Probing Protein Hydration and Conformational States in Solution

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ABSTRACT The addition of polyethylene glycol (PEG), of various molecular weights, to solutions bathing yeast hexokinase increases the affinity of the enzyme for its substrate glucose. The results can be interpreted on the basis that PEG acts directly on the protein or indirectly through water activity. The nature of the effects suggests to us that PEG's action is indirect. Interpretation of the results as an osmotic effect yields a decrease in the number of water molecules, ΔN_w, associated with the glucose binding reaction. ΔN_w is the difference in the number of PEG-inaccessible water molecules between the glucose-bound and glucose-free conformations of hexokinase. At low PEG concentrations, ΔN_w increases from 50 to 326 with increasing MW of the PEG from 300 to 1000, and then remains constant for MW-PEG up to 10,000. This suggests that up to MW 1000, solutes of increasing size are excluded from ever larger aqueous compartments around the protein. Three hundred and twenty-six waters is larger than is estimated from modeling solvent volumes around the crystal structures of the two hexokinase conformations. For PEGs of MW > 1000, ΔN_w falls from 326 to about 25 waters with increasing PEG concentration, i.e., PEG alone appears to "dehydrate" the unbound conformation of hexokinase in solution. Remarkably, the osmotic work of this dehydration would be on the order of only one kT per hexokinase molecule. We conclude that under thermal fluctuations, hexokinase in solution has a conformational flexibility that explores a wide range of hydration states not seen in the crystal structure.

INTRODUCTION

It has been known for a long time that hydration plays a major role in protein conformation and conformational changes, in substrate binding and enzyme catalysis, and in molecular recognition. Protein solvation has been measured directly, primarily from its preferential interaction with water compared to its interaction with a variety of other solutes (see Timasheff, 1993, for a current review). But water's contribution to the energetics of protein conformational change has been difficult to estimate.

One universally applicable strategy for probing water's energetic role is to measure its effects on the equilibrium between protein conformations, just as one would for any other small molecule (Parsegian et al., 1995). The osmotic stress of lowering water activity has been used to measure energies of interaction between large molecular surfaces and the change in the number of surface-associated waters. Osmotic stress has the unique advantage of being able to detect even extremely weakly perturbed water molecules near membrane (Rand and Parsegian, 1989) and polymer (Rau et al., 1984; Rau and Parsegian, 1990) surfaces, perturbations weaker than can be detected by NMR or other structural methods. This sensitivity results whenever the surfaces are large, and the weak perturbations of many water molecules sum simultaneously to give measurable total interaction energies (Leikin et al., 1993). This approach has shown that the cost of removing many waters whose energies differ from bulk water by as little as 1 calorie/mol can be measured, and that the cost of fully dehydrating, or the benefit of fully hydrating, most hydrophilic surfaces is very high, from 1.5 to 15 kcal/mol per 100 Å^2 (Rand and Parsegian, 1989).

Osmotic stress has been used to measure changes in the number of water molecules associated with single macromolecules undergoing conformational changes. Membrane channels, in the face of decreased water activity in their vicinity, open (hydrate) with increased difficulty (Rayner et al., 1992; Zimmerberg and Parsegian, 1986) and change their gating activity (Bezrukov and Vodyanoy, 1993; Vodyanoy et al., 1993). Changes in hydration have been measured in the reduction of cytochrome oxidase (Kornblatt and Bon Hoa, 1990), in the binding of oxygen to hemoglobin (Colombo et al., 1992), in the binding of ligands (Sidorova and Rau, 1995) and proteins (Garner and Rau, 1995; Robinson and Sligar, 1995) to DNA, and in the binding of substrates and inhibitors to adenosine deaminase (Dzingeleski and Wolfenden, 1993).

Hexokinase (HK) structures led to the original "induced-fit" mechanism of substrate specificity (Koshland, 1970). HK has a site in a cleft whose domains close on glucose binding (Bennett and Steitz, 1978), and the enzyme discriminates against water as a substrate (Bennett and Steitz, 1980). Recently we reported measurements of the effects of water activity on both glucose equilibrium binding and on hexokinase turnover (Rand et al., 1993). Under both conditions, the affinity for glucose increases with decreasing water activity, suggesting that water behaves like a competitive inhibitor of glucose binding.

Here we describe the effects of polyethylene glycol (PEG) on glucose binding to HK. Fig. 1 shows a schematic

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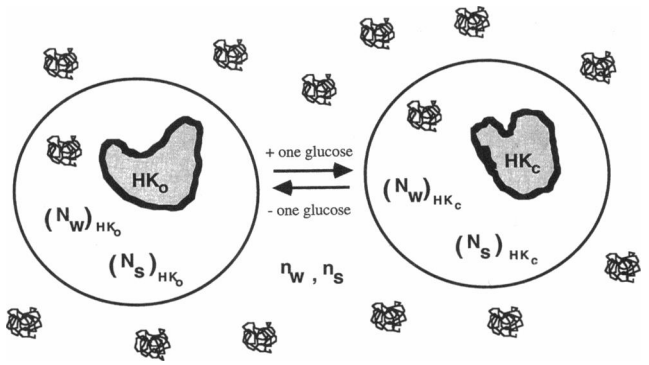

