Functionally Linked Hydration Changes in *Escherichia coli* Aspartate Transcarbamylase and Its Catalytic Subunit†

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ABSTRACT: Aspartate transcarbamylase (ATCase) is a highly regulated, dodecameric enzyme that catalyzes the first committed step in pyrimidine biosynthesis. Upon ligation, ATCase undergoes a conformational transition from a low-activity T-state to a high-activity R-state. This transition involves major changes in the molecular architecture, including structural rearrangements of several intersubunit interfaces and a 12 Å expansion of the molecule along its 3-fold axis. Solute-induced osmotic stress experiments report that ∼208 solvent waters are taken up by ATCase as it binds substrate. Solvent-accessible surface area calculations conducted on the T and R conformers of ATCase agree very well with this result, predicting that ∼189 waters are taken up during this conformational change. Both osmotic stress measurements and surface area calculations on the catalytic trimer of ATCase predict water release upon ligation of the trimer. Specific aspects of the application of osmotic stress to ATCase are also discussed, including solute size effects, and an assessment of potential alternative explanations for these results.

A key issue in resolving the molecular mechanism of a macromolecular process is understanding the involvement of solvent water. Many biomolecular processes, from ligand binding to conformational switching, necessarily involve movement of water. For example, water must be displaced from the active site of a protein when a ligand binds. Similarly, water must solvate surface area that is exposed as the result of a conformational transition. This involvement of water can both profoundly influence the equilibria of the molecular processes involved and be exploited as a tool to better understand these processes. We have used neutral solute-induced osmotic stress (Colombo et al., 1992; Parsegian et al., 1995) and structure-based surface area calculations (Connolly, 1983, 1985, 1993) to examine the involvement of water in the function of the holoenzyme and catalytic trimer of *Escherichia coli* aspartate transcarbamylase (ATCase).[1]

ATCase is a canonical model system for studying allosteric regulation and functional conformational equilibria (for reviews, see Allewell, 1989; Hervé, 1989; Kantrowitz & Lipscomb, 1990; Wild & Wales, 1990; Lipscomb, 1992, 1994; and Allewell & LiCata, 1995). ATCase catalyzes the first committed step in pyrimidine biosynthesis, the condensation of aspartate and carbamyl phosphate to produce carbamyl aspartic acid. ATCase is a highly regulated enzyme that binds aspartate cooperatively, is heterotropically regulated by nucleotide triphosphates, binds CTP with negative cooperativity, is synergistically inhibited by CTP and UTP in combination, and has a Bohr effect. The enzyme is a dodecamer consisting of six catalytic chains, organized as two catalytic trimers, and six regulatory chains, organized as three regulatory dimers. Upon ligation, ATCase undergoes a T to R transition in which the enzyme expands by 12 Å along its 3-fold axis, and a number of inter- and intrasubunit interactions change.

Osmotic stress is an experimental strategy that uses the thermodynamic linkage between osmotic pressure and the equilibrium of a macromolecular process to calculate the change in the amount of water associated with that process (Zimmerberg & Parsegian, 1986; Parsegian et al., 1986; Colombo et al., 1992). When the osmotic pressure of the bulk solution is increased in a controlled manner, movement of water away from osmotically stressed regions of the macromolecule is favored, so that macromolecular processes involving water release to the bulk are favored while those involving water uptake are inhibited. Several recent studies have used osmotic stress to examine the quantitative or qualitative participation of water in a wide variety of macromolecular processes [reviewed in Parsegian et al. (1995) and Robinson and Sligar (1995)]. The introduction of "membrane-free" applications, where the solutes that produce osmotic stress are added directly to the solution, has greatly decreased the technical difficulty of the experiment, but has simultaneously introduced new questions concerning excluded volume effects, the potential for direct solute interaction, and solute size effects [see Parsegian et al. (1995)]. In this study, we examine the hydration changes in ATCase during function as probed by osmotic stress, as well as examining some of these methodological questions in the context of ATCase.

Solvent-accessible surface area and volume calculations based on X-ray crystal structures provide a complementary approach for examining potential changes in hydration. As the exposed surface area of a macromolecule increases or decreases when the molecule switches from one conformation to another, solvent, mostly water, must bind or be released. Hence, the change in hydration should be proportional to the calculated change in molecular surface area. The presence of interior cavities and the irregularities of protein surfaces often complicate such calculations and

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[1] Abbreviations: ATCase, aspartate transcarbamylase; c_3, catalytic trimer; PALA, *N*-phosphonoacetyl-L-aspartic acid; PEG, polyethylene glycol; atm, atmospheres.

