss of interfacial water is not the only process contributing to the volume changes measured in the hydrostatic pressure experiments, we sought a more direct method to evaluate the role of water at interfaces. In this work we have used high concentrations of glycerol to lower the activity of water (Parsegian et al., 1986; Rand and Parsegian, 1989). Exposed protein surfaces tend to be hydrated, whereas most buried portions of proteins exclude water (Chothia and Janin, 1975; Arakawa and Timasheff, 1982; Miller et al., 1987). Protein-protein interfaces can behave as though they are separated from the bulk solution by a semipermeable membrane, the protein itself. In cases where the buried surface and exposed surface can exist in equilibrium, increasing the concentration of water in the surrounding solution should force the equilibrium toward the hydrated surface. Similarly, decreasing water activity in the bulk solution should force this equilibrium toward dehydration. High osmotic pressures are capable of dehydrating surfaces, thereby causing the formation of water-free interfaces (Prouty et al., 1985; Rand and Parsegian, 1989; Colombo et al., 1992). In the work described here we show that two different cytochrome complexes behave in opposite ways to glycerol-induced osmotic stress. Lowered water activity promotes formation of the complex between cytochrome $b_5$ and porphyrin-cytochrome *c* and inhibits that between porphyrin-cytochrome *c* and cytochrome *c* oxidase. In the latter case it may be that glycerol binds to the oxidase thereby disrupting the cytochrome *c* binding site or it may be that water is required at the interface.

## MATERIALS AND METHODS

The preparation of horse porphyrin-cytochrome *c* has been described (Robinson and Kamen, 1968). The preparation and measurement of bovine cytochrome *c* oxidase (Kornblatt et al., 1973) and trypsin-solubilized bovine cytochrome $b_5$ (Reid and Mauk, 1982) have also been described. 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (Bis-Tris) was from Fluka (Buchs, Switzerland). Glycerol was purchased from Carlo Erba (Milan, Italy) and was free of detectable reductants. Tween 80 was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available.

The studies on both systems were carried out in buffer containing 5 mM Bis-Tris, variable KCl, 1% (ca. 8 mM), Tween-80 (CMC 12 μM), and variable glycerol, pH 7.5. The fluorometer was maintained at 15°C. The pH of the buffer is relatively insensitive to glycerol; the dielectric constant is similarly insensitive (Douzou, 1977). The nonionic detergent Tween 80 was maintained in both systems in order to diminish any specific contributions from the detergent. Both oxidase and cytochrome $b_5$ are membrane proteins. The oxidase following its extensive purification is highly delipidated (1 cardiolipin/heme), while the cytochrome $b_5$ is the tryptic fragment. The oxidase has an absolute requirement for detergent, whereas the tryptic fragment of cytochrome $b_5$ does not.

The activity of water has been varied using glycerol as osmolyte. All solutions containing glycerol were prepared gravimetrically and their osmolalities obtained from Wolf et al. (1981). We have measured the osmolalities of glycerol containing solutions by freezing point depression up to concentrations of 20% and have validated the data in the Handbook of Chemistry and Physics at higher concentrations in the past (Kornblatt and Kornblatt, 1992).

The choice of osmolyte was limited by several considerations. The monohydric alcohols, propylene glycol, dimethylformamide and dimethyl sulfoxide all denature the oxidase at relatively low concentrations. Salts could not be used since they alter the binding constants for the cytochrome pairs. Polyethylene glycol, dextran, cyclodextran, and polyvinyl pyrrolidone could not be used since they precipitate the oxidase. Common sugars such as glucose, sucrose, and mannitol develop high osmotic pressures, such as required in this study, only at high concentrations and viscosities; the Nicholls' group has used these sugars to study the kinetics of the cytochrome *c* oxidase reaction (see discussion). Glycerol was chosen for this study, because it did not suffer from the above problems. It does not noticeably perturb the individual components, the cytochrome *c*, the cytochrome $b_5$, or the cytochrome *c* oxidase (Kornblatt and Hui Bon Hoa, 1990), at concentrations up to 50%. Additionally, very high purity glycerol can be obtained from commercial sources. 0.01% contamination, when one is working with 4 M solutions, is still 400 μM contaminant or 500 times the concentration of the cytochromes.