FIGURE 1 In the osmotic stress strategy, water activity, determined by measured osmotic pressure, Π, is controlled through the concentration of a solute that is preferentially excluded from an equilibrating aqueous domain around the protein, delineated here by the enclosing line. n_w/n_s and N_w/N_s are the number ratios of water and solute molecules in the bathing solution and within the protein domains. In terms of preferential hydration, $n_w/n_s < N_w/N_s$. Experimentally, differences in preferential hydration between the glucose-bound and unbound conformations are detected by a shift in their equilibrium, effected by a change in n_w/n_s, using PEG as solute.

diagram of the glucose binding equilibrium reaction

$$[HK_c \cdot glu] \leftrightarrow [HK_o] + [glu]$$

and illustrates the equilibrium partitioning of water and PEG between assay solution and local domains around the open, HK_o, and closed, HK_c, conformations of the protein. We consider both the direct and indirect effects of PEG and describe why we believe the effects of PEG are indirect and act by changing water activity. That interpretation provides a measure of the decreases in HK-associated waters that result on glucose binding to HK. These numbers are large for large MW-PEG, and decrease with MW, suggesting that different aqueous spaces around the protein can be probed. The sensitivity to osmotic stress suggests that hexokinase has very large conformational flexibility compared to its crystal conformation and occupies a wide range of hydration states. Osmotic work to remove the higher hydration states provides a measure of the energies of conformational change in proteins.

MATERIALS AND METHODS

Hexokinase (EC 2.7.1.1) was type C-302 from baker's yeast (Sigma), essentially fraction II of Kaji et al. (1961). The assay solution was for monomeric HK, in 20 mM glycylglycine buffer (pH 8.7), 200 mM KCl. The protein concentration was constant at 38.4 μg/ml assay solution. Polyethylene glycol (Fluka and Pharmacia) of vendor molecular weights 300 to 10,000 was added to the assay solutions up to 30 wt%.

Glucose dissociation constants (K_d) were measured through a decrease in intrinsic fluorescence, ΔF (λ_{ex} = 290 nm, λ_{em} = 340 nm), that occurs when glucose binds to hexokinase (Hoggett and Kellett, 1976; Rand et al., 1993). Glucose concentrations are expressed in molality; experimentally they spanned the range around K_d. There was no detectable effect of the PEG itself on the level of hexokinase fluorescence in the absence of glucose, i.e., the polymer alone does not induce the fluorescence changes in the protein. PEG has no detectable effect on the maximum decrease of fluorescence at high glucose concentration, i.e., PEG does not interfere with the glucose-hexokinase interaction at saturating levels of glucose.

The osmotic pressures of assay solutions were measured either directly with pressure gauges (Parsegian et al., 1986), by vapor pressure osmometry (Wescor 1500), or by secondary "osmometry." For the latter, x-ray diffraction measurements of multilamellar phases of stearoyloleoylphosphatidylcholine, hydrated under known osmotic pressures (Rand and Parsegian, 1989), were calibrated against those hydrated in the polymer solutions used in this study (Reid, 1995). Osmotic data have been collected for many solutes from many laboratories and are available on the Internet (at http://aqueous.brocku.ca) for readers wanting to do osmotic stress experiments.

Measured osmotic pressures were nonideal. PEG, particularly the larger PEGs, behaved osmotically as if it had an effective molecular weight (MW_{eff}) considerably smaller than "vendor" or number-average MW. Assuming that the nonideal osmotic pressure resulted from the "binding" of water to PEG effectively removing it from activity (Fullerton et al., 1992), we estimated MW_{eff} by fitting the measured osmolality to

$$\Pi = G/MW_{eff}/(1000 - mG)$$

over the whole range of measured pressures, where G = g PEG/1000 g water, and m is the grams of water per gram of PEG effectively removed by PEG from being active.

RESULTS

Table 1 provides our complete set of experimental data for 10 different PEGs, giving their vendor and effective molecular weights, effective molalities, measured osmotic pressures, and their effect on K_d, the hexokinase dissociation constant for glucose.

Osmotic pressures

Osmotic pressures can be far from ideal and require empirical measurement. Fig. 2 shows, as an example, the measured osmotic pressure for PEG of vendor MW 1500. The fit to the experimental data, imagining that m grams water "binds" to 1 g PEG and becomes inactive, yields an effective molecular weight of 857, and m = 0.956. Such a large value of m suggests that PEG removes a weight of water nearly equivalent to its own weight from being active. Table 1 shows that osmotic pressures of high-MW PEG solutions of fixed weight percentage become insensitive to number-averaged molecular weight. Both number-averaged and effective molecular weights and m for all the PEGs used in this study are provided in Table 1.

PEG increases the affinity of HK for glucose

All PEG solutions cause an increase in the affinity of hexokinase for glucose, causing a shift in the equilibrium reaction toward the glucose-bound conformation. Fig. 3 shows the relation between K_d and osmotic pressure for lower MW PEGs. For PEGs of MWs 1,000 to 10,000, similar relations are shown in Fig. 4.