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correlations. In addition, a number of fundamental assumptions in the calculation of accessible surface area continue to be the focus of active investigation. In this paper, we report the calculated accessible surface area for the two major conformations of ATCase and find good agreement for the hydration changes predicted by these calculations with those determined with the osmotic stress approach. Osmotic stress measurements and solvent-accessible surface area calculations for the catalytic trimer of ATCase both report the release of water from the trimer upon ligation, but differ quantitatively. The results of this study agree with previous studies of the conformational changes in ATCase and its catalytic trimer, but provide unique molecular information about the magnitude of the structural changes in terms of the involvement of solvent water.

METHODS

ATCase was purified as described previously (Nowlan & Kantrowitz, 1985); catalytic trimers were prepared following Yang et al. (1978), with the modifications of Burz and Allewell (1982). Plasmids for the expression of *E. coli* ATCase were provided by Dr. E. R. Kantrowitz (Department of Chemistry, Boston College). Solutes (polyethylene glycols and dextrans) for osmotic stress experiments were purchased from Fluka Chemika-Biochemika (Milwaukee, WI) and from Sigma Chemical Co. (St. Louis, MO). All other chemicals were from Sigma.

Enzymatic assays were performed with the colorimetric assay developed by Prescott and Jones (1969), as modified by Pastra-Landis et al. (1978), with further minor modifications as described in Yuan et al. (1996). ATCase and its catalytic trimer were assayed at a concentration of either 0.1 or 0.15 μg/mL (depending on the relative osmotic pressure-induced inhibition) in a buffer containing 20 mM Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride], 20 mM Bis-Tris (bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane], 20 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], 0.2 mM dithiothreitol, and 0.2 mM EDTA (ethylenediaminetetraacetic acid), pH 8.3 (Pastra-Landis et al., 1978; Yuan et al., 1996). All aspartate titrations were performed with saturating carbamyl phosphate (4.8 mM; 3 orders of magnitude above the K_d). Stressing solutes in the same buffer were added directly to the assay solutions to produce the desired osmotic pressure, as described previously (Colombo et al., 1992; Parsegian et al., 1995). pH was carefully controlled. Addition of solutes caused little or no change in pH: ΔpH before readjustment was at most 0.2 for the 30% (w/v) solute stock solutions. Osmotic pressures were measured on a Wescor vapor pressure osmometer. The osmotic pressure of the assay buffer is 2.1 atm, which thus is the lowest osmotic pressure (effective origin) for all experiments.

Nonlinear regression was performed using the nonlinear least-squares program NONLIN (Johnson & Frasier, 1985) on an IBM RS/6000 Model 550. ATCase holoenzyme data were fit using an equation that takes into account the substantial substrate inhibition of the enzyme (LiCata & Allewell, 1997):

$$\text{velocity} = \frac{V_{max} + V_i([S]^2/K_i^2)}{1 + (K^{n_H}/[S]^{n_H}) + ([S]^2/K_i^2)} \qquad (1)$$

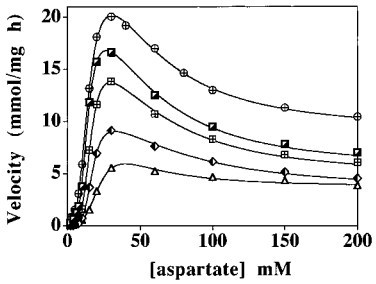

FIGURE 1: Representative aspartate titration curves for ATCase in the presence of increasing osmotic pressure. Osmotic pressure is induced by addition of 8000 molecular weight polyethylene glycol (PEG 8000) for all data in this figure. Crossed circles (⊕) show the titration in the absence of added solute. Titration curves at osmotic pressures of 2.5, 3.4, 4.8, and 6.5 atm are represented (top to bottom) by half-filled squares, crossed squares, half-filled diamonds, and open triangles, respectively. Solid lines show the fits of the data to eq 1.

Here, $K = K_m^{app} \approx [S]_{0.5}$; K_i is the apparent dissociation constant for substrate inhibition, V_i is the plateau velocity at maximal substrate inhibition, V_{max} has its usual definition, and n_H is the Hill coefficient. The catalytic trimer exhibits very mild substrate inhibition, and so all catalytic trimer data were fit using the standard Hill equation.

Osmotic stress results were analyzed using thermodynamic relationships developed previously (Colombo et al., 1992; Rand et al. 1993; Parsegian et al., 1995). Briefly, the change in the number of associated water molecules is obtained from the slope of the plot of ln (K_m^{Π}/K_m^0) versus osmotic pressure using relationships developed previously (Colombo et al., 1992; Rand et al., 1993; Parsegian et al., 1995):

$$\frac{\partial kT \ln (K^{\Pi}/K^0)}{\partial \Pi_{osm}} = \Delta V_w = \Delta N_w(30\ \text{Å}^3) \qquad (2)$$

Here K_m^{Π} is the K_m at osmotic pressure Π, K_m^0 is the K_m in the absence of added solute, ΔV_w is the linked change in volume, 30 Å^3 is the molecular volume of water, and ΔN_w is the linked change in the number of associated waters.