Equilibrium constants for complex formation between porphyrin-cytochrome *c*, and its acceptor cytochromes were estimated from fluorescence titrations (Kornblatt et al., 1984, 1988, 1992; Kornblatt and Luu, 1986; Kornblatt and Laberge, 1988). The measurement of porphyrin-cytochrome *c*, the pitfalls and errors involved in working with it, as well as the use of this derivative to estimate dissociation constants have been described in detail (Kornblatt et al., 1984, 1988, 1992; Kornblatt and Luu, 1986; Kornblatt and Laberge, 1988). Porphyrin-cytochrome *c* is fluorescent; the emission peak is at 616 nm and excitation is conveniently at 500 nm. The fluorescence is efficiently quenched on complex formation with either of the two acceptors. The observed fluorescence intensity (*asterisk* in Fig. 1, for instance) is the sum of the fluorescence of free porphyrin-cytochrome *c* and of bound porphyrin-cytochrome *c*. $K_d$ for the interaction between cytochrome $b_5$ and porphyrin-cytochrome *c* was evaluated by methods described previously and by computer simulations in which the data were compared

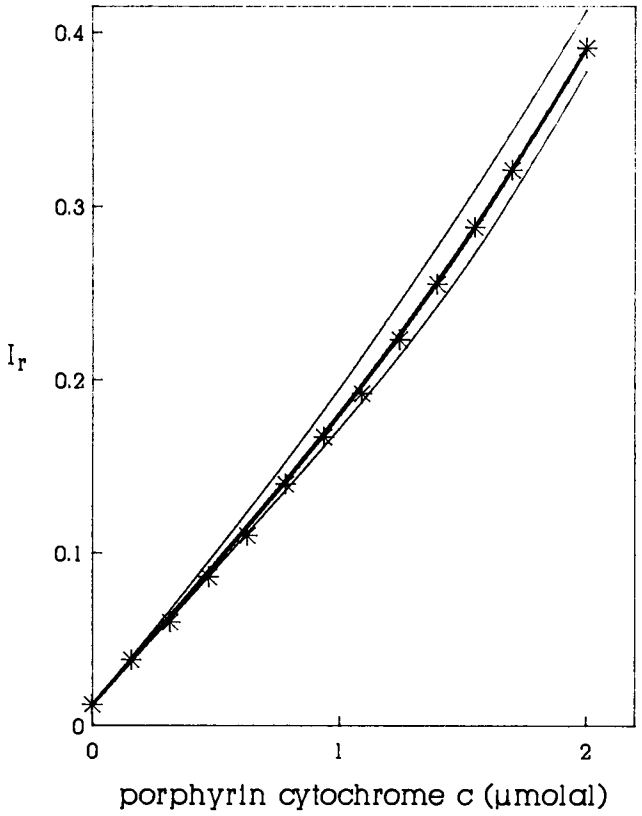


FIGURE 1 The fluorescence of porphyrin-cytochrome *c* is quenched by cytochrome $b_5$. Fluorescence quenching titration of cytochrome $b_5$ (1.6 μmolal) with porphyrin-cytochrome *c* (pH 7.5; 5 mM Bis-Tris, 5 mM KCl, 1% Tween-80) at 15°C. The observed fluorescence intensity (*), $I_r$, is the sum of the fluorescence of free porphyrin-cytochrome *c* and of bound porphyrin-cytochrome *c*. $K_d$ ($1 \times 10^{-7}$ molal) was evaluated by fitting to Eq. 1. The upper and lower curves show the data that would have been obtained with $K_d$ values of $3 \times 10^{-7}$ (*upper curve*), $1 \times 10^{-7}$ (*middle curve*), and $0.3 \times 10^{-7}$ (*lower curve*). All simulations leading to the data of Fig. 2 gave fits that were as good as that shown by the central curve in Fig. 1.

to the following equation:

$$I_{calc} = I_O + c_T FF - \Delta F\{(K_d + b_{5T} + c_T) - [(K_d + b_{5T} + c_T)^2 - 4(b_{5T}c_T)]^{1/2}\}/2 \quad (1)$$

Most simply, the total fluorescence, $I_{calc}$ is the algebraic sum of three contributions: that from the background ($I_O$) plus the total contributed by the porphyrin ($c_T FF$) minus the amount lost on binding of the porphyrin by the acceptor ($\Delta F\{(K_d + b_{5T} + c_T) - [(K_d + b_{5T} + c_T)^2 - 4(b_{5T}c_T)]^{1/2}\}/2$). Each term is defined as follows: $I_{calc}$ is the value of the calculated fluorescence intensity (solid lines in Fig. 1); $I_O$ is the background fluorescence intensity in the absence of porphyrin-cytochrome *c*; $b_{5T}$ and $c_T$ are the total molal concentrations of cytochrome $b_5$ and porphyrin-cytochrome *c*; $K_d$ is the estimated dissociation constant; *FF* is the experimentally determined fluorescence intensity of the free porphyrin-cytochrome *c*, and $\Delta F$ is the difference between the fluorescence emission intensity of the free and bound porphyrin. The values of $K_d$ determined by past methods and that described here agree within a factor of two. Similar agreement was found when the acceptor was cytochrome *c* oxidase interacting with porphyrin-cytochrome *c*.