ANALYSIS

We have recently described (Parsegian et al., 1995) the formulation of osmotic stress and its connection to prefer-

TABLE 1 Experimental data for 10 different PEGs

PEG Molecular Weight$_{Vendor}$ ($MW_{Effective}$, m = g H_2O "bound"/g PEG)

10000 (1426, m = 1.196)			8000 (1419, m = 1.193)			6000 (1353, m = 1.145)			3000 (1159, m = 1.091)			2000 (995, m = 1.025)		
[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.037	0.117	0.116	0.037	0.096	0.137	0.039	0.120	0.140	0.045	0.128	0.164	0.053	0.160	0.121
0.078	0.146	0.190	0.078	0.142	0.263	0.082	0.171	0.260	0.096	0.310	0.280	0.112	0.243	0.267
0.124	0.281	0.363	0.124	0.304	0.375	0.130	0.325	0.367	0.152	0.572	0.415	0.177	0.507	0.386
0.175	0.564	0.446	0.176	0.591	0.520	0.185	0.616	0.477	0.216	0.941	0.577	0.251	0.812	0.548
			0.235	1.012	0.659	0.246	1.040	0.531	0.288	1.460	0.671	0.335	1.340	0.666
									0.370	2.220	0.805	0.431	2.090	0.815

PEG Molecular Weight$_{Vendor}$ ($MW_{Effective}$, m = g H_2O "bound"/g PEG)

1500 (857, m = 0.956)			1000 (699, m = 0.887)			600 (491, m = 0.753)			400 (342, m = 0.561)			300 (281, m = 0.450)		
[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$	[PEG] molal effective	Π ($\times 10^7$ dynes/cm^2)	$\log\left(\frac{K_d^o}{K_d^\Pi}\right)$
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.061	0.153	0.162	0.075	0.186	0.167	0.107	0.267	0.137	0.154	0.357	0.118	0.188	0.542	0.077
0.130	0.328	0.266	0.159	0.347	0.293	0.226	0.634	0.237	0.325	0.848	0.260	0.396	1.000	0.232
0.206	0.573	0.413	0.252	0.729	0.491	0.359	1.020	0.404	0.515	1.390	0.336	0.629	1.690	0.378
0.292	0.945	0.557	0.358	1.120	0.576	0.509	1.600	0.458	0.730	2.150	0.471	0.891	2.580	0.506
0.389	1.500	0.694	0.477	1.800	0.729	0.679	2.380	0.663	0.974	3.140	0.620	1.188	3.740	0.558
0.500	2.280	0.795	0.613	2.660	0.772	0.873	3.420	0.752	1.252	4.480	0.768			

PEG Molecular Weight$_{vendor}$ is the number average molecular weight determined by end-group titration analysis (Fluka).
$MW_{Effective}$ and m values are derived from least-squares fit to Π (osmol) = $(G/MW_{effective})/1000 - m \times (G)$, where G is g PEG/1000 g H_2O, and m is g H_2O "bound"/g PEG.
molal effective is $(G/MW_{effective})/1000$ g H_2O.
Π is the measured osmotic pressures of the PEG solutions. We use 1 dyne/cm^2 = 4.11×10^{-8} osmolal (T = 20°C) for conversion.
K_d^o/K_d^Π is the ratio of the glucose equilibrium dissociation constants measured at osmotic pressures zero and Π (K_d^o = 0.19 ± 0.01 mmolal).
For further possible analysis, rows represent 0, 5, 10, 15, 20, 25 and 30 wt% PEG (g PEG/g PEG + water).

ential interactions, and compared them to "molecular crowding" (Zimmerman and Minton, 1993). In the formulation of preferential interactions, the equilibrium partitioning of water and solute (here PEG) between solution and protein is considered in terms of two domains, the bulk solution and a local domain around each isolated protein, as

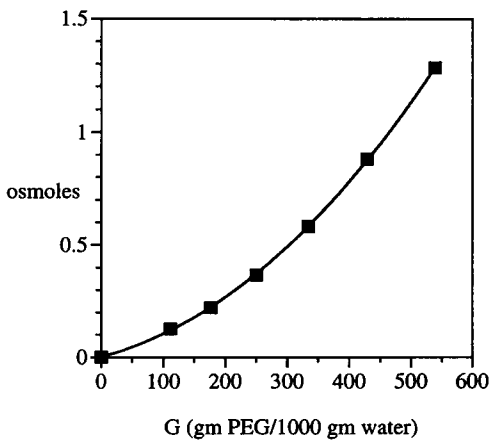

FIGURE 2 Plot of measured osmotic pressure of PEG (MW 1500) solutions. The fit to the data was made using Π (osmoles) = $G/MW_{eff}/(1000 - mG)$. This yielded an effective MW_{eff} of 857 and m = 0.956 g water/g PEG, an amount of water apparently removed by PEG from being active.