Surface area and volume calculations were performed on an IBM RS/6000 using the program PQMS from Michael Connolly's Molecular Surface Package (Connolly, 1983, 1985, 1993), which is based on the Lee and Richards' algorithm (Lee & Richards, 1971). Calculations were performed on the unligated (Stevens et al., 1990) and PALA-ligated (Krause et al., 1985, 1987; Ke et al., 1988) crystal structures (Brookhaven Protein Data Bank entries 6at1 and 8atc, respectively) using the default probe radius of 1.5 Å. Holoenzyme coordinates were generated from the asymmetric unit, and the structures were energy-minimized as described previously (Oberoi et al., 1996). As the structure of the isolated catalytic trimer has not been determined, calculations for ligated and unligated c_3 were performed by extracting coordinates for the trimer from the ligated and unligated holoenzyme coordinates.

RESULTS

Figure 1 shows representative aspartate titration curves for the holoenzyme in the absence and presence of neutral solute-induced osmotic pressure. In this figure, the stressing solute is polyethylene glycol with a molecular weight of 8000 (PEG 8000). Since ATCase exhibits significant substrate inhibition, here we show the full aspartate titration curves for the

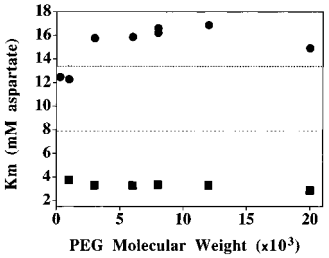

FIGURE 2: Osmolyte size dependencies at constant osmotic pressure for ATCase and its catalytic subunit. All data on this plot were collected at 4.5 (±0.5) atm osmotic pressure as induced by PEGs of different molecular weights. Closed circles are data for ATCase; closed squares are data for c_3. The two horizontal dashed lines show the K_m's in the absence of added osmotic pressure (13.3 mM for ATCase; 7.9 mM for c_3).

enzyme, including substrate inhibition. Increasing osmotic pressure produces a significant shift of the entire titration curve. The curves remain well described by eq 1 under all osmotic pressures, and the solid lines in this figure show the fits to eq 1.

Since added solutes osmotically stress only regions of the protein from which they are excluded, it is important to determine the solute size range that reports the total hydration change for the system. A solute size variation in osmotic pressure dependence has been reported in both systems where it has previously been assayed: the alamethicin channel (Vodyanoy et al., 1993), and the hexokinase system (Parsegian et al., 1995). Figure 2 shows the dependencies of K_m on osmolyte size for both ATCase and the catalytic trimer. All points are at the same osmotic pressure (4.5 ± 0.5 atm). Figure 2 shows that while the holoenzyme exhibits a marked dependence on solute size, the catalytic trimer displays very little solute size dependence. An important aspect of determinations such as those shown in Figure 2 is to demonstrate that the size effect tends toward a plateau (or that there is no significant size effect as in the case of the catalytic trimer). A leveling off of the solute size dependence indicates that (1) solutes in the plateau region are assaying the full hydration change for the macromolecule (i.e., they are fully excluded from all cavities and crevices on the protein), and (2) molecular crowding is not the primary effect of added solute (as this effect would continue to increase with solute size). These issues will be examined further under Discussion.

Figure 2 also shows that ATCase has a fairly sharp transition for K_m at an osmolyte size near 2000. The horizontal dashed line at 13.3 mM aspartate shows the K_m of ATCase for aspartate in the absence of applied osmotic pressure. Hence, one would expect a reversal in the measured change in hydration for ATCase depending on the solute size used. As is show in Figure 3, and discussed below, this is exactly what is observed. On the other hand, c_3 shows only a slight drift toward lower K_m values with increasing solute size. Thus, any stressing solute across this size range should report the total hydration change for c_3, whereas only solutes $>\sim$3000 molecular weight report the total hydration change for the holoenzyme.