The dissociation constants were determined as a functions of water activity, expressed as osmotic pressure, and as functions of salt concentrations expressed as ionic strength. Since the latter term is independent of activity coefficient and since the salt, KCl, is soluble in glycerol, no effort was made to correct for its concentration. The activity of water in any given solution was obtained as follows:

$$P_{os}(V_{H_2O}) = RT \ln X_{H_2O} \quad (2)$$

where $X_{H_2O}$ is the mole fraction of water determined from freezing point data (Handbook of Chemistry and Physics, 62nd Ed.). The relation between $P_{os}$ and $K_d$ was evaluated from

$$\partial \ln K_d / \partial P_{os} = -\Delta V^0 / RT \quad (3)$$

Where $\Delta V^0$ is a standard reaction volume associated with the osmotically available water in the two states of the equilibrium. $K_d$ was also evaluated as a function of salt concentration using the extended form of the Debye-Huckel equation (Kornblatt and Luu, 1986).

## RESULTS

Fig. 1 shows a typical titration curve for formation of the cytochrome *c*-cytochrome *b*5 complex at relatively low ionic strength; $K_d$ was determined to be $1 \times 10^{-7}$ molal. The addition of 30 and 40% glycerol had a small effect on $K_d$. It changed to $1 \times 10^{-7}$ molal and $0.58 \times 10^{-7}$ molal, respectively. When the same experiment was performed with porphyrin-cytochrome *c* and cytochrome *c* oxidase under identical conditions of pH, and detergent, the effects were considerably greater. The addition of glycerol increased $K_d$ from $0.4 \times 10^{-7}$ molal (0% glycerol) to $1.14 \times 10^{-7}$ molal and $1.83 \times 10^{-7}$ molal (30 and 40% glycerol, respectively).

As the equilibrium constant for complex formation for both pairs is highly dependent on ionic strength, a neutral electrolyte was added to shift the equilibrium, and the titrations were repeated at 0, 30, and 40% glycerol. The results of these experiments are shown in Fig. 2 as Debye-Hückel plots. Regardless of the glycerol concentration, increasing ionic strength destabilizes both complexes. Regardless of the presence or absence of glycerol, $K_d$ scales as a linear function of ionic strength. While the addition of glycerol to these samples changes the equilibrium for complex formation in each case, the effects of glycerol are opposite for the two systems. At constant ionic strength, glycerol decreases the stability of the cytochrome *c* oxidase/porphyrin-cytochrome *c* complex and increases the stability of the cytochrome $b_5$/porphyrin-cytochrome *c* complex.

In Fig. 3, the data of Fig. 2 have been plotted as a function of osmotic pressure; the $K_d$ values at each ionic strength have been normalized relative to the 0% glycerol sample. For both complexes, $K_d$ scales as a linear function of osmotic pressure; while the data are not extensive there are no discontinuities and no apparent curvatures to the plots. This indicates that glycerol is primarily probing one aspect of the interaction of the cytochrome couples. For the cytochrome *c*-cytochrome $b_5$ complex increasing osmotic pressure (decreasing water activity) stabilizes this complex as water is removed from the hydrated surfaces. Osmotic stress yields a value for $\Delta V^0$ of −47 ml/mol; in our study of the hydrostatic sensitivity of this complex we found a value of −50 ml/mol, while Rodgers and Sligar found −122 ml/mol. Even though it is clear that osmotic and hydrostatic pressure are not probing the same aspects of complex formation (Balny et al., 1989), it is also clear that both pressures are currently thought to exert their major forces via water (Morild, 1981; Balny et al., 1989;