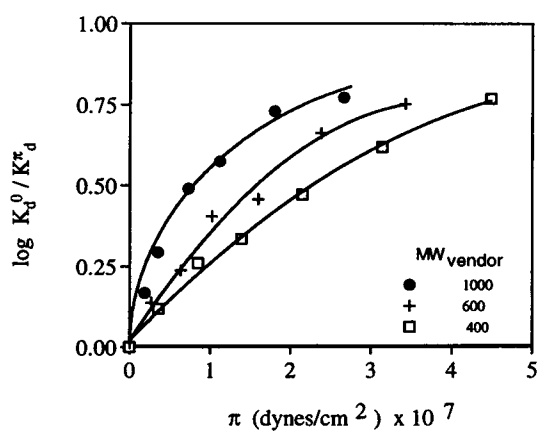

FIGURE 3 Sensitivity to osmotic pressure of hexokinase affinity for glucose. K_d^o is the dissociation constant measured without PEG and K_d^Π that measured in PEG solutions of molecular weights 400, 600, and 1000. Slopes were estimated from the lines that are best-fit second-order polynomials.

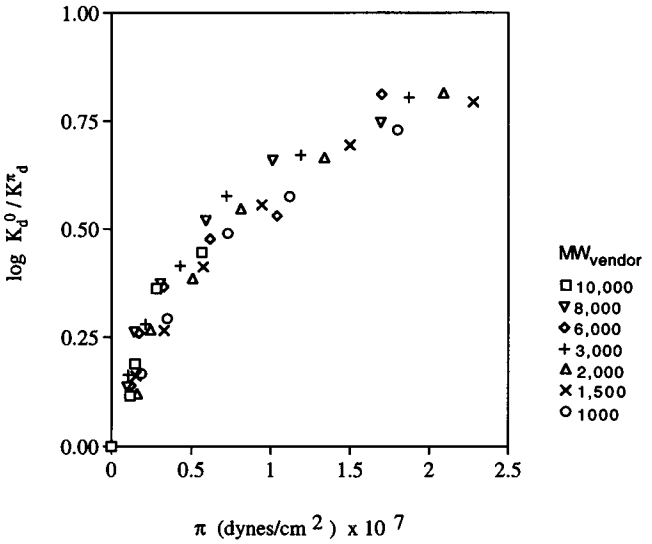

FIGURE 4 Sensitivity to osmotic pressure of hexokinase affinity for glucose. K_d^o is the dissociation constant measured without PEG, and K_d^Π is that measured in PEG solutions of vendor molecular weights 1000, 1500, 2000, 3000, 6000, 8000, and 10,000.

shown in Fig. 1. A similar two-domain approach has been used in the past (see, for example, Na and Timasheff, 1981) and more recently in an analysis of preferential interactions (Record and Anderson, 1995). n_w/n_{peg} and N_w/N_{peg} are the number ratios of water to PEG molecules in the assay solution and within the domains around the protein. Those domains can be of any size, as long as they enclose all the differences the protein makes on its local composition, and are small enough that they do not intersect other domains. Only a difference in partitioning of water and PEG into the domains of the unbound/open, HK_o, and bound/closed, HK_c, conformations can act to shift the equilibrium between them. In this formulation (Parsegian et al., 1995),

$$kT\, d \ln (K_d)/d\mu_w = \{(N_w)_{HK_o} - (N_w)_{HK_c}\} + n_w/n_{peg}\{(N_{peg})_{HK_o} - (N_{peg})_{HK_c}\}$$

$$kT\, d \ln (K_d)/v_w d\Pi_w = \Delta N_w + n_w/n_{peg}(\Delta N_{peg}), \quad (1)$$

or conversely,

$$kT\, d \ln (K_d)/d\mu_{peg} = \Delta N_{peg} + n_{peg}/n_w\,(\Delta N_w). \quad (2)$$

v_w is the molecular volume of water, 30 Å^3; μ_w and μ_{peg} are the chemical potentials of water and PEG.

In terms of the equilibrium reaction,

$$\Delta N_w + [HK_c \cdot glu] \cdot \Delta N_{peg} \leftrightarrow [HK_0] \cdot \Delta N_w + [glu] + \Delta N_{peg}.$$

Only differences in either N_w or in N_{peg}, or both, between HK_o and HK_c contribute to the shift in glucose binding, K_d, shown in Figs. 3 and 4. We consider the possible extreme cases, $\Delta N_w = 0$, and $\Delta N_{peg} = 0$.

PEG binding along with glucose

On the basis of the equilibrium reaction and in terms of Eq. 2, we examined the change in glucose dissociation constant as it varies with μ_{peg}. We evaluated μ_{peg} from the Gibbs-Duhem relation,

$$n_w\, d\mu_w + n_{peg}\, d\mu_{peg} = 0,$$

using the empirical measure of water activity and solution composition as

$$\mu_{peg} = v_w \int n_w/n_s\, d\Pi.$$

Fig. 5 shows two examples of the change in K_d with μ_{peg}. The empirical least-squares fits to the data are used to derive values of the slope which, assuming $\Delta N_w = 0$, gives a value for ΔN_{peg}. Values for ΔN_{peg} were similarly determined for all molecular weight PEGs, pooling all of the data from MW 1,000 to 10,000 that appeared indistinguishable (Fig. 4). ΔN_{peg} at low PEG concentrations as it varies with PEG molecular weight is plotted in Fig. 6. By this interpretation, PEG binds to the extent of approximately 0.5 to 0.05 more molecules to HK_c than HK_o as the molecular weight changes from 300 to 10,000. Fig. 7 shows the dependence of ΔN_{peg} as μ_{peg} increases for the examples of Fig. 5. Binding apparently increases in the case of PEG MW 400, and decreases in the case of PEG MW 1000.

