Biochemistry

 Volume 32, Number 23 June 15, 1993

Accelerated Publications

Measured Change in Protein Solvation with Substrate Binding and Turnover†

R. P. Rand,* N. L. Fuller, P. Butko, G. Francis, and P. Nicholls

Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada

Received March 12, 1993; Revised Manuscript Received April 14, 1993

ABSTRACT: Osmotic stress is used to measure solvation changes that accompany the conformational changes of an active enzyme. For hexokinase both the equilibrium dissociation constant and the kinetic Michaelis–Menten constant for glucose vary linearly, and to the same extent, with the activity of water in the protein medium, as adjusted with large molecular weight (>2000) osmolytes. The variation over the whole osmotic pressure range studied indicates that glucose binding is accompanied by the release of at least 65 ± 10 water molecules, and this is reversed on enzyme turnover. The results indicate that near the physiological range of pressures the number may be higher. Most of this water, which behaves like an inhibitor, likely comes from the cleft which is induced to close around the substrate. Such large dehydration/rehydration reactions during turnover imply a significant contribution of solvation to the energetics of the conformational changes. Osmotic stress is a method of general applicability to probe water's contribution to functioning molecules.

Although solvation has been used to guide thinking about protein structure, the contribution of hydration to protein conformational stability and enzyme catalysis remains an enigma. Extensive studies show a "hydration shell" around most proteins that excludes many solutes (Arakawa & Timasheff, 1985a,b), and extended networks of water have been seen within protein crystal structures (Meyer, 1992). Hydrostatic pressure-induced changes in protein association (Weber & Drickamer, 1983) and in enzyme catalysis (Low & Somero, 1975) strongly implicate changes in protein solvation through the electrostriction of water, but they remain difficult to separate from other possible density changes (Plager & Nelsestuem, 1992). Hydrostatic pressure methods would not identify water that moves without density change. The induced-fit mechanism of substrate specificity (Koshland, 1959) implies that substrate binding changes the hydration of the protein. Protein solvation has, however, been difficult to measure directly and its contribution to the energetics of conformational change difficult to estimate.

One direct and universally applicable strategy for probing water's role is to measure the effects of varying its activity, through osmotic stress (OS). OS has been used to measure forces of interaction between large molecular surfaces (Parsegian et al., 1986). It has the unique feature of detecting even extremely weakly perturbed water molecules near membrane (Rand & Parsegian, 1989) and polymer (Rau et al., 1984; Rau & Parsegian, 1990) surfaces, perturbations which sum to give large interaction energies when many such water molecules are simultaneously moved. These measurements also show that the costs of fully dehydrating, or the benefit of fully hydrating, such a variety of different surfaces are all within an order of magnitude and very high from 1.5 to 15 kcal/mol per 100 Å^2 of surface area (Rand & Parsegian, 1989).

OS can also be used to probe the hydration of single macromolecules. It has been used to show that membrane channels open (hydrate) with increased difficulty in the face of decreased water activity in their vicinity (Zimmerberg & Parsegian, 1986; Rayner et al., 1992). More recently, OS has been used to measure the hydration that accompanies the reduction of cytochrome oxidase (Kornblatt & Hui Bon Hua, 1990) and the binding of oxygen to hemoglobin (Colombo et al., 1992). The large number of waters accompanying these reactions is unexpectedly high and suggests that solvation energy contributes far more to the energetics of protein

† R.P.R. and P.N. are supported by the Natural Sciences and Engineering Research Council of Canada. R.P.R. is a Research Fellow of the Canada Council's Killam Program.

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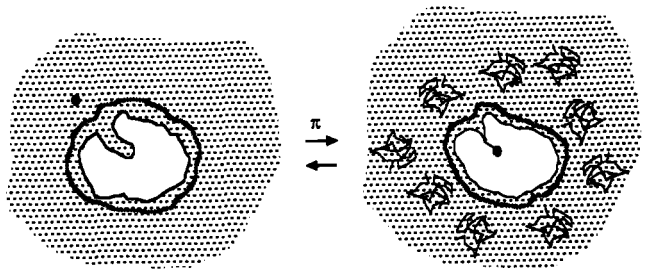

FIGURE 1: In the osmotic stress (OS) strategy, water activity is controlled through the concentration of an excluded solute, such as a polymer. Any equilibrating aqueous compartment that is inaccessible to that solute, delineated here by the enclosing line, has its water activity controlled by solute osmotic pressure, π. Since smaller solutes may have access to aqueous clefts, it is the solute–protein excluded volume that defines the size of the inaccessible compartment. Under such osmotic stress, proteins undergoing reversible transitions are shifted toward their more dehydrated conformation. A measure of the sensitivity of that shift to water activity gives the difference in the volume of water associated with each conformation (see Experimental Procedures). In the present example of hexokinase, glucose induces the conformational change required for catalysis, the classical example of an "induced-fit" mechanism of enzyme specificity. The OS strategy then measures the change in protein solvation accompanying that fit.

conformational changes than usually thought.