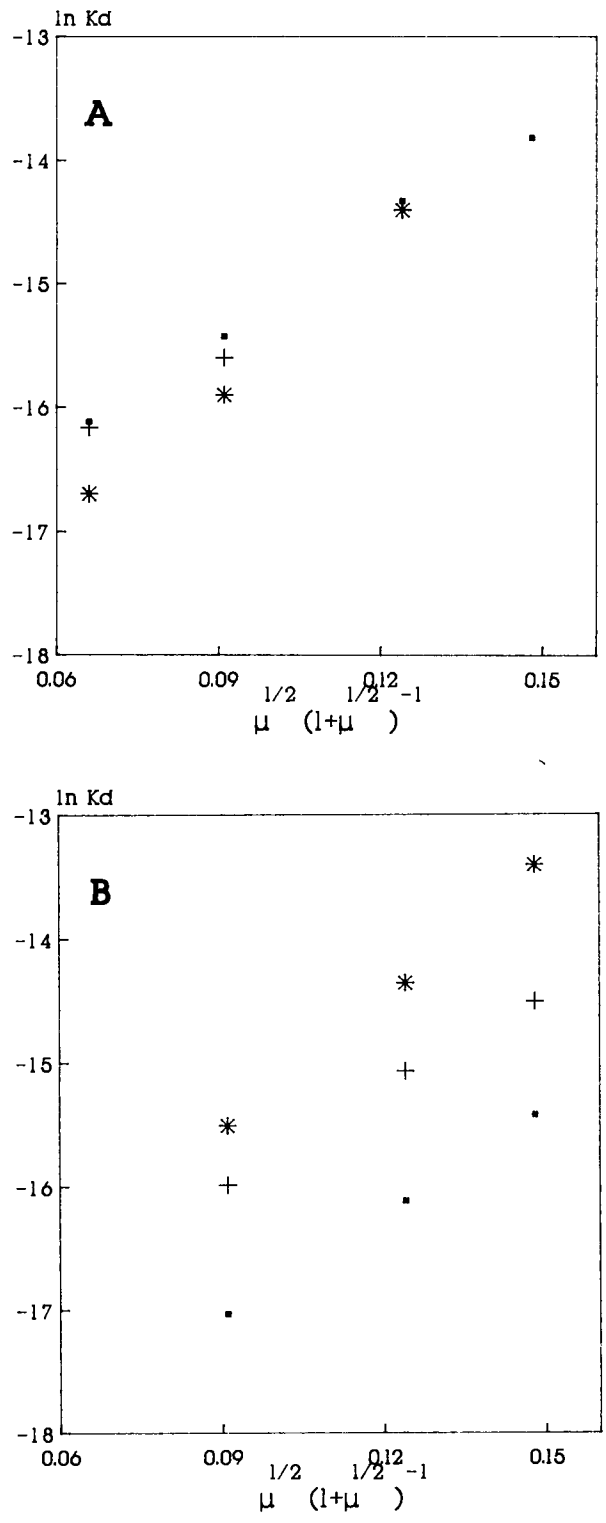


FIGURE 2 The effect of ionic strength on the dissociation constant of the cytochrome complexes. (*A*) The cytochrome $b_5$/porphyrin-cytochrome *c* equilibrium: (■) 0% glycerol; (+) 30.3% (w/w) glycerol; (*) 40% (w/w) glycerol. In addition to glycerol, all solutions contained 5 mM Bis-Tris, 1% Tween 80, and KCl for adjustment of ionic strength. (*B*) The cytochrome *c* oxidase/porphyrin-cytochrome equilibrium. Conditions and symbols are the same as in *A*. Extended Debye-Hückel theory was used to evaluate the relation between dissociation constant and ionic strength. The charge interaction term, *ZZ*, was evaluated by regression analysis to be ca. −14 in all cases.