Data similar to those in Figure 1 were collected for ATCase and its catalytic subunit using several different solutes to raise the osmotic pressure. Figure 3 shows the osmotic pressure dependence of K_m in these titrations. Two different types of stressing solute (carbohydrates and PEGs)

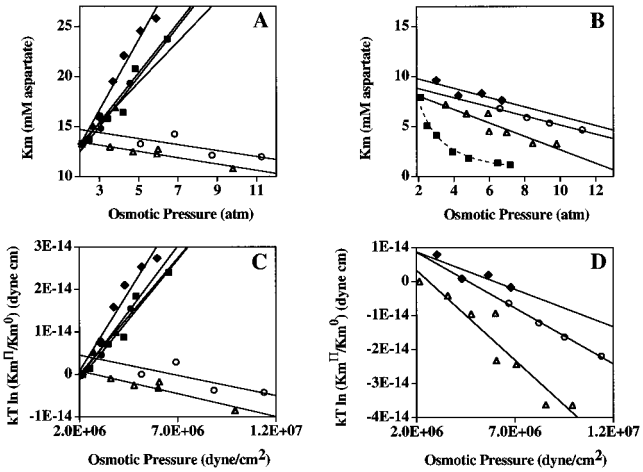

FIGURE 3: Solute-induced osmotic pressure dependence of K_m for ATCase (panels A and C) and its catalytic subunit (panels B and D). Panels C and D show the data as analyzed with eq 2 in the text. Symbols are the same in all panels, and the *y*-axes for panels A and B are adjusted to the same scale. The osmotic pressure of the assay buffer in the absence of added solute is 2.1 atm. The solutes used to raise the osmotic pressure are as follows: dextran 11 000 (closed diamonds, ♦); PEG 8000 (closed squares, ■); PEG 10 000 (ATCase only, closed circles, ●); PEG 12 000 (ATCase only, closed triangles, ▲); PEG 300 (open triangles, △); and sucrose (open circles, ○).

Table 1: Change in Associated Water Measured by Osmotic Stress[a]

stressing solute	ΔH_2O ATCase	ΔH_2O catalytic trimer
dextran 11 000	249 ± 48	−74 ± 57
PEG 8 000	189 ± 34	nd[b]
PEG 10 000	208 ± 70	nd
PEG 12 000	184 ± 106	nd
PEG 300	−36 ± 10	−176 ± 39
sucrose	−32 ± 39	−109 ± 10

[a] ΔH_2O and associated errors are calculated by fitting to eq 2 in the text. [b] Not determined.

and two different size ranges are shown. The slopes of these dependencies, analyzed via eq 2 (see panels C and D of Figure 3), yield the change in associated waters linked to substrate binding, and these values are reported in Table 1. There is generally a fair amount of scatter in osmotic stress data, probably as a result of the difficulties of working with high concentration solute solutions, especially the higher molecular weight solutes, and this is reflected in the associated errors for the change in hydration. For the holoenzyme, several titrations were performed with two additional large solutes, PEG 10 000 and 12 000. The scatter is even larger for these two smaller data sets, but the calculated water association is the same. In the case of the catalytic trimer, there is a quantitative discrepancy in the hydration changes observed with the different solutes, although all solutes report a release of water from the trimer. Potential sources of the quantitative differences seen for the catalytic trimer are examined below and under Discussion.

The results for the holoenzyme illustrate the importance of determining the dependence of the osmotic stress effect on solute size. Small solutes yield an apparent change in hydration that is opposite in sign to that reported by the large solutes. As predicted by Figure 2, the larger solutes measure the total hydration change for the holoenzyme, while the smaller solutes do not. Note, however, that there is little or no effect of the chemical nature of the solute (sugar/dextran

Table 2: Accessible Surface Area Calculations

molecule	ASA[a]	ΔÅ^2	ΔH_2O^{ASA} [b]	ΔH_2O^{OS} [c]
ATCase[d]				
unligated (T-state)	98660			
PALA-ligated (R-state)	100219	1559	189 ± 33	208
catalytic trimer[e]				
unligated (T-state)	32858			
PALA-ligated (R-state)	30894	−1964	−239 ± 42	−120

[a] ASA = accessible surface area in Å^2 using a 1.5 Å radius probe. [b] Calculated using a 7−10 Å^2 surface area coverage per water molecule (mean of ΔÅ^2/7 + ΔÅ^2/10) (Colombo et al., 1992). [c] Numbers are the means of the values in Table 1. [d] see Methods for details on the structural data used for the calculations. [e] Catalytic trimer coordinates were extracted from the holoenzyme coordinates.