We report here measurements of the effect of water activity, both on substrate equilibrium binding and on enzyme turnover. We selected hexokinase (HK) for a number of reasons. First, HK discriminates against water as a substrate which led to the original induced-fit mechanism of substrate specificity (Koshland, 1959). Second, HK is active as a single polypeptide, eliminating the complications of quaternary structure and changing protein–protein interfaces. Third, the glucose binding site is in a large cavity that closes on glucose binding (Steitz et al., 1981). Fourth, the crystal structures of the unbound (open) and bound (closed) conformations have been determined (Bennett & Steitz, 1978, 1980). Finally, both the equilibrium binding constant and the kinetic Michaelis–Menten constant for glucose can be independently measured. Their dependence on water activity can then be compared and related to the role of hydration and dehydration reactions in enzyme turnover.

EXPERIMENTAL PROCEDURES

Using Osmotic Stress. The effect of an inert solute molecule that is excluded from a hydration compartment around HK is to lower the water activity (W) outside that compartment and aid in water removal from it (Figure 1). If the osmotic pressure of the outside compartment is Π, and n is the number of water molecules of total volume ΔVw that is removed as a result of glucose binding, then an osmotic work equal to $\Pi\Delta Vw$ would appear as a contribution to the free energy, ΔG, of glucose binding. Thus

$$\Delta G = -RT \ln K_d + nRT \ln(W) = \text{constant}$$

where

$$nRT \ln(W) = \Pi\Delta Vw$$

so that

$$\Delta Vw = RT\, \Delta \ln K_d/\Delta\Pi = RT \ln(K_d^{\circ}/K_d^{\pi})/\Delta\Pi \quad (1)$$

Here K_d° and K_d^{π} are the dissociation constants measured at osmotic pressures of 0 and Π.

Therefore, a measure of the change in K_d with the change in osmotic pressure gives the change in volume of water that occurs on glucose binding. (This is equivalent to the relation used in hydrostatic pressure studies where the applied mechanical pressure is isotropic and ΔVw is a volume change that results from density changes in the system.) Assuming no density changes (but see below) the number of water molecules, n, is derived from ΔVw and the volume of a water molecule.

Similarly, any osmotic dependence of the K_m for glucose in the kinetic turnover of the enzyme would reflect a contribution by water to the free energy of the reaction. If K_m reflects the dissociation constant of the HK–glucose complex, then substitution of K_m for K_d above would also yield the volume and number of waters involved in the glucose binding step during enzyme turnover.

Polymers used to control the activity of water were poly(ethylene glycol) (PEG, Fluka and Pharmacia) of molecular weights 2000, 4000, 8000, and 20 000 and methylated PEG of molecular weight 2000. The osmotic pressures of the polymer solutions were measured directly (Parsegian et al., 1986) or by secondary "osmometry". For the latter, X-ray diffraction of multilamellar phases of stearyloleoylphosphatidylcholine, hydrated under known osmotic pressures (Rand & Parsegian, 1989), was calibrated against those hydrated in the polymer solutions used in this study.

Equilibrium Binding. Hexokinase (EC 2.7.1.1) was type C-302 from bakers' yeast (Sigma), essentially fraction II of Kaji et al. (1961). The assay solution was HK (14.5 μg/mL) in 20 mM glycylglycine buffer (pH 8.7) and 200 mM KCl.

For the equilibrium binding of one glucose (Glu) molecule to the single site on hexokinase (HK)

$$[HK^*\cdot Glu] \underset{k_1}{\overset{k_2}{\rightleftharpoons}} [HK] + [Glu]$$

the dissociation constant $K_d = k_2/k_1 = [HK][Glu]/[HK^*\cdot Glu]$ and HK and HK* are taken as the two conformations of the protein.

