Role of Macromolecular Hydration in the Binding of the *Escherichia coli* Cyclic AMP Receptor to DNA†

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ABSTRACT: The osmotic stress technique was used to measure the changes in macromolecular hydration that accompany binding of the *Escherichia coli* CAP protein to its transcription-regulatory site (C1) in the lactose promoter and that accompany the transfer of CAP from site C1 to nonspecific genomic DNA. Formation of the C1 complex is accompanied by the net release of 79 ± 11 water molecules. If all water molecules were released from macromolecular surfaces, this result would be consistent with a net reduction of solvent-accessible surface area of 711 ± 189 Å^2. This area is only slightly smaller than the solvent-inaccessible macromolecular interface in crystalline CAP−DNA complexes. The transfer of CAP from site C1 to nonspecific sites is accompanied by the net uptake of 56 ± 10 water molecules. Taken with the water stoichiometry of sequence-specific binding, this value implies that formation of a nonspecific complex is accompanied by the net release of 2−44 water molecules. The enhanced stabilities of CAP−DNA complexes with increased osmolality (decreased water activity) may contribute to the ability of *E. coli* cells to tolerate dehydration and/or high external salt concentrations.

The control of transcription initiation involves the binding of gene regulatory proteins to both regulatory and competing genomic DNA sequences. The stability and specificity of these interactions depend on the solution environment in which they take place. Variables that have been shown to be important include salt concentration and identity (Record *et al.*, 1976; Leirmo *et al.*, 1987; Record & Mossing, 1987), pH (Barkley *et al.*, 1981; Overman & Lohman, 1994), pressure (Royer, 1995; Robinson & Sligar, 1995), and accessible volume (Cayley *et al.*, 1991; Zimmerman & Minton, 1993). In addition, changes in hydration accompany macromolecular interactions [reviewed by Timasheff (1993), Parsegian *et al.* (1995), and Robinson and Sligar (1995)]. For those interactions in which the hydration change is large, the free energies of interaction depend sensitively on the activity of water (a_{H_2O}).

The intimate association of complementary protein and DNA surfaces is accompanied by the displacement of water molecules associated with those surfaces. In addition, allosteric changes that extend beyond the contact surfaces can alter the solvent-accessible surface areas of protein and DNA and, thus, the number of associated water molecules. Any water molecules bound or released in these transactions must be considered reactants or products, respectively, in the binding reaction. Change in the number of thermodynamically associated water molecules can be detected and quantitated by the osmotic stress technique (Parsegian *et al.*, 1986; Colombo *et al.*, 1992; Parsegian *et al.*, 1995) using small, neutral solutes (osmolytes) that are typically excluded from the volumes immediately adjacent to macromolecular surfaces (Arakawa & Timasheff, 1985; Timasheff, 1993). With three caveats, the dependence of the equilibrium affinity of protein for DNA (K_{obs}) on water activity is a measure of the net change in the number of water molecules that are associated with the participating macromolecules. These caveats are (i) that K_{obs} should not be significantly perturbed by the differential interaction of osmolytes with either reactants or products, (ii) that volume exclusion by osmolytes should not significantly alter K_{obs}, and (iii) that changes in solvent properties that might indirectly affect binding affinity (for example, the dielectric coefficient) should not account for changes in K_{obs}.

Here, we report use of the osmotic stress approach to measure the water stoichiometries of binding reactions in which the *Escherichia coli* cyclic AMP receptor protein (CAP)[1] associates with its regulatory site in the lactose (*lac*) promoter and with nonspecific DNA. In the presence of cAMP, CAP activates *lac* transcription by binding its regulatory site (C1) and by engaging in direct contact with promoter-bound RNA polymerase (Ebright, 1993; Kolb *et al.*, 1993). In the cell, the sequence-specific DNA interactions of CAP take place in the presence of a large molar excess of competing nonspecific DNA sites (Stickle *et al.*, 1994b). It is the distribution of CAP protein between regulatory and nonspecific sequences that determines the occupancy of *lac* site C1 and hence the response of the *lac* promoter to cAMP. As shown below, under standard *in vitro* conditions, this distribution depends on the water activity.

In principle, the value of the water stoichiometry may contain information about structural changes that accompany binding. If the water stoichiometry of a reaction reflects the net association or dissociation of water molecules from

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[1] Abbreviations: CAP, the *Escherichia coli* cyclic AMP receptor protein (also called the catabolite gene activator protein); cAMP, cyclic adenosine 3′,5′-monophosphate; bp, base pairs; DNase, deoxyribonuclease.

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the macromolecular surface, its value should be proportional to the sum of the changes in solvent-accessible surface areas of reaction participants. For proteins, it has been estimated that an inner-hydration layer water molecule occupies 9 ± 1 Å^2 of surface (Colombo *et al.*, 1992). We use this value, with several caveats, to obtain an upper-limit estimate of the change in solvent-accessible surface area accompanying CAP−DNA interaction. This estimated change in solvent-accessible surface area is similar to one calculated for an isosteric model of the interaction, using the protein and DNA structures present in the CAP−DNA cocrystal solved by Steitz *et al.* (1991). The similarity in these values is surprising, because both CAP and DNA have been shown to undergo significant allosteric transitions during sequence-specific binding [reviewed in Kolb *et al.* (1993)].

EXPERIMENTAL PROCEDURES

Reagents. Sucrose, ethylene glycol, triethylene glycol, poly(ethylene glycol)s 400 and 8000, acrylamide, *N*,*N*′-methylenebis(acrylamide), bacterial alkaline phosphatase, and cyclic AMP were purchased from Sigma. Acetamide and poly(ethylene glycol) 1450 were from Eastman. Phage T4 polynucleotide kinase was purchased from New England Biolabs; [γ-^{32}P]ATP was from DuPont-New England Nuclear.

