Thermodynamics of Ligand Binding to *trp* Repressor†

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ABSTRACT: The thermodynamics of L-tryptophan and operator DNA binding to the tryptophan repressor of *Escherichia coli* were analyzed by titration microcalorimetry and van't Hoff analysis of footprinting titrations, respectively. At 25 °C in 10 mM sodium phosphate, pH 7.6, and 0.1 M NaCl, the binding of L-tryptophan to the repressor is characterized by values of $\Delta G° = -6.04$, $\Delta H° = -14.7$, and $T\Delta S° = -8.67$ kcal/mol. The temperature dependence of $\Delta H°$ yields $\Delta C_p° = -0.46 \pm 0.08$ kcal/(mol·K) per dimer. The binding is noncooperative at all temperatures studied. At 23 °C in 2.5 mM sodium phosphate, pH 7.6, and 25 mM NaCl, the binding of operator DNA to the repressor is characterized by values of $\Delta G° = -13.3$ kcal/mol, $\Delta H° = -1.55$ kcal/mol, $T\Delta S° = 11.8$ kcal/mol, and $\Delta C_p° = -0.54 \pm 0.10$ kcal/(mol·K). Changes in water-accessible surface areas upon binding of L-tryptophan or DNA were calculated from X-ray crystal structures. The experimentally observed $\Delta C_p°$ values were compared with $\Delta C_p°$ values calculated according to several methods based on various proposed relationships between surface area changes and heat capacity changes. Regardless of which method is used, we find poor agreement between the calorimetric results for L-tryptophan binding and the surface areas calculated from X-ray data; the direction of the discrepancy is that the X-ray data underestimate the value of $\Delta C_p°$. Better agreement is obtained by incorporating solution data on repressor flexibility, suggesting that $\Delta C_p°$ measurements may report on protein dynamical transitions accompanying ligand binding. For the case of DNA binding there is apparently fortuitous agreement between the measured and calculated $\Delta C_p°$ values, despite clear limitations in calculating $\Delta C_p°$ for this type of reaction.

Many processes involving proteins are observed to have large standard heat capacity changes, $\Delta C_p°$ (Sturtevant, 1977; Privalov & Gill, 1988; Ha et al., 1989). These processes include folding, small-ligand binding, aggregation/association, and DNA binding. These large $\Delta C_p°$ values imply strong temperature dependence of the enthalpy change, $\Delta H°$, for the reactions. Such reactions are often accompanied as well by compensatory $\Delta H°$ and $T\Delta S°$ contributions, resulting in nearly temperature-independent free energy changes. The molecular origins of these effects in protein processes have been the subject of intense experimental [e.g., Privalov (1989) and Ha et al. (1989)] and theoretical [e.g, Baldwin (1986), Murphy and Gill (1991), and Baldwin and Muller (1992)] effort.

Edsall (1935) was among the first to point out the contribution of nonpolar groups to the large $\Delta C_p°$ of dissolving small molecules in aqueous solution. Privalov and co-workers observed a correlation between the $\Delta C_p°$ of protein folding and the number of nonpolar contacts made in the native state (Privalov et al., 1974; Privalov, 1979). Gill and co-workers proposed a relationship between the number of waters in the first hydration shell of gaseous nonpolar compounds and the $\Delta C_p°$ for transfer of these compounds to water (Dec & Gill, 1985). Record and co-workers (Spolar et al., 1989) derived a quantitative relationship between available literature values of $\Delta C_p°$ and ΔA_{np}, the change in water-accessible nonpolar surface area: $\Delta C_p°/\Delta A_{np} = 0.28 \pm 0.05$ cal/(mol·K·Å^2). They demonstrated that this relationship holds for the transfer of model nonpolar solutes from water to their pure liquid phase and for the folding of small globular proteins. By using a much larger data set and uniformly calculated surface areas, they later arrived at an improved average value for both hydrocarbon transfer and protein folding, $\Delta C_p°/\Delta A_{np} = 0.32 \pm 0.09$ cal/(mol·K·Å^2) (Livingstone et al., 1991), and proposed that this relationship may apply to other processes involving proteins.