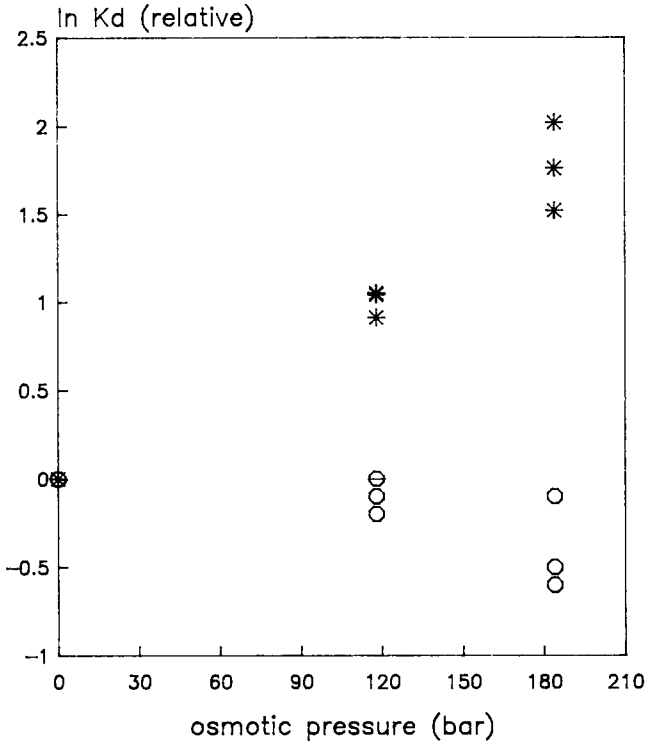


FIGURE 3 The equilibrium dissociation constant for the complexes formed by porphyrin-cytochrome *c* with cytochrome *c* oxidase and cytochrome $b_5$ as a function of osmotic pressure. The pressures of the solutions of Fig. 2, *A* and *B*, were evaluated using Eq. 2; $\Delta V^0$ was evaluated from Eq. 3. The dissociation constants were normalized by dividing the constants for the 30.3 and 40% solutions at each ionic strength by the value of the constant at 0% glycerol at the same ionic strength. (*) Cytochrome *c* oxidase/cytochrome *c*; (○) cytochrome $b_5$/cytochrome *c*. The volume changes that occur with complex formation as determined by regression analysis are 224 ml/mol for the upper set and 47 ml/mol for the lower.

Rodgers and Sligar, 1991). How much water is removed on formation of the cytochrome *c*-cytochrome $b_5$ complex?

For the purposes of the calculation we assume that water at the protein surface is completely inaccessible to osmolyte (Arakawa and Timasheff, 1982) but that its partial molal volume is 18 ml/mol, the same as that of the bulk (Columbo et al., 1992). Accordingly, the cytochrome *c*-cytochrome $b_5$ complex excludes 3 ± 1 water molecules from the protein-protein interface compared to the free molecules.

Interestingly, Fig. 3 shows that increased osmotic pressure destabilizes the cytochrome *c* oxidase-porphyrin-cytochrome *c* complex. The volume change is 224 ± 16 ml/mol. The significance of this change is discussed in the next section.

## DISCUSSION

The results reported here concern two related systems, viz. porphyrin-cytochrome *c* complexing to either cytochrome $b_5$ or to cytochrome *c* oxidase. The two complexes were probed using glycerol as a perturbant. Phenomenologically, glycerol strengthens the interactions between porphyrin-cytochrome *c* and cytochrome $b_5$ and weakens the interactions between porphyrin-cytochrome *c* and cytochrome *c* oxidase.

The cytochrome $b_5$ data do not differ substantially from that which we had anticipated. The surface of the both reactants is primarily hydrophilic; the interface that forms must exclude water to some degree. The earlier studies conducted by Sligar's group as well as our own showed that the cytochrome $b_5$, (porphyrin)-cytochrome *c* complex could be dissociated by hydrostatic pressure. Both groups found comparable reaction volumes for the equilibrium. The interpretation of the volume change was that hydrostatic pressure promotes solvation of the interfacial surfaces. If hydrostatic pressure can promote solvation of a buried interface, lowered water activity should promote removal of water from potential interfacial surfaces; i.e., complex formation such as is seen here. In the case of the cytochrome $b_5$, (porphyrin)-cytochrome *c* complex it appears as though both hydrostatic and osmotic pressures give consistent results which agree with expectations.

The cytochrome *c* oxidase, porphyrin-cytochrome *c* case is quite different from the cytochrome $b_5$, porphyrin-cytochrome *c* case where lower water activity increases the strength of the interaction. In the oxidase case, the addition of glycerol weakens the interaction between the cytochromes. One possible explanation is that glycerol is a poor choice for an osmotically active compound; it could—and probably does to some extent—bind preferentially to the oxidase either displacing water from the primary hydration shell of the protein or ordering the water that would otherwise be present. Timasheff (1992) suggests that either the Wyman (1964) plot (lg $K_d$ versus lg glycerol) or the linear extrapolation of Greene and Pace (1974), where $\Delta G$ is plotted versus glycerol concentration, should give a straight line if the primary influence of glycerol is binding to the protein. Colombo et al. (1992) have used this approach to show that sucrose, stachyose, and polyethylene glycol influenced HbA by something other than binding of the three perturbants. When plotted by either of the two methods cited, the cytochrome *c* oxidase data does not show a linear relationship. We do not wish to assert that there is no binding of the perturbant to the oxidase or to cytochrome $b_5$, but if the Wyman/Greene and Pace plots are indicative, $K_d$s are not being displaced by glycerol in this work.