Protein and DNA Samples. The *E. coli* cyclic AMP receptor protein (CAP) was purified as described (Fried, 1982) from strain pp47/pHA5 (Aiba *et al.*, 1982). Judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, the protein was >95% pure. The preparation used in these experiments was 37% active in cAMP-dependent binding to DNA containing the wild-type *lac* promoter. The isolation of the 216 bp wild-type *lac* promoter fragment used in these experiments has been described (Stickle *et al.*, 1994a). This DNA contains two high-affinity sites for CAP, designated C1 and C2, respectively. Site C1 is the binding site from which CAP activates the *lac* promoter (Reznikoff, 1992a,b). It is bound ~40-fold more tightly than C2 (Hudson & Fried, 1991). *Lac* 216 DNA was labeled at 5′ termini with ^{32}P according to the method of Maxam and Gilbert (1977). Calf thymus DNA was purchased from Sigma and sonicated and deproteinized as described (Fried & Bloomfield, 1984). All DNA samples were dialyzed extensively against 10 mM Tris-HCl (pH 8.0 at 21 °C) and 1 mM EDTA prior to use. CAP and DNA concentrations were determined spectrophotometrically, using $\epsilon_{280} = 3.5 \times 10^4$ M^{-1} cm^{-1}/CAP dimer (Anderson *et al.*, 1971; Fried, 1982) and $\epsilon_{260} = 1.3 \times 10^4$ M^{-1} cm^{-1}/base pair, respectively. The protein concentrations given throughout this paper refer to the species active in sequence-specific DNA binding.

Formation and Detection of Protein−DNA Complexes. Binding reactions were carried out at 21 (±1) °C in solutions containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM KCl, 20 μM cAMP, and 0.1 mg of bovine serum albumin/mL, supplemented with osmolyte (acetamide, ethylene glycol, glycerol, sucrose) to obtain desired final concentrations. Mixtures were equilibrated at 21 (±1) °C for 1 h. In binding competition experiments, competing DNA was directly added to tubes containing protein−DNA complexes, mixed gently, and allowed to equilibrate at 21 (±1) °C for 1 h. In all cases, duplicate samples incubated for longer periods gave identical results, indicating that equilibrium had been attained. Following incubation, samples were analyzed by native gel electrophoresis (Fried & Crothers, 1981; Garner & Revzin, 1981).

Polyacrylamide gels (nominal concentration 10% w/v) were cast as described (Hudson & Fried, 1990) using an initial acrylamide:*N*,*N*′-methylenebis(acrylamide) proportion of 75:1. Both gel and electrophoresis buffers contained 20 mM Tris−acetate and 2 mM EDTA; electrophoresis buffer contained, in addition, 20 μM cAMP. Gels were prerun at 10 V/cm for 30 min to ensure that the cAMP was distributed uniformly. Electrophoresis of samples was carried out at 8 V/cm. For the CAP−DNA system, these conditions have been shown to allow accurate quantitation of free and bound DNA species (Fried, 1989; Fried & Liu, 1994). In the experiments reported here, gels containing identical samples run for different intervals gave binding distributions that were, within error, indistinguishable (results not shown), indicating that dissociation of complexes during electrophoresis was insignificant. Autoradiographs of developed gels were obtained with DuPont Reflection film, exposed at 4 °C. Gel segments containing individual electrophoretic species were excised using the developed film as a guide and counted in a scintillation counter. In a few cases, film densitometry was performed using a Hewlett-Packard scanner. When this method was used, care was taken to ensure that film exposure was within the linear range of dose−response (Fried, 1989).

Analysis of Binding. For determination of K_{obs}, the simple binding mechanism

$$C + D \overset{K_{obs}}{\rightleftharpoons} C{\cdot}D \tag{1}$$

was assumed. Here C represents CAP, D the DNA, C·D the CAP−DNA complex, and $K_{obs} = [C{\cdot}D]/[C][D]$. Values of [D] and [C·D] were determined by quantitation of the ^{32}P associated with the corresponding gel bands. Values of K_{obs} were obtained by fitting eq 2 to the binding data. Here

$$\frac{[D]}{[D]_{tot}} = 1 - \frac{a - \sqrt{a^2 - 4b}}{2} \tag{2}$$

$a = 1 + ([C]_{tot} + 1/K_{obs})/[D]_{tot}$, $b = [C]_{tot}/[D]_{tot,}$ and $[C]_{tot}$ and $[D]_{tot}$ are the total concentrations of CAP and DNA molecules in the sample, respectively (Stickle *et al.*, 1994a).

Binding competition assays were performed as follows. CAP−DNA complexes with CAP occupying *lac* site C1 were titrated with unlabeled calf thymus DNA, establishing the equilibrium

$$C{\cdot}P + D \leftrightarrows C{\cdot}D + P \tag{3}$$

in which C, P, and D represent CAP, site C1, and competing DNA sites, respectively. At the *i*th step of the titration, the specificity ratio is given by

$$\frac{K_S}{K_N} = \frac{[C{\cdot}P][D]}{[P][C{\cdot}D]} \tag{4}$$

Here [C·P] and [P] are measured quantities, $[C{\cdot}D] = [C{\cdot}P]_o - [C{\cdot}P]_i$ and $[D] = [D]_o - m[C{\cdot}D]$, with $[C{\cdot}P]_o$ and $[D]_o$ equal to the initial concentrations of C·P and D, $[C{\cdot}P]_i$ equal to the measured concentration of C·P at the *i*th step, and *m* equal to the number of base pairs occupied by the protein (Fried & Crothers, 1981; Fried, 1989).