Although the nonpolar surface area change is the main contributor to the observed heat capacity change for protein folding, recent work (Makhatadze & Privalov, 1990; Murphy & Gill, 1991; Spolar et al., 1992; Yang et al., 1992; Privalov & Makhatadze, 1992) shows that burial of polar surface area upon protein folding contributes positively to the heat capacity change. Makhatadze and Privalov (1990) showed that the ratio of heat capacity change to area change for the chemically distinct polar groups of amino acid side chains ranges from 0.94 to –0.87 cal/(mol·K·Å^2) at 25 °C. Spolar et al. (1992) have recently derived an empirical linear relation that includes the average contribution of polar groups: $\Delta C_p° = (0.32 \pm 0.04)\Delta A_{np} + (-0.14 \pm 0.04)\Delta A_p$. Murphy et al. (1992) have derived a similar relation: $\Delta C_p° = (0.45 \pm 0.02)\Delta A_{np} + (-0.26 \pm 0.03)\Delta A_p$.

These treatments suggest that the large heat capacity changes observed in protein folding and ligand binding may provide a quantitative measure of the reduction in water-accessible surface area accompanying such processes. This suggestion implies the reflexive property that the water-accessible surface area, typically calculated from the crystal structures of products and reactants (Lee & Richards, 1971) can be used to derive the heat capacity change for a reaction. Indeed, this approach has been successful for certain protein-folding and peptide–antibody-binding reactions (Murphy & Freire, 1992; Murphy & Gill, 1991; Murphy et al., 1993). However, the paucity of data on complex systems makes it difficult to validate deriving thermodynamic results from structural data generally, particularly for ligand-binding reactions. Thus, while recent years have witnessed a proliferation of theoretical and empirical treatments of both the

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hydrophobic effect and the thermodynamics of processes involving biopolymers, a complete molecular understanding of such processes includes relating the thermodynamics to the structure and dynamics. Yet few systems to date meet the stringent criteria to furnish all the required data, especially for DNA and small-ligand binding by proteins [but see Connelly et al. (1990), Varadarajan et al. (1992), and Connelly and Thomson (1992)].

The tryptophan repressor (TrpR) of *Escherichia coli* is well-suited to studies of this kind. TrpR is a dimeric regulating protein that is the direct sensor of tryptophan levels in the cell. Upon binding of two molecules of L-tryptophan (L-Trp) per dimer, the repressor becomes activated for operator-specific DNA binding in at least four operons involved in aromatic amino acid metabolism: *aroH*, *trpEDCBA*, *trpR*, and *mtr* (Zurawski et al., 1981; Rose et al., 1973; Gunsalus & Yanofsky, 1980; Heatwole & Somerville, 1991; Sarsero et al., 1991). Cloning of the gene for TrpR in an overexpressing system (Paluh & Yanofsky, 1986) has enabled a wide range of physical studies. The X-ray crystal structures of the aporepressor (ApoR; Zhang et al., 1987), the holorepressor (HoloR; Schevitz et al., 1985), and the HoloR/operator DNA complex (Otwinowski et al., 1988) have been solved to 1.8-, 2.6-, and 2.4-Å resolution, respectively, by Sigler and coworkers. The structures of ApoR and HoloR have been determined in solution using NMR by Jardetzky and coworkers (Arrowsmith et al., 1990, 1991a,b; Zhao et al., 1993). The DNA- and L-Trp-binding equilibria have been studied intensively (Lane, 1986; Arvidson et al., 1986; Marmorstein et al., 1986; Kumamoto et al., 1987; Carey, 1988; Chou et al., 1989; Staacke et al., 1990; Carey et al., 1991; Haran et al., 1992). The dynamic features of the repressor in various ligation states have been examined theoretically as well (Komeiji et al., 1991; Guenot & Kollman, 1992; Howard & Kollman, 1992). In this paper, we report determination of the standard heat capacity change for both L-Trp binding and operator binding by TrpR, measured by titration microcalorimetry and van't Hoff analysis of footprinting titrations, respectively. We compare the results to the changes in water-accessible surface area for both binding reactions calculated from X-ray and NMR structures.