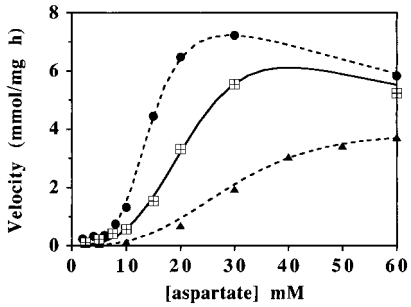

FIGURE 4: Regulation of ATCase by nucleotides at 6.5 atm osmotic pressure, induced by PEG 8000. Titrations are shown in the absence of added nucleotides (crossed squares), and in the presence of 2 mM ATP (closed circles, ●) or CTP (closed triangles, ▲).

versus PEG), an indication of the absence of significant solute specific effects. In the presence of large solutes (i.e., those in the plateau region of Figure 2), the K_m of ATCase increases with increasing osmotic pressure, indicating that more water becomes associated with the molecule upon substrate binding. This effect can be attributed to the conformational expansion of the molecule as it shifts from the low-affinity T form to the high-affinity R conformation, since water must associate with the newly exposed surface area. The accessible surface area calculations (Table 2) reinforce this conclusion.

Results for both large and small solutes indicate that water is released from the catalytic trimer as substrate binds (Figure 3). Also, as Figure 2 predicts, there is little or no solute size dependence for the measured hydration change (Table 1). However, the results for c_3 are more solute-specific that those for the holoenzyme. For example, the PEG 8000-induced osmotic pressure dependence is distinctly nonlinear. A similar nonlinearity has been observed for the PEG 8000-induced osmotic pressure dependence of hexokinase (Parsegian et al., 1995). The origins of this nonlinearity are unclear. For the other solutes, while the slopes remain somewhat similar, the position (i.e., *y*-intercept) of each line shifts. The stressing solutes thus appear to exert two effects on c_3: (1) an osmotic pressure effect that reports a release of water from the trimer upon ligation, and (2) some solute-specific shifts in substrate binding. The larger scatter in the calculated change in associated water for c_3 may be partly due to these overlapping solute-specific effects.

ATCase is heterotropically regulated by nucleotide triphosphates. Figure 4 shows the activation of ATCase by ATP and the inhibition by CTP at an osmotic pressure of 6.5 atm. The portions of the titrations before the onset of significant substrate inhibition are shown. All three curves have been shifted to higher aspartate concentrations by the application of osmotic stress. However, the regulation of ATCase by nucleotides (i.e., the relative relationships of the curves) is nearly identical to that observed by us and others in the absence of osmotic stress (Stebbins & Kantrowitz, 1989; LiCata & Allewell, submitted for publication). At the concentration of aspartate corresponding to the K_m in the absence of effectors, the ATP activation is ∼195% and the CTP inhibition is ∼66%. In other words, the ratio of K_m^{Π}/K_m^0 at 6.5 atm is nearly the same in all three cases. Figure 4 importantly demonstrates that the holoenzyme is not dissociated by osmotic stress, and that it appears to be functioning normally.

DISCUSSION

The hydration behavior of ATCase as revealed by osmotic stress measurements and accessible surface area calculations provides a unique quantitative perspective on the function and conformational equilibrium of the enzyme, and, as will be discussed below, correlates well with previous results. Internal consistency and correlation with other methods argue that osmotic stress data are an accurate reflection of the hydration behavior of ATCase. However, a systematic examination of the applicability of osmotic stress to ATCase, including evidence regarding other possible interpretations of the osmotic stress data, is in order.

Applicability of Osmotic Stress to ATCase

The fundamental assumption of the osmotic stress approach is that solute-induced osmotic pressure is directly linked to the binding or release of solvent water. The derivation of this direct linkage has been discussed by Parsegian and associates (Parsegian et al., 1995; Colombo et al., 1992). Below, we discuss the potential contributions of other effects of stressing solutes on ATCase function.

(A) Molecular Crowding Effects. Addition of inert solute to solution effectively increases the concentrations of all reaction species via molecular crowding. Molecular crowding effects have been studied in detail by Minton and associates [see Zimmerman and Minton (1993) for a review]. There will be some molecular crowding in any osmotic stress experiment; thus, it is important to ask if these effects dominate the observed results. At constant osmotic pressure, increasing solute size will consistently increase the solute-occupied volume fraction of the system. Figure 2 shows, however, that the solute size dependence of ATCase plateaus, while there is almost no solute size dependence for c_3. Both cases are inconsistent with the possibility that crowding effects are dominant.

Molecular crowding increases the activities of all reaction components, thus favoring substrate binding, but addition of stressing solute decreases the aspartate binding affinity of ATCase. Conversely, the data in Figures 2 and 3B for the catalytic trimer are directionally consistent with a crowding effect. However, in addition to the solute size independence mentioned above, the c_3 data are inconsistent with crowding effects in other respects. (1) The fact that dextran 11 000 shows the smallest quantitative effect (see Table 1) is the opposite of what would be expected from crowding, and (2) estimation of the increase in substrate activities due to solute volume exclusion by using molal substrate concentrations (although this is only a first ap-

proximation) predicts only an ~10% change in the slopes of the large solute data in Figure 3.