It is possible that PEG changes its binding in this way as HK changes its conformation, and that line of analysis could be followed using the set of data provided in Table 1. To more rigorously evaluate any such changes in direct PEG binding awaits direct measures of the interaction of PEG with HK_o and HK_c. A number of reasons discussed in the following, in addition to the unusual binding characteristics shown in Fig. 7, suggest to us that PEG's action is indirect, and we choose to follow an osmotic interpretation.

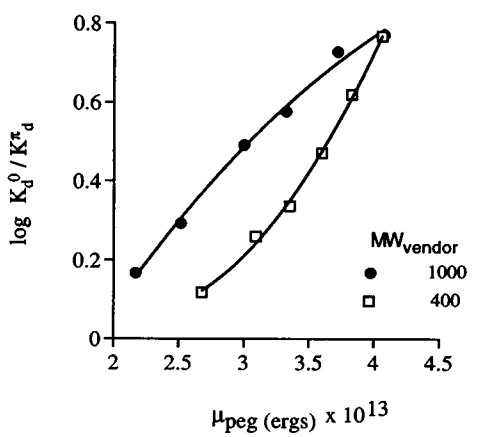

FIGURE 5 Sensitivity of hexokinase affinity for glucose to the chemical potential of PEG, μ_{peg}. K_d^o is the dissociation constant measured without PEG, and K_d^Π is that measured in PEG solutions of molecular weights 400 and 1000. Slopes were estimated from the lines that are best-fit second-order polynomials.

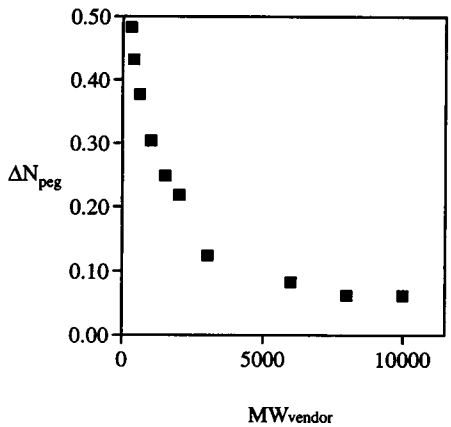

FIGURE 6 ΔN_{peg}, estimated from the initial slopes (lowest μ_{peg}) of the relations similar to those shown in Fig. 5, as it varies with the MW of the PEG. Data for MW's 1000 to 10000, shown in Fig. 4, were pooled for this estimate.

Bhat and Timasheff (1992) have shown that PEG is a solute that is strongly preferentially excluded from all native proteins measured. Changes in PEG binding have been observed only with large conformational changes such as protein denaturation and the exposure of hydrophobic areas (Timasheff, 1992). In this study we are considering two modestly different conformations of an active enzyme. Glucose binding involves a rigid rotation (Bennett and Steitz, 1978) or a shearing (Gerstein et al., 1994) of the two protein domains that form the cleft, without significant conformational change of the domains themselves. There was no detectable effect of PEG itself, even at its highest concentrations, on the level of hexokinase fluorescence in the absence of glucose, i.e., the polymer alone does not induce fluorescence changes in the protein. PEG has no detectable effect on the maximum decrease in fluorescence at high glucose concentration, i.e., PEG does not interfere with the glucose-hexokinase interaction at saturating levels of glucose.

Under all experimental conditions described here, we have shown that the enzyme maintains a constant activity and turnover rate, and that only its affinity for substrate is affected by PEG (Rand et al., 1993). The effect of PEG on enzyme kinetics was qualitatively the same on the hexokinase dimers as on the monomers (unpublished observations). The dissociation constant K_d and K_m are equal (Viola et al., 1982; Woolfitt et al., 1988) under control conditions, and this equality is maintained at all PEG molecular weights and concentrations used here. Methylated and unmethylated PEG have identical effects; one would expect effects of binding to be chemically specific.

For these reasons, in the following we have further interpreted the data in terms of PEG acting indirectly through water activity, with ΔN_{peg} equal to zero.

Water release with glucose binding

Assuming then that ΔN_{peg} is zero and referring to Eq. 1, the initial slopes of such lines as shown in Figs. 3 and 4 give ΔN_w, at low PEG concentrations. For all of the PEGs investigated, ΔN_w is shown in Fig. 8. As the molecular weight increases from 300 to 1000, ΔN_w increases from 50 ± 5 to 326 ± 35 and remains constant thereafter, independent of molecular weight.