MATERIALS AND METHODS

Materials. Aporepressor was isolated from *E. coli* strain CY15071/pMS421/pJPR2 [Paluh and Yanofsky, 1986; J. L. Paluh and C. Yanofsky, personal communication), using a French press instead of lysozyme to open the cells. The concentration of ApoR was determined by using an extinction coefficient of 1.2 mL mg^{-1} cm^{-1} at 280 nm (Joachimiak et al., 1983; see below for discussion of error in the extinction coefficient). The protein was homogeneous (>98%) as determined by electrophoresis in an overloaded polyacrylamide gel containing urea and sodium dodecyl sulfate. L-Trp was purchased from Sigma and used without further purification. The concentration of L-Trp was determined by using an extinction coefficient of 5.47 mM^{-1} cm^{-1} at 278 nm (Marmorstein et al., 1986).

Plasmid pRK9 containing the *Serratia marcescens trpEDCBA* operator (Nichols & Yanofsky, 1983) was transformed into DH1 cells and isolated by CsCl ultracentrifugation. A DNA fragment (~100 bp) bearing the operator region was obtained by *Eco*RI cleavage of pRK9, end filling using the large fragment of DNA polymerase I and [α-^{35}S]dATP, and *Bam*HI cleavage (Carey, 1988). The labeled fragment was purified from an agarose gel by electrophoresis onto NA45 paper (Schleicher & Schuell) and recovered by elution with 2 M NaCl. The sequence contacted by TrpR on this fragment is 5′-GAACCAGTTAACTAGTAC-3′ (Carey, 1989).

Titration Microcalorimetry. Calorimetric measurements were carried out using an OMEGA titration calorimeter from Microcal, Inc. (Northampton, MA) in the laboratory of Dr. Kenneth Breslauer, Rutgers University. This instrument has been described in detail by Wiseman et al. (1989). The reference cell was filled with water containing 0.03% azide. The calorimeter was calibrated electrically at each temperature. Previous electrical and chemical reaction calibration gave virtually the same calibration constants (E. Plum, personal communication). Just prior to a titration experiment, both ApoR and L-Trp solutions were thoroughly degassed by stirring under vacuum, and the 1.3605-mL sample cell was filled with ApoR solution. L-Trp solution was drawn into a syringe, which was then mounted into a stepper motor for delivery into the sample cell. The syringe was rotated at 400 rpm during the experiment. For titration experiments at different temperatures, syringes of 50-, 100-, or 250-μL volume, ApoR concentrations of 113 or 571 μM in dimer, and L-Trp concentrations of 10.0 or 25.0 mM were used. Reaction buffer for L-Trp binding contained 10 mM sodium phosphate (pH 7.6), 0.1 M NaCl, and 0.1 mM EDTA. Under these conditions, TrpR is a dimer (K. S. Martin et al., submitted for publication). DNA-binding could not be measured by titration microcalorimetry, principally because of the magnitude of the dissociation constant, K_d (subnanomolar); the relative insensitivity of K_d to solution conditions (Carey, 1988; Carey et al., 1991) precluded shifting K_d into an accessible range. At TrpR and DNA concentrations far above K_d, multiple binding modes with different stoichiometries are used (Carey, 1988; Carey et al., 1991; Haran et al., 1992), complicating interpretation of calorimetric data.