(B) Solute Interactions with the Enzyme. The fact that chemically different solutes yield the same results argues against any significant solute-specific interaction with the enzyme. The fact that the holoenzyme and the trimer show opposite hydration behavior also argues against this possibility. Further, for this interpretation to hold: (1) dextran 11 000 and PEG 8000 would have to act as nearly identical allosteric effectors of the holoenzyme, (2) they must be nearly as effective as CTP, the enzyme's natural allosteric inhibitor, and (3) they must bind tightly, as neither exceeds a concentration of 25 mM in these experiments. In addition, the molecular weight of the PEG would have to have a significant effect on its regulatory properties, switching from inhibition to activation at lower molecular weights. In the case of the catalytic trimer, as discussed above, specific solute−protein interactions may be involved in the relative displacement of the osmotic dependencies for the different solutes and the increased scatter in the determinations of hydration changes in c_3.

(C) Correlation with Other Solution Properties. The data of Figure 3 show no correlation among solutes with measured viscosity (data not shown). None of the solutes used is charged, and there are no pH changes in the experiment; hence, ionic strength and pH effects are ruled out. The expected changes in the solvent dielectric (ϵ) are quite small ($\pm<3\%$), and are not correlated in sign with the observed functional changes, nor correlated among solutes (data not shown; Arnold et al., 1985; Mathlouthi & Reiser, 1995). Thus, while addition of the stressing solutes may alter other aspects of the solution properties, the results are most consistent with the interpretation that the change in osmotic pressure is the dominating factor in the common modulation of function by the different solutes.

(D) Dissociation or Aggregation of the Enzyme. Nucleotide regulation of ATCase requires that the holoenzyme be intact; thus, osmotic stress induced dissociation of the enzyme is inconsistent with the data of Figure 4 which show that the relative regulatory response to nucleotides is not affected by osmotic stress. Further, dissociation of the holoenzyme would decrease the K_m, while dissociation of c_3 would not affect the K_m (since c monomers are inactive).

Molecular crowding favors macromolecular aggregation (Zimmerman & Minton, 1993). The plateauing of the solute size dependence (Figure 2) argues against this possibility, but crowding is not the only possible source of aggregation. Aggregation would decrease accessible surface area, and thus is inconsistent with the results for the holoenzyme. For aggregation to explain the c_3 results, a small amount of aggregation (~3%) would have to produce a disproportionate increase in substrate affinity (≥ 40%). Further, aggregation of c_3 is not observed even at protein concentrations 5 orders of magnitude higher than those of the present experiments (Gerhart & Schachman, 1965).

(E) Use of K_m. The linkage between osmotic pressure dependence and the change in associated water is rigorous for processes where the chemical potentials of the reactants are related to and altered by the change in the chemical potential of water induced by the addition of inert solute (Parsegian et al., 1995). Although any measured parameter can be inserted into the normalized ratio of eq 2, the quantitative relationship between hydration and the volume change for a kinetic parameter (e.g., k_{cat}) is unclear, and there is no significant dependence of n_H on osmotic pressure, so we have limited the current discussion to the osmotic pressure dependence of K_m. Aspartate binding to the catalytic trimer of ATCase has been determined to be nonsticky ($k_{-1} >> k_2$) (Turnbull et al., 1992), and is generally considered to be nonsticky for the holoenzyme [see Yuan et al. (1996)], although this question has not been definitively resolved. Nonsticky substrate binding would indicate that $K_m = K_s$ for aspartate, i.e., $K_m = k_{-1}/k_1$. Since V_{max} (k_{cat}) decreases with increasing osmotic pressure (see Figure 1), even if there is some slight sticky character to the binding of aspartate, increasing the osmotic pressure will further shift the enzyme toward the nonsticky limit, $K_m = K_s$.

Solute Size Dependence

The holoenzyme of ATCase shows a significant solute size dependence that the catalytic trimer does not. The apparent direction of water movement is reversed in the presence of large versus small stressing solutes. Osmolyte size dependence is interpreted to be caused by partial accessibility of the smaller solutes to spaces on the molecular surface where movement of water is occurring (Vodyanoy et al., 1993; Parsegian et al., 1995). When the solute size increases to the point where it is fully excluded from the differentially hydrating molecular spaces, the size effect should plateau. Osmotic stress data collected with solutes smaller than those in this plateau region will report hydration changes for the subset of hydrated molecular spaces from which they are excluded. In the case of the holoenzyme, small solutes apparently monitor some subset of the molecular surface that releases water upon ligation. The molecular volume of the active site ligands, calculated using the PALA-ligated crystal structure, predicts that ~8.5 waters should be displaced by substrate binding at each active site. The total of 50 displaced waters for all 6 active sites of ATCase is reasonably close to the ~34 waters reported to be released from the holoenzyme by small solute-induced osmotic stress, and it is possible that this is the hydration change monitored by the small solutes. In contrast to the holoenzyme, the data for the catalytic trimer (Figure 2) exhibit the least dependence on osmolyte size of the three systems in which, to our knowledge, solute size dependence has been directly examined (Vodyanoy et al., 1993; Parsegian et al., 1995; and this study). These results emphasize the importance of empirically determining the solute size dependence, or independence, of each system examined.