Changes in water release with osmotic pressure

For MWs of 1,000 to 10,000 the relation between K_d over the full osmotic pressure range is nonlinear. Examples of the changes in ΔN_w with osmotic pressure, estimated from the slopes of the fitted data of Fig. 3, are shown in Fig. 9. For PEGs of molecular weight 600 and less, ΔN_w changes little with osmotic pressure, compared to the higher molecular weights. We suggest in the discussion that PEG "dehydrates" the protein before the glucose binding step. Fig. 10 shows a schematic of this interpretation.

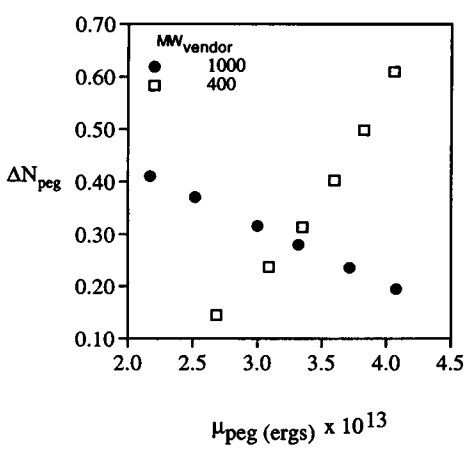

FIGURE 7 ΔN_{peg} as it varies with μ_{peg} estimated from the slopes of the curves shown in Fig. 5.

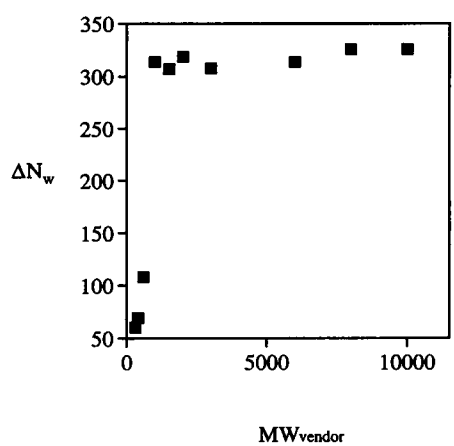

FIGURE 8 ΔN_w, determined from the data of Figs. 3 and 4 as it varies with the molecular weight of PEG.

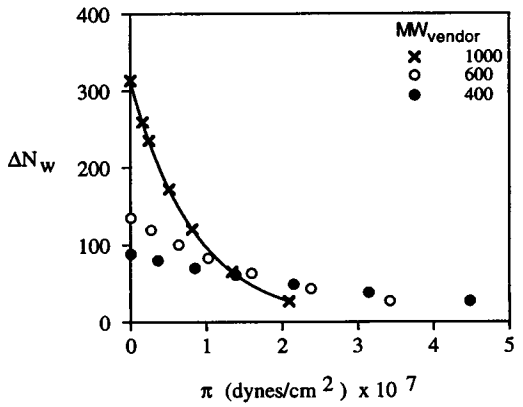

FIGURE 9 ΔN_w as it varies with the osmotic pressure of the assay solution, determined from the least-squares fit to the data of Fig. 3.

On this basis, Fig. 9 allows a measure of the osmotic work that would be done in transforming the unbound or open conformation of hexokinase from one hydrated state to the other, i.e., from *A* to *C* in Fig. 10. The variation of ΔN_w with Π for PEG MW 1000 can be described as

$$\Delta N_w = 312 \cdot e^{-1.186\Pi}.$$

The osmotic work is

$$W = \int_0^{2.28\times10^7} v_w \cdot \Delta N_w \cdot d\,\Pi.$$

Over the pressure range shown in Fig. 3, that osmotic work is 4×10^{-14} ergs, or 1 *kT*. Remarkably, only 1 *kT* osmotic work would be done on the protein in reducing the number of polymer-inaccessible waters around it by about 300.

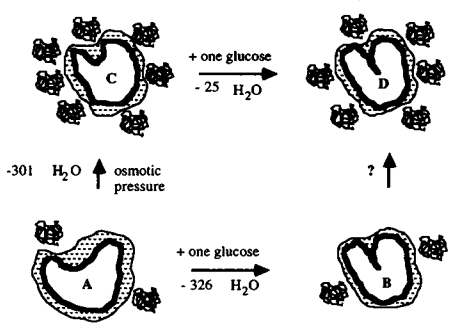

FIGURE 10 Schematic representation of the results based on the interpretation that PEG acts indirectly through water activity. Glucose binding induces the detectable conformational change required for catalysis (*A–B, C–D*). For PEG of MW 1,000 to 10,000 and for low osmotic pressures, ΔN_w for that conformational change is 326 ± 10%. However, ΔN_w decreases with PEG concentration. We suggest that these larger MW PEGs are excluded from the same maximum size aqueous compartment around the protein and act to osmotically shrink that compartment (*A–C*).

DISCUSSION

It remains to be more firmly established how PEG and water combine to affect glucose binding. Identical results with chemically different solutes is usually interpreted as meaning that their action is indirect and takes place through water activity (Parsegian et al., 1995). In this system, methylated PEG was found to give the same results for the active enzyme (Rand et al., 1993). For practical reasons a number of other solutes have been found not to work (Rand et al., 1993). Readers might use these data to construct a model of differential PEG binding to account for the results. In the following we discuss the implications of what we consider to be the more likely case, as interpreted in many other systems, that PEG does not bind differently to the two HK conformations and effects change through water binding.