Analysis of calorimetric data was carried out with the Origin software provided with the OMEGA instrument (Wiseman et al., 1989). A description of the analytical procedures and equations used in this software is available from Microcal, Inc., or from the authors. This program package uses the relationship between the heat generated for each injection and $\Delta H°$, n (the number of binding sites), K_a (the association binding constant), the total protein concentration, and the free and total ligand concentrations. The process of fitting experimental data involves the following steps: initial guesses of n, K_a, and $\Delta H°$; calculation of heat for each injection from these guessed values and comparison with the measured heat for the corresponding experimental injection; and improvement of the initial values of n, K_a, and $\Delta H°$ followed by iteration of the above procedure until no further improvement in fit is achieved. The errors reported in n, K_a, and $\Delta H°$ are generated by the Origin program, and although they do not represent standard errors, they do reflect the quality of the fit between the nonlinear least-squares curve and the experimental points in Figure 1b.

We report up to three significant figures for all experimental and derived values, even though the extinction coefficient for TrpR has been reported to have an error of up to 20% (Joachimiak et al., 1983). However, we believe the extinction coefficient is much more accurate than this because of the good match between the values of n calculated from fitting (1.97–2.01) and the value expected from the known crystal structure of TrpR (2.00). In fitting the experimental calorimetric data to obtain values of n, K_a, and $\Delta H°$, the accuracy of the n value is directly dependent on the accuracy of the TrpR concentration in the reaction cell; in fact, the theoretical

n value known from independent evidence (such as crystallography) can be used by the Origin program to determine the actual TrpR concentration. Errors for $\Delta G°$ and $T\Delta S°$ propagated from fitting errors of K_a and $\Delta H°$ are at most 0.3% and 1.4%, respectively. Errors reported on $\Delta C_p°$ are 2 SD (95% confidence interval).

DNase I Footprinting Titrations. Quantitative DNase I footprinting titration experiments (Brenowitz et al., 1986; Carey, 1988; Carey et al., 1991) were conducted to measure the affinity of TrpR for its operator DNA at different temperatures. The binding buffer for complex formation and TrpR dilution contained 2.5 mM sodium phosphate, pH 7.6, 25 mM NaCl, and 0.4 mM L-Trp. Reaction mixtures of 250 μL containing ^{35}S-labeled operator DNA (<10^2 pM, constant) and TrpR at various concentrations were incubated for 30 min at the experimental temperature prior to DNase I exposure. DNase I in 10 μL of a buffer containing 0.1 M NaCl, 1 mM $MnCl_2$, and 10 mM $CaCl_2$ was then added to a final concentration of 4 ng/mL to the TrpR/DNA mixture. To maintain a constant extent of DNase I digestion (<10% of intact DNA digested) at different temperatures, the incubation time was varied from 4 to 15 min. Digestion was quenched by adding 700 μL of 89 mM sodium acetate in 92% ethanol and 5 ng of tRNA and chilling in dry ice/methanol. Precipitated material was resuspended in formamide loading buffer, heated to 90 °C for 4 min, and loaded onto 6% acrylamide/50% urea sequencing gels. Gels were fixed in methanol/acetic acid, vacuum dried, and exposed to X-ray film.

Because the concentration of operator DNA in these experiments is negligible relative to the amount of TrpR at half-saturation, binding affinity expressed as the dissociation constant, K_d, is approximately equal to the protein concentration required to reach the midpoint of the titration. With very small increments in TrpR concentration, K_d can be estimated with very good accuracy and precision. Autoradiograms were quantitated by computerized densitometric scanning using a false-color imaging system in the laboratory of Dr. Michael Brenowitz, Albert Einstein College of Medicine. Three independent footprint titrations were quantitated at each temperature. Average assocation constants ($K_a = 1/K_d$) for the binding reaction were plotted as a function of temperature, and $\Delta H°$ and $\Delta C_p°$ were determined from the following equations: $\ln K_a = -\Delta H°/RT + \Delta S°/R$ and $\ln K_a = (\Delta C_p°/R)[T_H/T - \ln(T_S/T) - 1]$, where T_H and T_S are the temperatures at which $\Delta H°$ and $\Delta S°$ are 0, respectively (Baldwin, 1986; Ha et al., 1989).