Hydration Behavior of ATCase

The osmotic stress dependence of ATCase predicts that substrate binding in ATCase is associated with the uptake of approximately 208 water molecules (the mean for the 4 large solutes in Table 1). This uptake can be attributed to the solvation of newly exposed surface area as the enzyme expands from the low affinity T-state to the high-affinity R-state. Accessible surface area calculations on the T- and R-state holoenzyme structures, when translated into a change in associated water, predict a nearly identical change in hydration: the association of ~189 waters (Table 2). This represents a calculated 1.6% increase in the total hydration of the molecule. In addition to the crystallographic data, the conformational expansion of ATCase upon ligation has been observed by difference sedimentation centrifugation

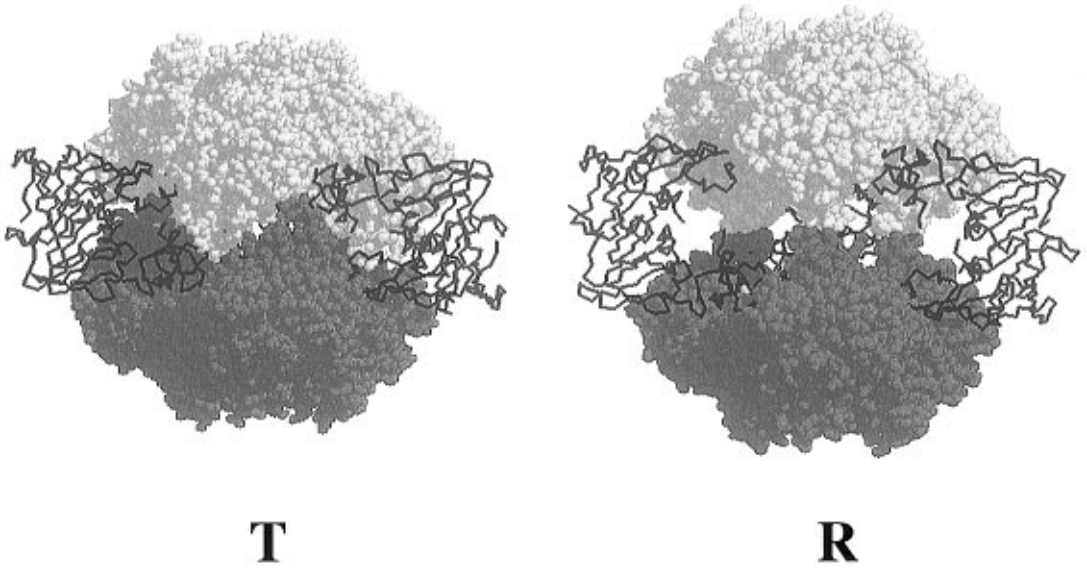

FIGURE 5: Views along the molecular 2-fold axis of the T- and R-states of ATCase emphasizing the exposure of new surface area in the interior of the molecule. Shown are PDB entries 6AT1 (unligated; Stevens et al., 1990) and 8ATC (PALA-ligated; Krause et al., 1985, 1987; Ke et al., 1988). The upper and lower catalytic trimers are shown in space-filled format. The regulatory dimers are shown as backbone traces.

(Gerhart & Schachman, 1968; Howlett & Schachman, 1977), differential optical mixing spectrophotometric determination of the change in the translational diffusion coefficient (Dubin & Cannell, 1975), X-ray solution scattering (Moody et al., 1979), and analytical gel chromatography (Bromberg et al., 1990). The hydrodynamic and spectrometric approaches each yield a unique perspective on the conformational change that would be difficult or impossible to translate into a quantitative change in water association. However, the directional consistency with osmotic stress measurements, and the fact that all methods similarly report conformational changes of a few percent, lends further support to the osmotic stress results. The associated uptake of water can also account for the observed large unfavorable entropy of substrate binding to ATCase (Knier & Allewell, 1978; Hoffman et al., 1979; LiCata & Allewell, submitted for publication).