Osmometry and Densimetry. Sample osmolalities were measured with a vapor pressure osmometer. In aqueous solutions, osmolality (Os) and water activity are related by $\ln a_{H_2O} = -\mathrm{Os}\bar{V}$, in which $\bar{V}$ is the molal volume of water and osmolality is given in units of osmoles per kilogram. Solution densities were measured as a function of osmolyte concentration using a Mettler densimeter, operating at 21 °C. Apparent partial specific volumes, $\overline{v_{app}}$, were calculated using the relationship (Casassa & Eisenberg, 1964)

$$\overline{v_{app}} = \left(\frac{1}{\rho_o}\right)\left[1 - \left(\frac{\rho - \rho_o}{c}\right)\right] \quad (5)$$

in which ρ is the density of the solution in grams per milliliter, ρ_o is the density of the solvent, and c is the osmolyte concentration in grams per milliliter. Values of $\overline{v_{app}}$ were plotted as a function of osmolyte concentration, and the value extrapolated to infinite dilution was taken as the partial specific volume, $\bar{v}$. Molar volumes were calculated according to $V_{mol} = \bar{v}M_r$.

Error Analysis. Confidence intervals for fitted parameters were estimated by the method of Broderson *et al.* (1987). Values of fitted parameters were multiplied by random variables with mean values of 1.0. The resultant values were retained if they gave fits to the experimental data that were within the 95% probability limit according to the *F* test (Sprague *et al.*, 1980). The largest and smallest members of a population of 200 acceptable values were taken to represent the 95% confidence limits for that parameter.

RESULTS

Effects of Neutral Solutes on the Binding of CAP to Its lac Promoter Regulatory Site. The binding of CAP (C) to a DNA site (D) in an aqueous solution containing a neutral solute (S) can be represented by

$$C + D \leftrightarrows C{\cdot}D + \nu_{H_2O}H_2O + \nu_S S \quad (6)$$

in which ν_{H_2O} and ν_s are the stoichiometric coefficients of water and solute, respectively. At constant temperature and pressure, a change in the experimentally observed equilibrium constant can be expressed as

$$d \ln K_{obs} = \left(\frac{\partial \ln K_{obs}}{\partial \ln a_{H_2O}}\right)_{a_S} d \ln a_{H_2O} + \left(\frac{\partial \ln K_{obs}}{\partial \ln a_S}\right)_{a_{H_2O}} d \ln a_S \quad (7)$$

Wyman (1964) has shown that

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln a_i}\right)_{j \neq i} = \nu_i \quad (8)$$

in which ν_i is the difference in the numbers of molecules of component *i* associated with reactants and products. Combining eqs 7 and 8 and rearranging gives

$$\frac{d \ln K_{obs}}{d \ln a_{H_2O}} = \nu_{H_2O} + \nu_S\left(\frac{d \ln a_S}{d \ln a_{H_2O}}\right) \quad (9)$$

Timasheff and co-workers have shown that a variety of low molecular weight osmolytes are excluded from the volumes immediately adjacent to proteins (i.e., that these volumes are occupied preferentially by water molecules (Arakawa & Timasheff, 1985; Timasheff, 1993)). Thus, for a process involving a large change in solvent-accessible surface area, taking place in a solution containing such a "noninteracting" solute, one might expect ν_{H_2O} to contribute more importantly to $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ than does ν_S. As described below, this expectation can be tested by comparing values of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ obtained with different structurally and chemically distinct osmolytes.

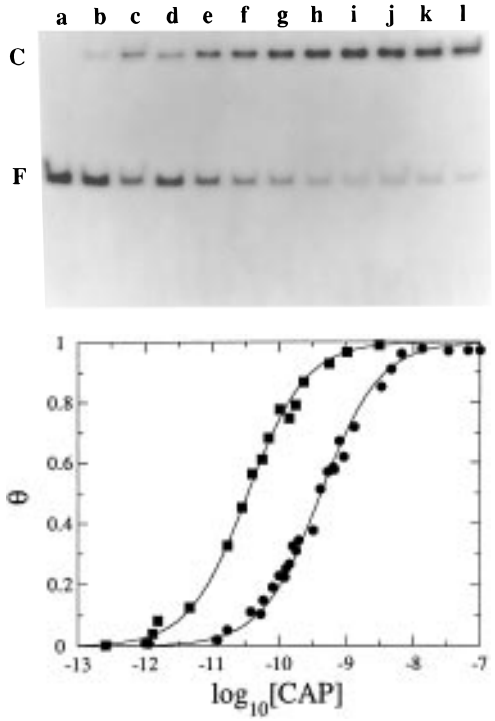

FIGURE 1: (A, top) Electrophoretic mobility shift analysis: titration of *lac* 216 DNA with CAP protein. Addition of CAP to a solution containing the free ^{32}P-labeled *lac* promoter fragment (F) resulted in the formation of the 1:1 CAP−DNA complex (C). All reactions were carried out in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, 20 μM cAMP, 0.8 M sucrose, and 0.1 mg of bovine serum albumin/mL. All samples contained 1.2×10^{-10} M *lac* promoter DNA. Samples b−l contained, in addition, 9.5×10^{-12}, 1.9×10^{-11}, 3.8×10^{-11}, 5.7×10^{-11}, 7.6×10^{-11}, 9.5×10^{-11}, 1.1×10^{-10}, 1.5×10^{-10}, 1.9×10^{-10}, 2.3×10^{-10}, and 2.8×10^{-10} M CAP, respectively. (B, bottom) Representative isotherms for the binding of CAP protein to the 216 bp *lac* DNA fragment. The fraction of *lac* 216 DNA bound (θ) is plotted as a function of the log of the free [CAP]. Symbols: (●) binding reactions carried out in the absence of added osmolyte; (■) binding reactions carried out in the presence of 0.8 M sucrose. The solid curves were calculated using values of $K_{obs} = 2.3 \times 10^9$ M^{-1} (no added osmolyte) and $K_{obs} = 2.9 \times 10^{10}$ M^{-1} (0.8 M sucrose) that were obtained by fitting the binding data as described in the text.