The derived decrease in the number of water molecules, ΔN_w, with glucose binding can be viewed as a release of water from around the protein when HK changes conformation in the direction of cleft closure. ΔN_w increases from 50 molecules of water to about 326 waters per HK with increasing PEG molecular weight. We attribute this increase to the exclusion of PEG from an ever-increasing size of aqueous compartment around the protein as PEG itself increases in size, up to MW 1000. The common maximum value of ΔN_w, measured for all PEGs of molecular weight greater than 1000, suggests that all PEGs of sizes greater than this are sterically excluded from the same maximum size aqueous space around the protein. The obvious candidate for that space is the glucose-binding cleft. The increase in exclusion of PEG with increasing MW is strongly reminisent of the decreasing access to membrane channels (Vodyanoy et al., 1993). In the case of channels, however, one has the considerable advantage of independently measuring the solute accessibility of the channel through conductance measurements.

The present analysis shows that a maximum in ΔN_w is reached between PEG MW 600 and 1000, and remains constant for PEGs over a wide range of higher molecular weight. It is clear from the measured osmotic pressures that PEGs of larger molecular weight behave energetically as more than one osmotically active molecule. They appear to "bind" a mass of water nearly equal to their own mass, removing its contribution to water activity. In the past, geometrical arguments have been used to estimate excluded volume on the basis of the steric exclusion of the center of mass of PEG molecules from a protein surface, where each is considered to be spherical in shape (see, e.g., Bhat and Timasheff, 1992). These estimates predict no maximum in excluded volume with increasing PEG size, and it becomes unclear how the present results can be easily reconciled with these models.

Where could the estimated numbers of waters that are released on glucose binding come from? Fig. 11 shows the unbound and bound, low-resolution, protein crystal conformations of HK, along with scaled-down water molecules filled out to three layers from the protein surface. There

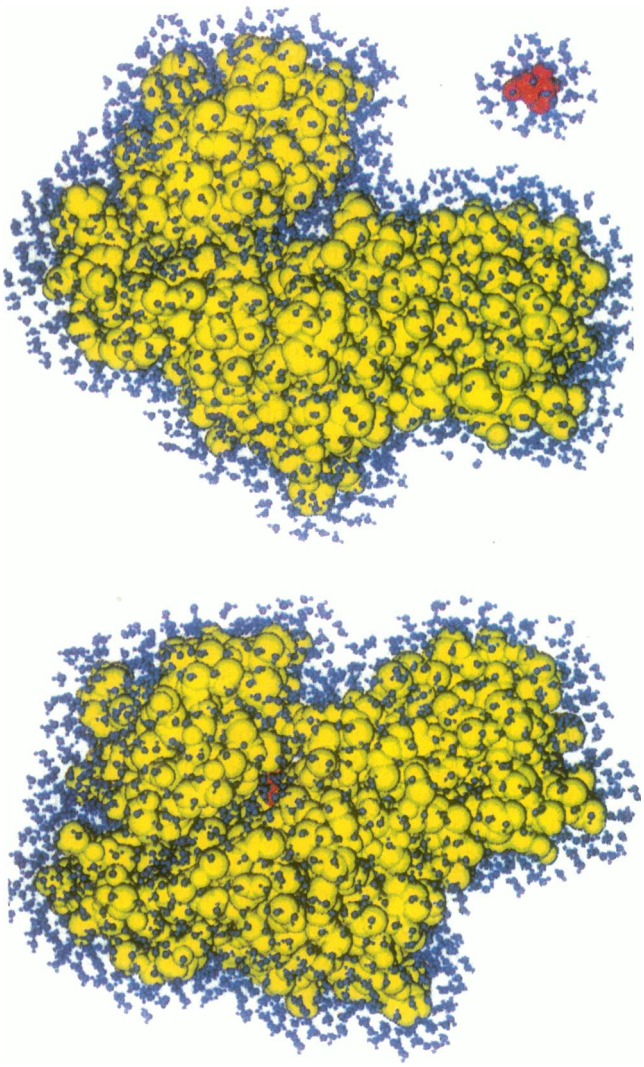

FIGURE 11 Space-filling models of the two crystallographic conformations of hexokinase and glucose. Superimposed are water molecules, on a smaller scale, placed to fill the volume around the protein out to three water layers. The protein structures were determined by Bennett and Steitz (1980). The image was created using Quanta (Molecular Simulations, Waltham, MA) with data from the Brookhaven protein data bank (Bernstein et al., 1977). 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.

could be several contributions to ΔN_w, including dehydration of glucose itself, the obvious decrease in cleft size in the protein, and changes in PEG accessibility to solvent-exposed protein surface groups.

Glucose is thought to have approximately six water molecules effectively associated with it that do not participate in changing the activity of water (Haldane, 1928). These six waters appear to be removed on glucose binding to HK (Bennett and Steitz, 1980). The reduction in cleft size involves a rigid rotation (Bennett and Steitz, 1978) or a shearing (Gerstein et al., 1994) of the two domains that form the cleft, without significant conformational change of the domains themselves. In that movement there is a decrease of approximately 163 Å^2 in protein area that is exposed to a single layer of water (Bennett and Steitz, 1978), and this would involve the removal of approximately 26 water molecules.