Figure 5 shows views of the T and R states of the ATCase holoenzyme that illustrate where the associating water may bind. The movement of the upper and lower catalytic trimers away from each other exposes new surface area in the interior of the molecule. This view (Figure 5) emphasizes the substantial increase in the size of the central cavity in the R form of the enzyme. It should be noted, however, that both the T and R forms have a central cavity. This central opening has multiple access points and resembles a central "pavilion" more than a cavity, making estimation of its volume quite difficult, as the boundary between the inside and the outside of the space is not clear. After newly exposed surface area within the central pavillion is solvated, additional water molecules may also be taken up into this space which do not actually cover new surface area. Access of the different stressing solutes to this interior space may be the principal source of the solute size dependence for the holoenzyme. Exposure of new surface area at the interfaces of the catalytic and regulatory subunits can also be seen in this view, and will also account for some of the uptake of water.

Correlation of the osmotic stress results with results from structure-based surface area and volume calculations is not straightforward. The accurate calculation of protein surface area and volume themselves is an area of active investigation. It is not entirely clear whether osmotic stress measurements should best correlate with changes in the solvent-accessible surface area, the solvent-excluded volume, the van der Waals molecular surface, or with some other parameter. For ATCase, we find strikingly good agreement between the solvent-accessible surface area calculations and the osmotic stress measurements, an agreement that has been absent in a number of other systems, but this agreement may be fortuitous. The exploration of different algorithms, different probe radii, and the comparison of the quantitative differences between the various surface and volume definitions is beyond the scope of the present investigation, although it should be noted that use of a 1.4 Å radius probe for the calculation of the change in accessible surface area gives the same answer, within error, for the calculated change in hydration reported in Table 2. The net exposure of surface area during the T to R switch reported in Table 2 is comprised of the burial of $\sim$1300 Å^2 of polar surface and the exposure of $\sim$2900 Å^2 of nonpolar surface.

Osmotic stress measurements and surface area calculations on the catalytic trimer of ATCase both report a release of water upon ligation. Because solute-induced osmotic pressure increases the apparent affinity for aspartate in the catalytic trimer, the approximately 120 waters released from the trimer upon ligation may reflect: (1) a conformational contraction of the trimer; (2) displacement of water from the active site when substrate binds; and (3) the result of an increase in ligand activity due to crowding effects of added solute. Volume calculations indicate that active site ligands in the trimer would displace about 25 waters. Further, as discussed above, the results for c_3 are inconsistent with molecular crowding effects in several ways. Thus, most of the osmotic stress effect observed with the catalytic trimer is probably due to the conformational contraction of the trimer. This contraction is also observed by difference sedimentation centrifugation (Kirschner & Schachman, 1971; Howlett & Schachman, 1977), and analytical gel chroma-

tography (Bromberg et al., 1990). The present measurements are the first nonhydrodynamic observation of the conformational contraction of the trimer upon ligation.

The isolated catalytic trimer of ATCase has not been crystallized, but, as shown in Table 2, it is possible to extract the trimer coordinates from those for the holoenzyme and calculate the change in surface area upon ligation. This calculation predicts approximately twice the water release reported by osmotic stress. The fact that the trimer coordinates have been extracted from holoenzyme coordinates and the scatter in the osmotic stress data for the trimer both contribute to this discrepancy. It is interesting, however, to ask if the trimers within the holoenzyme might undergo a larger conformational transition than in solution, and if this might be one of the molecular origins for the switch from noncooperative ligation of the isolated trimers to their cooperative ligation in the holoenzyme.

Escherichia coli maintains a standard intracellular osmotic pressure of approximately 7.6 atm unless grown under conditions of high osmotic stress (Stock et al., 1977; Kennedy, 1987). Our osmotic pressure titrations of ATCase extend up to or past this physiological pressure (Figure 3). Although a variety of other factors undoubtedly influence ATCase under *in vivo* conditions, our results indicate that both aspartate binding and the T to R transition of ATCase should change only slightly under physiological conditions ($\pm<0.5$ kcal/mol depending on the solute size equivalent exclusion inside the cell). Allosterically, the enzyme will clearly still function in largely the same manner as *in vitro*. Further, Figure 4 indicates that the heterotropic feedback regulation of ATCase by nucleotide triphosphates will be essentially unaffected by osmotic pressures within the cell.

After the base line functional hydration behavior has been characterized for a macromolecule, a potential extension of the osmotic stress approach is to examine differential conformational changes in that macromolecule. Alterations in the magnitude of the molecular conformational change due to mutation or binding of effector molecules etc. should be detectable as alterations in the hydration change for the molecule. Studies of this nature are currently in progress for ATCase.

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