The gel electrophoresis mobility-shift assay (Fried & Crothers, 1981; Garner & Revzin, 1981) was used to detect the binding of CAP to its primary *lac* promoter site, C1 (Figure 1A). Under conditions of low binding saturation, the predominant complex formed contained one dimer of CAP, occupying site C1 (Fried & Crothers, 1984). Previous studies have shown that the secondary site C2 is bound with substantially lower affinity than C1 and, on this DNA, is occupied in <5% of 1:1 complexes (Hudson & Fried, 1990, 1991). Isotherms for the binding of CAP in buffer containing 0 and 0.8 M sucrose are shown in Figure 1B. In the absence of added osmolyte, the value of K_{obs} [$(2.3 \pm 0.1) \times 10^9$ M^{-1}]

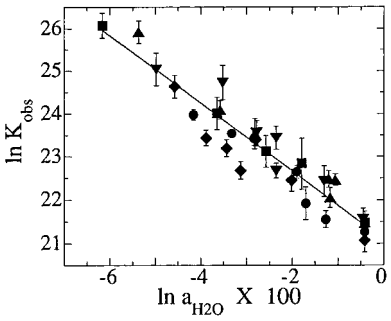

FIGURE 2: Dependence of K_{obs} for the CAP-*lac* 216 DNA binding reaction on the water activity of solutions containing the indicated osmolytes. Values of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ for solutions containing 0−2.5 M acetamide (●), 0−6.0 M ethylene glycol (◆), 0−4.1 M glycerol (■), 0−1.5 M sucrose (▲), and 0−2.3 M triethylene glycol (▼) were -80 ± 18, -76 ± 15, -78 ± 10, -84 ± 11, and -81 ± 16, respectively. Error bars represent 95% confidence limits, estimated as described in the text.

Table 1: Stoichiometry of Water Displacement and Change in Solvent-Accessible Surface Area for the Binding of CAP to *lac* Promoter DNA[a]

solute	M_r	V_{mol}[b] (mL/mol)	$\frac{\partial \ln K_{obs}}{\partial \ln a_{H_2O}}$ [c]	ΔA_W[d] (Å^2)
acetamide	59	55.1	-80 ± 18	-738 ± 242
ethylene glycol	62	54.1	-76 ± 15	-699 ± 211
glycerol	92	71.6	-78 ± 10	-712 ± 168
triethylene glycol	150	129.5	-81 ± 16	-745 ± 225
sucrose	342	214.4	-84 ± 11	-767 ± 183
global fit to all data for the solutes listed above			-79 ± 11	-711 ± 189
poly(ethylene glycol)	400	343.6	-518 ± 125	
poly(ethylene glycol)	1450	1225.3	-1034 ± 337	
poly(ethylene glycol)	8000	6760.0	-1677 ± 274	

[a] In addition to the indicated solute, reactions contained 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, and 20 μM cAMP. [b] Determined from apparent partial specific volumes as described in eq 5. [c] Error ranges represent 95% confidence limits. [d] Estimated with the assumption that a water molecule occupies 9 ± 1 Å^2 of macromolecular surface; experimental uncertainties in $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ are propagated into the error range for ΔA_W.

is in good agreement with values previously obtained under similar buffer conditions (Hudson & Fried, 1991).

The inclusion of acetamide, ethylene glycol, glycerol, sucrose, or triethylene glycol in the reaction mixture increases the value of K_{obs}. For these five solutes, ln K_{obs} is linearly correlated with ln a_{H_2O}, with the consensus value of $\partial \ln K_{obs}/\partial \ln a_{H_2O} = -79 \pm 11$ (Figure 2). In principle, several factors can contribute to the observed increase in K_{obs}, including changes in water activity, changes in solvent polarity, and direct solute−macromolecule interactions. An important criterion for discrimination between direct solute interactions with macromolecules and osmotic effects is the sensitivity of the value of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ to changes in the identity of the solute (Parsegian *et al.*, 1995). The number of solute molecules thermodynamically associated with each macromolecule and the change in that number with protein−DNA interaction (ν_S) should depend on the identity of the solute. On the other hand, a purely osmotic effect should be independent of solute identity (Colombo *et al.*, 1992; Parsegian *et al.*, 1995). Closely similar values of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ were obtained for the five low molecular weight solutes examined (Table 1). This could occur only if the product $\nu_S \cdot (\partial \ln a_S/\partial \ln a_{H_2O})$ was the same, within error, for all solutes tested or if all values of $\nu_S \cdot (\partial \ln a_S/\partial \ln a_{H_2O})$ were much less than that of ν_{H_2O} (eq 9). Since chemically distinct solutes associate to different degrees with proteins (Arakawa & Timasheff, 1985; Timasheff, 1993) and are likely to do so with nucleic acids as well, it seems improbable that the product $\nu_S \cdot (\partial \ln a_S/\partial \ln a_{H_2O})$ should be the same for sucrose, glycerol, ethylene glycol, acetamide, and triethylene glycol. For this reason we conclude that preferential macromolecule−osmolyte interactions, represented in aggregate by $\nu_S \cdot (\partial \ln a_S/\partial \ln a_{H_2O})$, are likely to contribute much less to $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ than do changes in macromolecular hydration, represented by ν_{H_2O}.

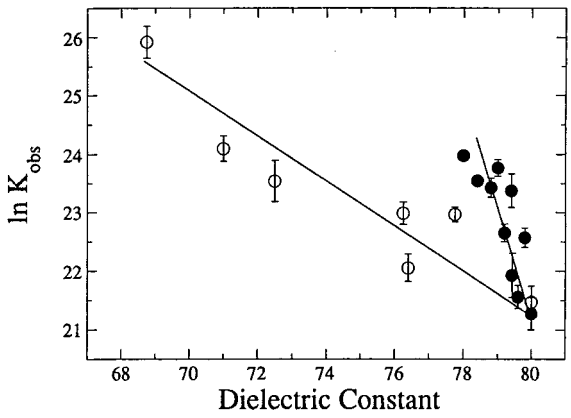

FIGURE 3: Dependence of K_{obs} for the CAP−*lac* 216 DNA binding reaction on the dielectric coefficients of solutions containing acetamide (●) and sucrose (○). Dielectric coefficients were calculated from dielectric increments tabulated by Cohn and Edsall (1943). Values of $\partial \ln K_{obs}/\partial$(dielectric coefficient) for solutions containing acetamide and sucrose were −7.5 and −0.8, respectively. Error bars represent 95% confidence limits.