K_d is measured through a decrease in HK intrinsic fluoresence, ΔF (λ_{ex} = 290 nm, λ_{em} = 335 nm), when glucose binds (Hoggett & Kellett, 1976). Titration of ΔF, normalized to that at saturating glucose, ΔF_{max}, yields the fraction of the hexokinase that has bound glucose

$$[HK^*\cdot Glu]/\{[HK] + [HK^*\cdot Glu]\} \text{ as } \Delta F/\Delta F_{max}$$

Glucose concentration is given by molality, to account for the significant volume fraction occupied by the polymer in samples at high osmotic pressure. Inasmuch as the osmotic pressures of glucose and polymer are additive (D. C. Rau and V. A. Parsegian, personal communication), we conclude that the glucose has effective access to all the water, and its activity is given by its molal concentration.

We attempted to do similar binding experiments with several other polymers. However, poly(vinyl alcohol), poly(vinylpyrrolidone), and hydroxyethyl starch, even after extensive dialysis, all had absorption levels at λ = 335 nm that precluded fluoresence measurements. Low molecular weight dextrans reduced both the level of HK fluoresence and its change on glucose binding and at high protein concentrations did not give linear double-reciprocal plots, all suggesting a dextran–hexokinase interaction. Solutions of higher molecular weight dextrans with sufficient osmotic pressure were too viscous. We have not attempted to describe more exhaustively all contributions to the equilibria between glucose and hexokinase: thermodynamically complete expressions including osmolyte–glucose and osmolyte–protein interactions as well as those involving water activity. But we believe that the

results show that the former are small and the major contribution is through water activity.

Kinetic Measurements. The Michaelis–Menten constant, K_m, for glucose was measured for the hexokinase reaction

$$HK + Glu \underset{k_1}{\overset{k_2}{\rightleftharpoons}} HK^*\cdot Glu + ATP \overset{k_3}{\rightarrow} HK + Glu\text{-}6\text{-}PO_4 + ADP + H^+ \quad (2)$$

$K_m = (k_2 + k_3) / k_1$, where k_3 would include several steps in getting back to the original conformation of the enzyme.

A single enzyme assay was used to preclude possible multiple osmotic effects on the more commonly used coupled enzyme reactions. The assay followed the time course of the pH change measured either with cresol red absorbtion (Darrow & Colowick, 1961) or by direct recording with a pH electrode. These methods gave identical results. The reaction was started by adding 50 μL of HK (10 units/mL) to 3 mL of the reaction mixture containing the indicated concentrations of glucose and PEG, plus 0.01 mM cresol red, 5 mM $MgCl_2$, 0.5 mM ATP, and 100 mM KCl in 1 mM glycylglycine buffer (~pH 8.7). No enzyme activity was detected in the presence of any of the polymers alone.

RESULTS

Polymer Increases the Affinity of HK for Glucose. Figure 2a shows examples of the effect of three different concentrations of PEG, MW 2000, on the glucose dissociation constant K_d. Linearity of the double-reciprocal plots is consistent with previous work in buffer solutions (Hoggett & Kellett, 1976; Woolfitt et al., 1988; Peters & Neet, 1978) and is maintained for all polymer solutions reported in these experiments. This shows that glucose binding can be described with a single dissociation constant. The polymer increases the affinity of HK for glucose.

No difference could be detected in the dependence of K_d on osmotic pressure for PEGs of molecular weights 2000, 4000, and 8000 and methylated PEG (MW 2000) (Figure 3).

Several observations indicate that the effect of these polymers on glucose binding is indirect. First, the polymer had no detectable effect on the maximum decrease of fluoresence at high glucose concentration. Second, there was no detectable effect of the polymer itself on the level of HK fluoresence in the absence of glucose; i.e., the polymer alone is not inducing any detectable conformational change in the protein, as glucose does. Third, the effect of PEG was the same for PEGs of different molecular weights and for methylated PEG MW 2000. Fourth, the effect of PEG was the same on the HK dimers as on monomers (legend, Figure 2a). We conclude that the polymer most likely exerts its effect by determining the water activity. This is consistent with the extensive measurements of protein stabilization by PEG (Lee & Lee, 1987) and the exclusion of PEG from the hydration shells of proteins (Rajiv & Timasheff, 1992).