Simple space-filling molecular modeling of one hydration layer around the two conformations of HK also gives this value. The number of water molecules required to fill two and three layers of water from the HK surface is reduced by 60 and 100 water molecules, respectively, as the conformation changes from the crystallographic open to glucose-bound conformations. Alternatively, rolling a spherical probe over the two crystal conformations generates contact surfaces whose enclosed volume is also greater for the open conformation. Fig. 12 shows that for spherical probes of radius 8–200 Å, the volume difference increases rapidly up to that equivalent to about 100 water molecules and changes little thereafter. The rapid increase reflects the increasing exclusion of larger probes from the obvious cleft in the open conformation. This strongly resembles Fig. 8, except for the maximum value of ΔN_w reached.

These various estimated differences in volumes and numbers of water molecules are consistent with those estimated in this work by the smaller molecular weight PEGs, which we assume have some access to the cleft, and by all of the larger MW PEGs at higher pressures. However, these numbers, derived from the crystallographic conformations, fall short of the approximately 326 waters estimated in dilute solution using high molecular weight PEGs. Why?

Although we judge it to be unlikely, we cannot rule out the possibility that ΔN_w is overestimated to the extent that PEG does change its binding between the two conformations of HK. However, it is likely that the protein under the conditions of crystallization is "dehydrated" by crystallizing solutions and stabilized by crystal packing forces. One

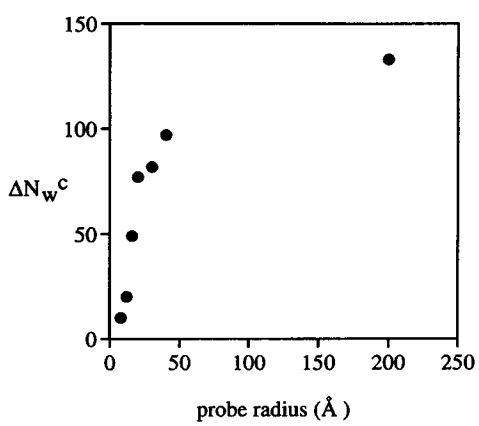

FIGURE 12 The volumes enclosed by the contact surface traced by spherical probes of radii from 8 to 200 Å rolling over the open and closed crystallographic conformations of HK were determined. ΔN_w^c is the difference in number of water molecules required to fill those volumes. It increases rapidly up to about 100 water molecules, reflecting the increasing inaccessibility of the probe to the open cleft, and changes slowly thereafter up to about 135.

might expect that the cleft could explore more open conformations in solution, particularly in the unbound conformation. In addition, the high mobility of the surface groups of proteins in solution seen by NMR suggests that their excluded volume would be higher than when constrained in crystals. A more open and flexible molecule with mobile surface groups in solution would increase the size of a PEG-excluding aqueous compartment beyond that in a crystal. We believe that the large size measured in this study is such a larger compartment, and that it includes more vicinal water than usually considered in structural studies.

That more vicinal water may contribute to the energetics of dehydration is consistent with measurements showing that interacting hydrophilic surfaces significantly perturb water beyond the first layer (Rand and Parsegian, 1989) and repel each other. Such vicinal waters are not detected by x-ray diffraction or NMR because their chemical potential differs so little from bulk water and the residency time is so short. 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 measurable.

Osmotic effects on hexokinase

There are a number of models that would give the nonlinearity of the log K_d dependence on osmotic pressure, seen particularly for PEGs of MW $>$ 1000. The equilibrium model considered here supposes only two conformation states with fixed numbers of "bound" PEG and water molecules. A third state or a continuum of states as we suggest in Fig. 10 would give such nonlinear behavior. Here we suggest that the larger PEGs in particular dehydrate the protein before the glucose binding, steps *A* to *C* in Fig. 10, i.e., they reduce the size of the preferentially hydrated space from which they are excluded as they reduce the surrounding water activity. So osmotically shrunk, fewer waters are then lost on glucose binding. To more firmly establish such a purely osmotic effect on the open state will require an independent measure of the osmotic sensitivity of protein conformation.

Gerstein et al. (1994) suggest that the dynamic equilibrium of protein conformational states can involve domain movements requiring very small energies. On the basis of the osmotic interpretaion, these results are consistent with this and provide an estimate of these energies. Of the 326 water molecules released on glucose binding by "unstressed" hexokinase (Fig. 10, *A* to *B*), the work needed to remove about 300 of them (Fig. 10, *A* to *C*), is remarkably small—on the order of 1 kT per hexokinase molecule. This suggests that unbound hexokinase in solution occupies a wide range of conformations. Although the open forms must allow specific substrate access to an active site, could they have such a wide range of conformations? Gerstein et al. (1994) have suggested recently that with substrate binding to only one domain, thermal fluctuations could bring the second domain into further contact and stabilize the closed conformation. Whatever the details of such binding steps, these osmotic stress results suggest a way of measuring such conformational flexibility.

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