A second possibility is that the increase in K_{obs} is a consequence of the decrease in the solution dielectric coefficient with osmolyte concentration. If this were the case, ln K_{obs} would be expected to scale identically with the dielectric coefficient for all osmolytes tested. Shown in Figure 3 are values of ln K_{obs} as a function of dielectric coefficient for the osmolytes acetamide and sucrose. Since the slopes differ markedly for these osmolytes, it follows that osmolyte-mediated changes in solvent polarity cannot account in a simple way for the common effects of different osmolytes on K_{obs}.

A third possibility is that the observed changes in K_{obs} are due to volume exclusion by the osmolytes. A hallmark of such effects is that they increase strongly with solute molar volume (Zimmerman & Minton, 1993). Shown in Figure 4 is the dependence of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ on ln V_{mol}, the natural log of solute molar volume, measured in our assay buffer, for solutes ranging from $M_r = 59$ (acetamide) to $M_r \sim 8000$ (poly(ethylene glycol) 8000). Values of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ depend strongly on ln V_{mol} for the large solutes but become essentially independent of ln V_{mol} for the small ones. As shown in the inset, a linear extrapolation of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ as a function of V_{mol} to $V_{mol} = 0$ gives a limiting value, -76 ± 26, that is indistinguishable from the experimental consensus value (-79 ± 11). This result suggests that volume exclusion affects the value of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ significantly only when the osmolytes are large (for this system, $V_{mol} > 214.4$ mL/mol).

Taken together, the results presented above support the interpretation that the consensus value $\partial \ln K_{obs}/\partial \ln a_{H_2O} = -79 \pm 11$, obtained with small osmolytes, is a measure of

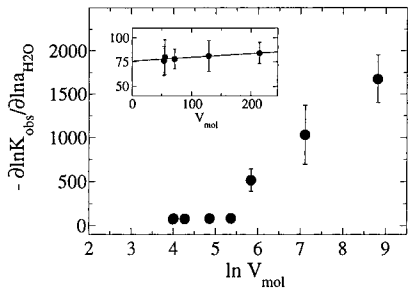

FIGURE 4: Dependence of the apparent water stoichiometry on solute molar volume. The data are for the binding of CAP to the 216 bp *lac* promoter fragment at 21 °C in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM KCl. Molar volumes of solutes were calculated from solution densities as described in the text. Error bars representing 95% confidence limits in $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ are plotted for all data but are obscured by the symbols for some points.

the net change in macromolecular hydration (ν_{H_2O}) that accompanies CAP's interaction with *lac* promoter site C1. This makes water by far the largest stoichiometric component of the binding reaction. If we assume that all water molecules contributing to the measured value of ν_{H_2O} occupy the inner hydration layer when associated with a macromolecule, and if a water molecule in the inner-hydration layer occupies 9 ± 1 Å^2 of macromolecular surface as proposed for the hydration of proteins (Colombo *et al.*, 1992), then the value of ν_{H_2O} specifies the change in solute-excluding, solvent-accessible macromolecular surface area (ΔA_W) that accompanies complex formation. As summarized in Table 1, the effects of low molecular weight solutes on K_{obs} are consistent with the estimate $\Delta A_W = -711 \pm 189$ Å^2. As shown below, this value is only slightly smaller than the area occluded in the CAP−DNA interface on formation of a sequence-specific complex.

Burial of Solvent-Accessible Surfaces in the CAP−DNA Interface. The 3.0 Å crystal structure of the CAP−DNA complex obtained by Steitz and co-workers (Schultz *et al.*, 1991) provides us with a model from which to estimate the amount of solvent-accessible surface area that is buried at the protein−DNA interface on complex formation. Atomic coordinates were obtained from the Brookhaven Protein Data Bank (PDB file 1CGP), and solvent-accessible surface areas were calculated using the Connolly algorithm (Connolly, 1983), as implemented in the program Insight from Biosym Technology. A probe radius of 1.4 Å (equal to that of the oxygen atom in a water molecule) was used. Models of the solvent-inaccessible surfaces of CAP and DNA present in the CAP−DNA complex are shown in Figure 5. The change in solvent-accessible surface area on formation of the CAP−DNA complex (ΔA_S) was calculated using

$$\Delta A_S = A_{CD} - (A_C + A_D) \qquad (10)$$

Here A_{CD} is the solvent-accessible surface area of the complex and A_C and A_D are the corresponding areas calculated for models of free CAP and DNA molecules in which their conformations are identical to those found in the complex. In consequence, this calculation does not take into account changes in the solvent-accessible surface areas of the protein and the DNA that result from conformational changes that occur during binding. The value of ΔA_S for the CAP−DNA binding reaction was found to equal −958 Å^2, slightly larger than that estimated from the water stoichiometry of the binding reaction ($\Delta A_W \sim -711$ Å^2; see above). The value of ΔA_S obtained for the CAP complex is considerably smaller than one previously calculated for a *trp* repressor−operator complex (−2900 Å^2; Otwinowski *et al.*, 1988; Lawson & Carey, 1993). This difference is intriguing, since both CAP and *trp* repressor are typical helix−turn−helix motif DNA binding proteins (Steitz *et al.*, 1982) that protect DNA regions of similar size from the actions of DNase I and hydroxyl radical [cf Schmitz (1981), Shannblatt and Revzin (1987), and Carey (1988, 1989)].