Polymer Decreases K_m for Glucose in Hexokinase Turnover. Figure 2b shows examples of Lineweaver–Burk plots for glucose for three different concentrations of PEG MW 2000. Linearity is maintained for the highest polymer concentrations reported here. K_m for the polymer-free assay solution is consistent with those previously described for HK monomers

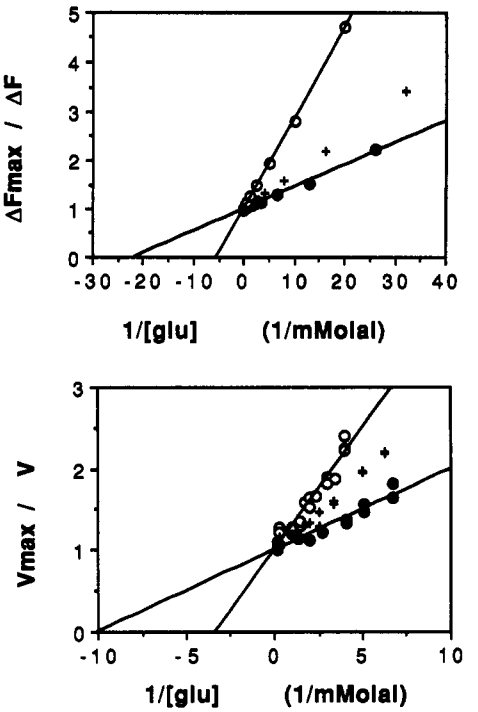

FIGURE 2: (a, top) Double-reciprocal plots of the normalized fluoresence decrease of hexokinase (HK) with glucose concentration are shown for 0 (O), 20 (+), and 35 (●) wt % solutions of PEG (MW 2000). Glucose concentrations bracketed the K_d for glucose, which was determined from least squares fits to the data. Scatchard plots (not shown) give one binding site at all concentrations of PEG. HK dimerization occurs at pH 6.5 and low ionic strengths (Hoggett & Kellett, 1976). Measured K_d for the dimer was higher than the monomer by an order of magnitude, consistent with previous studies (Hoggett & Kellett, 1976). Thus the polymer did not cause the dimerization of HK; its effect on K_d was in the opposite direction. In addition, the major effect of PEG was also to decrease the K_d of dimers to approximately the same extent as the monomer (results not shown). (b, bottom) Lineweaver–Burk plots are shown for 0 (O), 16 (+), and 32 (●) wt % solutions of PEG (MW 2000). Glucose concentrations bracketed the K_m for glucose, which was determined from least squares fits to the data. The polymer had no detectable effect on V_{max}.

(Darrow & Colowick, 1961; Viola et al., 1982; Rees et al., 1989). The effect of polymer is to decrease K_m.

The effects of PEG on the kinetics of the reaction are similar for molecular weights 2000, 8000, and 20 000 (Figure 3). This again suggests that there is no direct interaction of the polymer with the protein and that increased glucose affinity is effected by lowering the water activity. Water behaves as an inhibitor to glucose binding.

DISCUSSION

Under no osmotic stress K_d and K_m are equal, consistent with earlier isotope partitioning measurements (Viola et al., 1982) and with the similar binding and kinetic measurements (Woolfit et al., 1988). That K_m maintains this equality under lower water activity may appear surprising, considering that this is a two-substrate enzyme which, during turnover, undergoes several steps subsequent to glucose binding before regaining its original conformation. The single rate constants, k_1 and k_2, of the glucose-induced steps (eq 2) would be expected to be greater than k_3, which can be considered as a lumped rate constant that includes several subsequent steps back to the original conformation of the enzyme. These steps include HK rehydration, which itself would be slowed by osmotic pressure. This would reduce k_3 further, leaving $K_m = k_2/k_1$ at all osmotic pressures. A reduction in k_3 might be expected

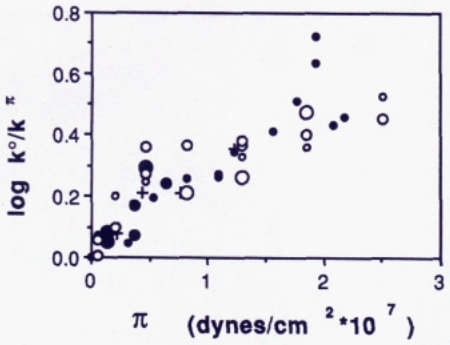

FIGURE 3: Plot of the relative change in log K_d (open circles and crosses), and of log K_m (closed circles), with osmotic pressure produced by all the polymer solutions used in this study (○, ○, ◯, and +, PEG MW 2000, 4000, and 8000 and MePEG MW 2000, respectively; •, ●, and ●, PEG MW 2000, 4000 and 20 000, respectively). Following eq 1, least squares fits to the data yield $\Delta Vw = 1710 \pm 300$ Å^3 (95% confidence intervals) for the equilibrium data and $\Delta Vw = 2340 \pm 420$ Å^3 for the kinetic data. These are not significantly different. Although there is a suggestion in the binding data that the change in equilibrium binding constant is greater at the lower pressures, until the scatter in the measurements is reduced we take it that the effect is uniform over the range of pressures measured. The pooled equilibrium and kinetic data yield a change in volume of 1950 ± 300 Å^3 (95% confidence limits), equivalent to 65 ± 10 water molecules of volume 30 Å^2.

to reduce V_{max}, but we could not detect such an effect within the precision of our experiments.