It has recently been found that glycerol can bind to the triple helices of collagen and specifically abolish *attractive* hydration forces (Leikin, Rau, and Parsegian, personal communication). In this instance, the glycerol is displacing water. The net result of the displacement is that the triple helices repulse one another. This is quite analogous to that which is occurring with the oxidase-cytochrome *c* system studied here.

Another possible explanation is that water loss from cytochrome *c* oxidase is associated with a conformational change similar to that seen in hemoglobin or hexokinase. This conformational change could result in oligomer formation as it does in HbS (Prouty et al., 1985), the burial of exposed surface as occurs in HbA (Colombo et al., 1992) or cleft closure as found with hexokinase (Rand et al., 1993). If oligomer formation were taking place or if there were a substantial conformational change, we might expect to find a change in the stoichiometry of porphyrin-cytochrome *c* binding to the oxidase; binding sites might become buried. The data indicate that the binding stoichiometry does not differ from $1 \pm 0.1$ porphyrin-cytochrome *c*/oxidase in any of the conditions studied here. Oligomer formation or a substantial conformational change might also be expected to influence the fluorescence coefficient of the bound porphyrin-cytochrome *c* since it is sensitive to both distance from the quencher and, to a lesser extent, orientation with respect to the quencher. The fluorescence coefficient of the oxidase bound porphyrin-cytochrome *c* shows no change throughout the experiments here. These observations indicate that the binding site for porphyrin-cytochrome *c* on the surface of cytochrome *c* oxidase and the binding geometry for this complex stay constant.

Nicholls and coworkers (personal communication) have studied the steady state kinetics of electron transfer from cytochrome *c* to cytochrome *c* oxidase to oxygen; they have measured the effects of glucose and sucrose on $K_m$ (cytochrome *c*) and $V_m$. The major effects are on $K_m$ which becomes larger (binding gets weaker) as the concentration of the two perturbants increase. $\Delta V$ associated with $K_m$ is substantially higher than that reported here and is dependent on the sugar, the larger the sugar the larger is $\Delta V$. They interpret the data to mean that larger molecules probe more of the cytochrome *c*, cytochrome *c* oxidase interaction site than do small molecules.

Three different polyols, glycerol, glucose, and sucrose, ranging in size from 92 to 342 daltons, all give qualitatively similar results. It is not likely that all three will be binding to the oxidase so as to disrupt the cytochrome *c* binding site but it is possible.

A final explanation of the oxidase results is that 224 ml/mol of *water* (about 12 waters) is trapped in a cavity at the interface of the porphyrin-cytochrome *c*-cytochrome *c* oxidase complex and that these waters are essential for binding. These molecules would represent an increase in the glycerol inaccessible space of the two proteins in their 1:1 complex over that of the two proteins at infinite separation. If the density of bound and bulk water is similar, the volume occupied by 12 waters corresponds to about three new amino acid side chains at the interface between the two proteins. Such packing is not impossible, but it would have to represent something rather unusual in association phenomena. To the best of our knowledge the only system to definitely show such behavior is that of HEL complexing to the Fv fragment, D1.3 (Bhat et al., 1993). Here, the data indicating water binding at the interface are the result of both the x-ray crystal structure at 1.8 Å and differential scanning calorimetry. In contrast to the formation of the cytochrome *c*, cytochrome $b_5$ complex which loses water to the bulk solution, the formation of the D1.3, lysozyme complex is enthalpically driven, not entropically. Both the antibody binding site and lysozyme are craggy structures. It should not surprise one that the packing at the interface is not perfect, that voids would be left were they not filled with water. These trapped

waters can act as bridges between groups. In the oxidase, cytochrome *c* case, if this were occurring it would mean that the fit of the two molecules is good but not perfect. One might expect this kind of complementarity considering that the reactants and products differ only in charge and, probably, conformation. In the final analysis, binding is necessary but so is dissociation; the fit should not be too good.

We thank Drs. Stephen G. Sligar, Pierre Douzou, Dimitry Davydov, and Tho-Trang Cao for their ongoing help and encouragement. Dr R. P. Rand rescued us from committing several errors; his aid is gratefully acknowledged. Dr. V. Adrian Parsegian provided valuable criticism of the manuscript. Supported by grants from the Natural Sciences and Engineering Research Council of Canada (to J. A. Kornblatt and M. J. Kornblatt) and by National Institutes of Health grant GM-33804 (to A. G. Mauk).

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