The Ratio of K_s/K_n Increases with Decreasing Water Activity. To regulate transcription, CAP partitions between regulatory and nonspecific DNA sites in a cAMP-dependent manner (Kolb *et al.*, 1993; Stickle *et al.*, 1994b). In the specific binding mode, CAP binds noncooperatively, occupies ~26 bp (Schmitz, 1981; Hudson & Fried, 1990), and bends the DNA by 90−120° (Zinkel & Crothers, 1990; Schultz *et al.*, 1991). In the nonspecific mode, binding is highly cooperative, requires only 13 bp (Saxe & Revzin, 1979), and may not bend the DNA (Hudson *et al.*, 1990). These contrasts suggest that the change in solvent-accessible surface area accompanying DNA binding should differ for sequence-specific and nonspecific binding reactions and hence that the water stoichiometries of specific and nonspecific binding reactions should differ. If the water stoichiometries differ, the distribution of CAP between regulatory (specific) and nonspecific sites will depend on water activity.

To test these notions, we performed binding competition assays (Fried & Crothers, 1981) to measure the specificity ratio K_S/K_N as a function of water activity. Here K_S is the association constant for *lac* promoter site C1 and K_N is the population-average association constant for genomic DNA. As shown in Figure 6A, solutions containing ^{32}P-labeled *lac* promoter DNA (1.4×10^{-10} M) and CAP (1.7×10^{-10} M) were titrated with unlabeled calf thymus DNA. The transfer of CAP from site C1 to competitor reduces the amount of ^{32}P-labeled CAP−*lac* DNA complex (C) and increases the amount of free ^{32}P-labeled DNA (F). Analyzed according to eq 4, these data gave a value of $K_S/K_N = 5.3\ (\pm 2.0) \times 10^5$, in good agreement with previous measurements (Fried & Crothers, 1984; Hudson & Fried, 1991). Results of competition experiments in which a_{H_2O} was varied by inclusion of acetamide, ethylene glycol, glycerol, and sucrose are shown in Figure 6B. The consensus value of $\partial \ln (K_S/K_N)/\partial \ln a_{H_2O}$ is -56 ± 10; values for individual osmolytes are summarized in Table 2. Taken with these competition data, the value $\nu_{H_2O} = -79 \pm 11$ for formation of the specific CAP−C1 complex implies that the formation of a nonspecific CAP−DNA complex is accompanied by net water release, with $-44 \leq \nu_{H_2O} \leq -2$. These values are significantly less negative than that for the specific CAP−C1 interaction and are consistent with the notion that the reduction in solvent-accessible surface area accompanying nonspecific binding (ΔA_W) is less than that for a specific interaction.

DISCUSSION

The binding of protein to nucleic acid brings together initially hydrated surfaces and may be accompanied by allosteric transitions in one or both molecules. Typically, these processes reduce the net surface area accessible to solvent (Ha *et al.*, 1989; Spolar & Record, 1994) and result