The equality of K_m and K_d under osmotic stress is strong evidence that, of all the steps in enzyme turnover, glucose binding is the step most affected by osmotic pressure. This is the step of major conformational change (Steitz et al., 1981; Bennett & Steitz, 1980).

Solvation of Hexokinase. On the basis of eq 1, Figure 3 shows the relative change in log K_d, and in log K_m, as a function of osmotic pressure. The combined data show that on glucose binding there is a decrease in the volume of water associated with the system of approximately 1950 ± 300 Å^3. This volume is equivalent to 65 ± 10 water molecules of normal density.

This volume is determined by pooling all the equilibrium and kinetic data over the whole osmotic pressure range explored. There is a suggestion in the results that this number might be higher under physiological conditions. Consideration of only the equilibrium points at lower osmotic pressures gives the impression of a higher slope and therefore of volume change. It would not be surprising that osmotic stress alone would "dehydrate" HK to some extent so that at the higher pressures less water is removed in the glucose-induced conformational change. However, unless and until the scatter in the data justifies this impression, we reserve judgment on this interesting possibility. (Note Added in Proof: Our more recent evidence supports this possibility. The curve appears steeper near the origin and so at the lower osmotic pressures the number of waters appears to be higher than the average derived using the whole pressure regime studied.)

Figure 4 shows the unbound and bound conformations of HK, along with water molecules filled out to three layers from the protein surface. There could be several contributions to the measured change in volume on glucose binding, including dehydration of glucose itself, the obvious decrease in cleft size in the protein (Steitz et al., 1981), and changes in hydration of solvent-exposed protein surface area (Colombo et al., 1992). Glucose is considered to have approximately six water molecules effectively associated with it which do not participate in changing the activity of water (Haldane, 1928). These appear to be nearly all removed on binding (Bennett & Steitz, 1980). That would leave an additional volume decrease of 1080 cm^3/mol, equivalent to about 60 water molecules. Isotropic hydrostatic pressure studies of proteins show that volume changes due to density changes account for a maximum of about 100–200 cm^3/mol (Low & Somero, 1975; Greaney & Somero, 1979). While volume changes measured osmotically and by hydrostatic pressure are not easily compared, any density changes that contribute to these osmotic measurements would be of this order and so a small proportion of the total. We conclude therefore that most of the volume decrease measured in these experiments comes from the bulk transfer of water, without density change, between the polymer-inaccessible space around the protein and the protein solution.