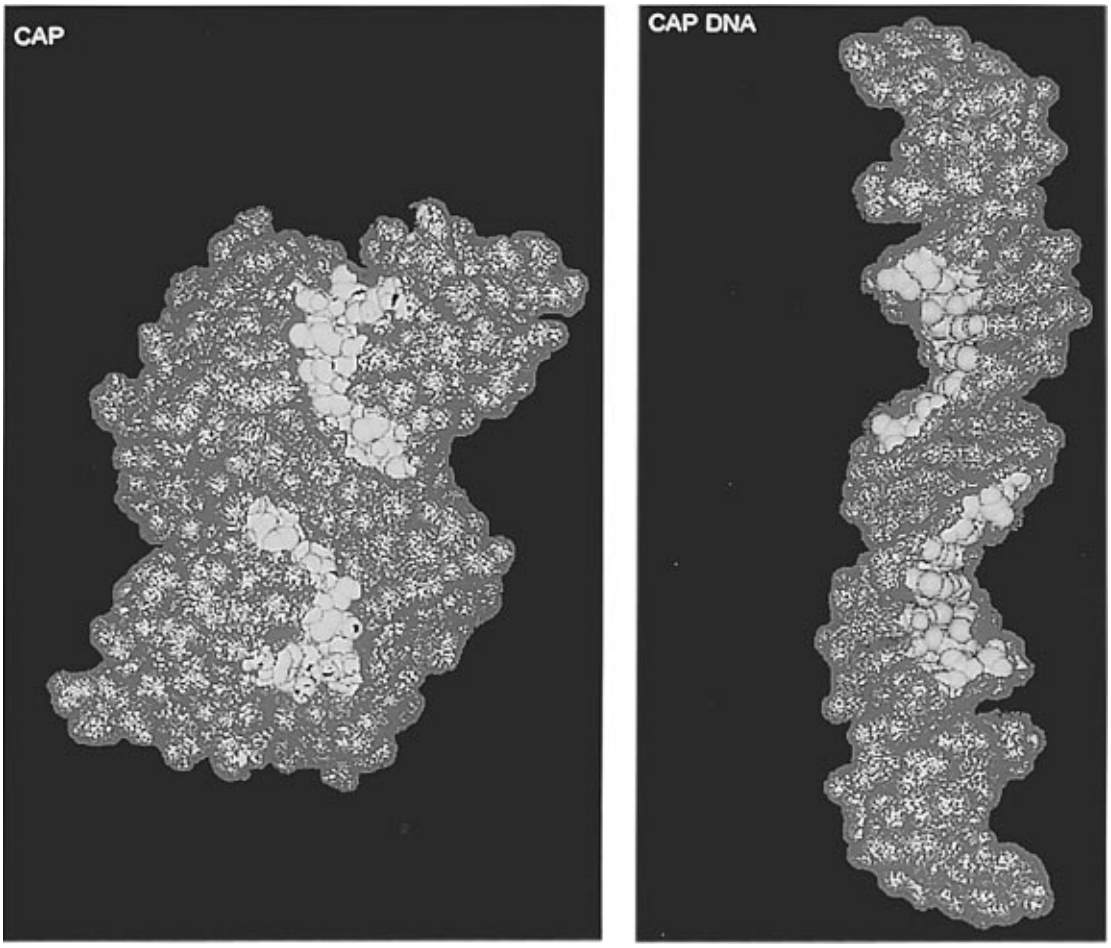

FIGURE 5: (A, left) Van der Waals surface of CAP in the complex with DNA. The red region represents the solvent-accessible surface of the CAP in the complex with DNA, determined using the Connolly algorithm (Connolly, 1983). The white region is the surface of the protein that is inaccessible to solvent in the complex. Coordinates of the CAP−DNA crystal structure (Schultz *et al.*, 1991) were obtained from the Brookhaven Protein Data Bank. (B, right) Van der Waals surface of DNA in the complex with CAP. The red region represents the solvent-accessible surface of DNA in the complex with CAP, determined using the Connolly algorithm (Connolly, 1983). The white region represents the surface of the DNA that is inaccessible to solvent in the complex.

in a release of water [although see Robinson and Sligar (1996) for an interesting exception]. The liberation of water molecules provides a powerful entropic driving force for the binding reaction. The osmotic stress method introduced by Parsegian and co-workers allows measurement of the change in the number of water molecules present in system compartments that are inaccessible to neutral solutes (Parsegian *et al.*, 1986, 1995). These compartments include cavities and molecular interfaces from which solutes are sterically excluded and surfaces that are preferentially hydrated (Parsegian *et al.*, 1995; Robinson & Sligar, 1995).

Using this method, we have found that $\partial \ln K_{obs}/\partial \ln a_{H_2O} = -79 \pm 11$ for the binding of *E. coli* CAP protein to lactose promoter site C1, in solutions containing low molecular weight osmolytes. This value was nearly independent of V_{mol} for small osmolytes (acetamide, ethylene glycol, glycerol, sucrose, and triethylene glycol). However, when larger osmolytes were tested (PEGs 400, 1450, and 8000), $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ increased with increasing V_{mol}, as expected if volume exclusion contributed significantly to K_{obs}. On this basis, we conclude that solute molar volume-dependent perturbations of K_{obs} can be detected by our assay method but that they are not significant, under our reaction conditions, when small osmolytes ($V_{mol} \leq 250$ mL/mol) are used.[2] We found that $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ exhibited a different dependence on solution dielectric coefficient in reactions in which sucrose was the osmolyte than it did in reactions in which acetamide was the osmolyte. This outcome is inconsistent with the notion that changes in K_{obs} resulted directly from changes in the solution dielectric coefficient. Finally, the agreement among values of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ obtained with chemically diverse osmolytes argues that direct solute−macromolecule interactions do not contribute significantly to K_{obs}. Together, these results support our interpretation that $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ is a measure of ν_{H_2O}, the change in the number of macromolecule-associated, solute-excluding water molecules that occurs on complex formation.

The water stoichiometry of the CAP−C1 interaction ($\nu_{H_2O} = -79 \pm 11$) is comparable to, if somewhat less negative than, water stoichiometries measured for the *gal* repressor−operator interaction ($-180 \leq \nu_{H_2O} \leq -100$; Garner & Rau, 1995), the *lac* repressor−operator interaction ($\nu_{H_2O} \leq -210$; Ha *et al.*, 1992), and the binding of *Eco*RI to its canonical

[2] The sharp transition in the dependence of $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ on V_{mol} that occurs at $V_{mol} \sim 350$ mL/mol is an intriguing feature that may reflect size-dependent solute exclusion from crevices in macromolecular surfaces; however, this possibility remains to be validated.

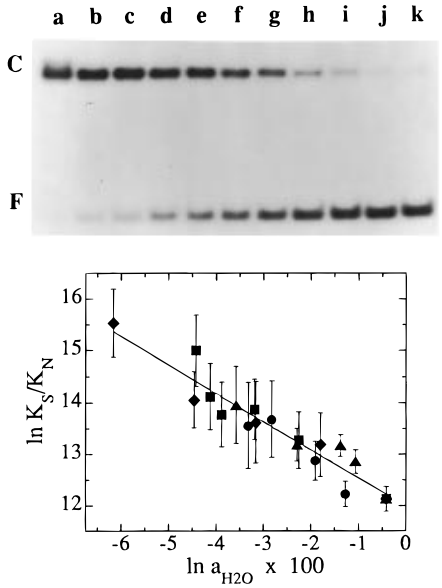

FIGURE 6: (A, top) Electrophoretic mobility shift analysis: binding competition assay. CAP−DNA complexes (C) were incubated with increasing concentrations of unlabeled calf thymus DNA. Transfer of CAP to the competitor results in the liberation of *lac* promoter DNA (F). All reactions were carried out in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, 20 μM cAMP, 0.9 M sucrose, and 0.1 mg of bovine serum albumin/mL. All samples contained 1.4×10^{-10} M *lac* promoter DNA and 4.4×10^{-10} M CAP protein. Competing DNA concentrations in samples b−k were 5.8×10^{-6}, 1.2×10^{-5}, 2.3×10^{-5}, 3.5×10^{-5}, 4.6×10^{-5}, 5.8×10^{-5}, 1.2×10^{-4}, 2.3×10^{-4}, 4.6×10^{-4}, and 5.5×10^{-4} M (base pairs), respectively. (B, bottom). The distribution of CAP between *lac* and nonspecific genomic DNAs depends on water activity. Graph of $\ln(K_S/K_N)$ as a function of $\ln a_{H_2O}$ in solutions containing the indicated osmolytes: acetamide (●), ethylene glycol (■), glycerol (◆), and sucrose (▲), respectively. Error bars represent 95% confidence limits.

Table 2: Changes in the Numbers of Water Molecules Thermodynamically Associated with Macromolecules as CAP Protein Is Transferred from Nonspecific DNA to CAP Site 1

solute[a]	ν_{H_2O}[b]	ΔA_W[c] (Å^2)
acetamide	-59 ± 11	-542 ± 158
ethylene glycol	-60 ± 12	-552 ± 168
glycerol	-55 ± 12	-507 ± 163
sucrose	-50 ± 12	-462 ± 158

[a] In addition to the indicated osmolyte, all reactions contained 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, and 20 μM cAMP. [b] The water stoichiometries (ν_{H_2O}) were determined from the slopes $\partial \ln(K_S/K_N)/\partial \ln a_{H_2O}$. Error ranges represent 95% confidence limits. [c] Estimated with the assumption that a water molecule occupies 9 ± 1 Å^2 of macromolecular surface; experimental uncertainties in $\partial \ln K_{obs}/\partial \ln a_{H_2O}$ are propagated into the error range for ΔA_W.

site ($\nu_{H_2O} \sim -160$; Robinson & Sligar, 1995). This makes water by far the largest known stoichiometric component in these binding reactions. The release of large numbers of water molecules in these reactions is compatible with the proposition (Ha *et al.*, 1989; Spolar & Record, 1994) that DNA binding is driven, at least in part, by the removal of large amounts of nonpolar surface from water. However, our current understanding of the relationship between the change in solvent-accessible surface area and the experimentally derived water stoichiometry is, at best, approximate. If all water molecules contributing to the measured value of ν_{H_2O} occupy the inner-hydration layer when associated with a macromolecule, and if a water molecule in the inner hydration layer occupies 9 ± 1 Å^2 of macromolecular surface (Colombo *et al.*, 1992), the value of $\nu_{H_2O} = -79 \pm 11$ obtained for CAP implies that the change in solute-excluding, solvent-accessible surface area (ΔA_W) is equal to -711 ± 189 Å^2. We attach to this estimate two caveats. First, not all water molecules need be released from macromolecular surfaces. If any water molecules are released from outer-hydration layers, the area buried on complex formation should be less than or equal to that occupied by a monolayer containing ν_{H_2O} water molecules. Second, the estimate that one water molecule occupies 9 ± 1 Å of macromolecular surface is based on results obtained with proteins (Colombo *et al.*, 1992, and results cited therein). The area occupied by a water molecule associated with a highly charged DNA surface may be smaller as a consequence of electrostriction. Both considerations suggest that the largest value of ΔA_W is an upper limit for the net change in solvent-accessible surface area that accompanies DNA binding.

Both allostery and the burial of complementary protein and DNA surfaces contribute to the net change in ν_{H_2O}. The area of the protein−DNA interface that becomes solvent-inaccessible on binding (ΔA_S) is 958 Å^2. A monolayer of water occupying a surface of this size would contain ~106 water molecules, a value that is closely similar to the experimentally determined number of water molecules released (79 ± 11). This similarity raises the intriguing possibility that the allosteric transitions that accompany site-specific binding (including those producing altered proteolytic susceptibility in CAP (Angulo & Krakow, 1986) and a 90−120° DNA bend (Liu-Johnson *et al.*, 1986)) result in little *net* change in the number of solute-excluding water molecules.[3] This possibility is currently under investigation.

The regulation of gene expression requires that CAP partition between regulatory and competing genomic DNA binding sites. Differences in binding site size and protein and DNA conformations [cf Majors (1977), Schmitz (1981), Hudson and Fried (1990), Saxe and Revzin (1979), and Hudson *et al.* (1990)] support the expectation that the water stoichiometries of sequence-specific and nonspecific DNA binding reactions should differ. The results presented above are consistent with a net release of water molecules with nonspecific DNA binding; however, the number of molecules released[4] ($-44 < \nu_{H_2O} < -2$) is smaller than that found for specific binding to the regulatory site C1. Garner and Rau observed a similar pattern in the DNA binding of *gal* repressor. Sequence-specific binding was accompanied by the net release of 100−180 water molecules, while nonspecific binding was accompanied by little or no water release (Garner & Rau, 1995). The differential sensitivity of these DNA binding reactions to changes in water activity suggests that water release may play an important role in regulating the specificity of protein−DNA interactions. While a reduction in intracellular water activity will stabilize all interactions with $\nu_{H_2O} < 0$, sequence-specific interactions will be stabilized to a greater degree if ν_{H_2O} (specific) $< \nu_{H_2O}$ (nonspecific).

E. coli cells are capable of surviving under conditions of high external osmolarity [reviewed in Csonka (1989)].

[3] These considerations are based on the premise that water molecules occupy ~9 Å^2 of macromolecular surface, on both protein and DNA. Should this premise be invalid, the similarity of ΔA_W to ΔA_S may be fortuitous.

[4] This range is large as a consequence of the propagation of uncertainties from the values of ν_{H_2O} used in its calculation.

Among the adaptive responses of these cells to increases in external osmotic pressure are the intracellular accumulation of potassium and glutamate ions (Epstein & Schultz, 1965; Measures, 1975; Meury *et al.*, 1985; Larsen *et al.*, 1987; Richey *et al.*, 1987; Dinnbier *et al.*, 1988) and loss of water to the environment, resulting in decreased cytoplasmic volume (Cayley *et al.*, 1991). A consequence of increased salt concentration is a significant reduction in the stability of protein−DNA complexes [cf Record *et al.* (1976, 1991) and Fried and Stickle (1993)]. In spite of this effect, gene regulatory proteins appear to function appropriately in osmotically stressed cells grown at high extracellular salt concentrations (Richey *et al.*, 1987). To resolve this apparent paradox, Record and co-workers have proposed that macromolecular crowding caused by the reduction of cytoplasmic volume favors regulatory protein−DNA interactions (Cayley *et al.*, 1991). The results presented here suggest an additional stabilizing mechanism, in which the reduction of cytoplasmic water activity (equivalent to elevated osmotic pressure) acts directly to stabilize protein−DNA assemblies.

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