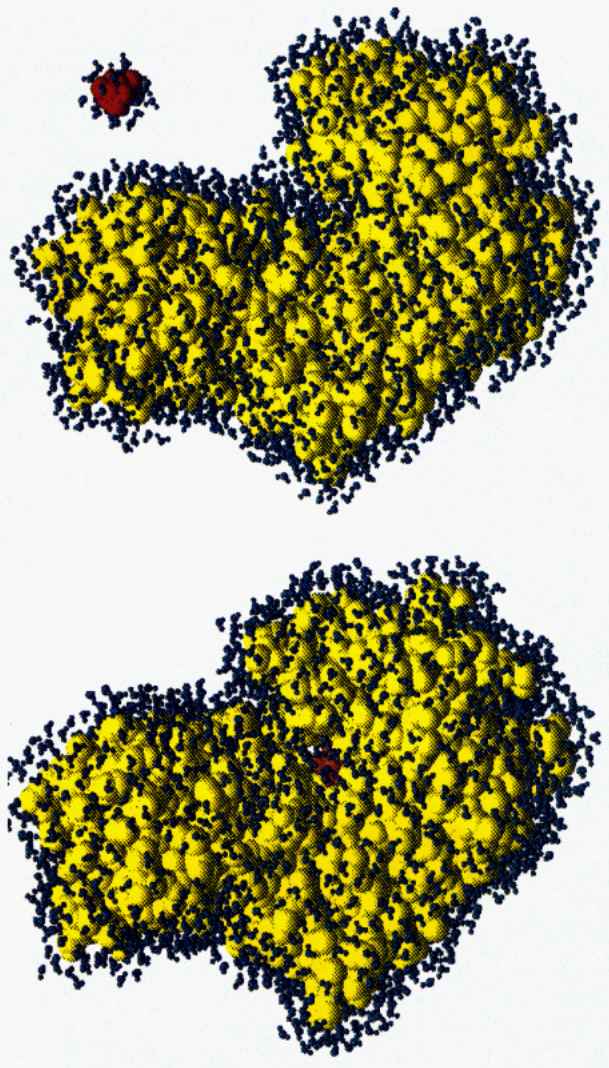

FIGURE 4: Space-filling models of the two conformations of hexokinase and glucose. Superimposed are water molecules, on a smaller scale, placed to fill the volume around the protein out to three water layers. The protein structures were determined by Bennett and Steitz (1980). The image was created using Biograf (Molecular Simulations, Waltham, MA) with data from the Brookhaven Protein Data Bank (Bernstein et al., 1977), substituting glycine for the unknown amino acids. The difference in number of waters, open minus closed conformations, is approximately plus 25, 60, and 100 for 1, 2, and 3 hydration layers, respectively.

The results show that the size of the polymer-inaccessible space is independent of molecular weight between 2000 and 20 000. PEG of molecular weight 600, on the other hand, shows little effect on glucose binding (in progress). This suggests that it is the large aqueous cleft that excludes the larger MW species and provides the major fraction of the

water that is measured in these experiments. Osmotic stress with several different size osmotic probes might provide the possibility of detecting different size aqueous compartments around the protein.

The measured water could come both from complete protein surface dehydration and from the removal of more vicinal water of the aqueous space in the cleft. The reduction in cleft size involves a rigid rotation of the two protein domains that form the cleft without significant conformational change of the domains themselves. Bennett and Steitz (1978) have calculated from their structures that on closing there is approximately 163 Å^2 decrease in area in the cleft exposed to a single layer of water. This decrease represents new protein–protein contacts involving the removal of approximately 26 water molecules. Simple space-filling molecular modeling of one hydration layer around HK also gives this value (Figure 4). On the other hand, the number of water molecules required to fill two or three layers of water from the HK surface is reduced by 60 or 100 water molecules, respectively, as the conformation changes from the open to glucose-bound state. These larger numbers are more consistent with the experimental measurements. Taken together, this suggests that of the approximately 60–65 waters that leave on glucose binding, 26 or approximately one-half come from new protein–protein contact that is made within the cleft, i.e., surface dehydration, and the other half comes from more vicinal layers.

That the more vicinal water may contribute to the energetics of dehydration is consistent with measurements showing that hydrophilic surfaces significantly perturb water well beyond the first layer (Rand & Parsegian, 1989). Even though these perturbations may be weak for any one water molecule, the simultaneous removal of large numbers of these weakly "bound" molecules becomes energetically important. That is the major lesson learned from the measurements of forces between large surfaces and points out the sensitivity of the OS method in detecting such waters. Such vicinal waters are unlikely to be detected by X-ray diffraction or NMR since their chemical potential is so little different from bulk water and the residency time so short. But, conversely, OS applied to X-ray crystals or used in NMR studies provides a unique way to determine any structural effect of removing these weakly perturbed waters.

This is the third solution protein whose conformational change has been probed with OS. Each one shows qualitatively different solvation reactions on substrate binding. Hemoglobin gains 60 water molecules with its complement of 4 oxygens (Colombo et al., 1992), HK loses about 60 water molecules for each glucose molecule, and it is proposed that cytochrome oxidase undergoes a hydration/dehydration cycle involving 10 water molecules as the protein is reduced and then transfers an electron internally (Kornblatt & Hui Bon Hoa, 1990). The quantitative features of the equilibrium and kinetic results with HK show particularly that such hydration/dehydration reactions are present during catalysis where water behaves like an inhibitor to glucose binding. One general feature of this work is that water in such quantity can be viewed as a variable part of protein structure, critical in protein function. Its extent can be measured using osmotic stress, a strategy which can, in principle, be applied to any of a variety of systems.

ACKNOWLEDGMENT

We are indebted to Adrian Parsegian, who has guided our thinking in the thermodynamics of the OS strategy. He and others, Serge Timasheff, George Somero, Jack Kornblatt, Steve Hand, and John Carpenter, have provided constructive suggestions during this work.

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