Surface Area Calculations. Coordinates for the crystal structures of ApoR and HoloR were obtained from the Brookhaven Protein Data Base (PDB files 3WRP and 2WRP, respectively). Coordinates for the crystal structure of HoloR/operator DNA (Otwinowski et al., 1988) were generously provided by Dr. Paul Sigler (file name 0TRO). The operator DNA in the crystal structure is a self-complementary duplex with the sequence 5′-TGTACTAGTTAACTAGTAC 3′. A few atoms or residues (see Table III footnotes) were absent from one or more X-ray structures due to disorder and thus were deleted from coordinate sets as necessary so that products and reactants in the same reaction contained the same atoms.

Calculations of water-accessible surface area were performed using the program ACCESS (Lee & Richards, 1971; version date March 6, 1983). Bound hydrogen atoms are included in the van der Waals (vdw) radii of all atoms. The list of atom types and vdw radii in the ACCESS program was extended to include values for atoms unique to DNA taken from Alden and Kim (1979) for phosphorus (1.80 Å) and phosphate oxygen (1.64 Å). A water probe radius of 1.4 Å and a slice size (*Z*-step) of 0.08 Å were used. Virtually the same areas were obtained in a trial calculation with a *Z*-step of 0.01 Å. We considered only carbon as a nonpolar atom and oxygen, nitrogen, and sulfur as polar atoms (Chothia, 1976). Summation of total nonpolar or polar surface areas for proteins was carried out using the program ACCFMT (F. M. Richards, version date October 31, 1990). Summation for DNA and for each chemically distinct protein functional group was done manually.

Estimation of $\Delta C_p°$ for Individual Protein Functional Groups. In order to treat each chemically distinct functional group of protein side chains separately, as advocated by Makhatadze and Privalov (1990), a value must be obtained for the $\Delta C_p°$ of transfer of each group from the liquid state to water. These values are not directly available for the relevant functional groups. Instead, they were derived from available literature data on heat capacities of evaporation (Privalov & Gill, 1988) and gaseous hydration (Makhatadze & Privalov, 1990) of the corresponding functional groups as explained in the following paragraph. The heat capacity changes per unit surface area thus derived for the processes of evaporation and hydration were added together to obtain heat capacity changes per unit area for transfer from the pure liquid state to water.

An average value for evaporation heat capacity change per unit surface area for nonpolar compounds [–0.05 cal/(mol·K·A^2), obtained by averaging values for 5 and 25 °C] was extracted from the literature (Privalov & Gill, 1988). As a necessary approximation due to the lack of data, the same value was used for both polar and nonpolar protein functional groups. Though only an approximation, it can be justified partially on the grounds that the heat capacity effect upon evaporation is dominated by the large increase in the number of degrees of freedom accompanying the phase change, and is thus largely entropic in origin. Rearrangements of solvent around solutes, which may differ for polar and nonpolar solutes, contribute relatively less. For NH_2 and COOH groups of the L-Trp ligand, the only available data are partial molar heat capacities (Makhatadze & Privalov, 1990). These were converted to hydration heat capacities by subtracting from them the heat capacity for the gas state, obtained by linear extrapolation from literature values (Benson, 1976) at higher temperatures. A table listing estimations of $\Delta C_p°$ values at 5 and 25 °C for each chemically distinct group is available from the authors upon request.

RESULTS

Figure 1a shows raw data obtained from a typical calorimetric titration of TrpR with L-Trp. The area of each peak represents the total heat evolved upon addition of a single aliquot of L-Trp. The integrated heat per injection is plotted in Figure 1b. Curve fitting to data in this form is used to extract values of $\Delta H°$, K_a (the affinity for each site), *n* (the number of sites), and ω (cooperativity). Results from experiments performed at four different temperatures are listed in Table I, along with the values for $\Delta G°$ and $T\Delta S°$ calculated from the experimental data. In the temperature range tested (~5–25 °C), the Gibbs free energy change, $\Delta G°$, for the binding of 1 mol of L-Trp per dimer ranges from –6.41 to –6.04 kcal/mol. At all temperatures tested, the net free energy of reaction contains a favorable contribution from changes in enthalpy and an unfavorable contribution from changes in

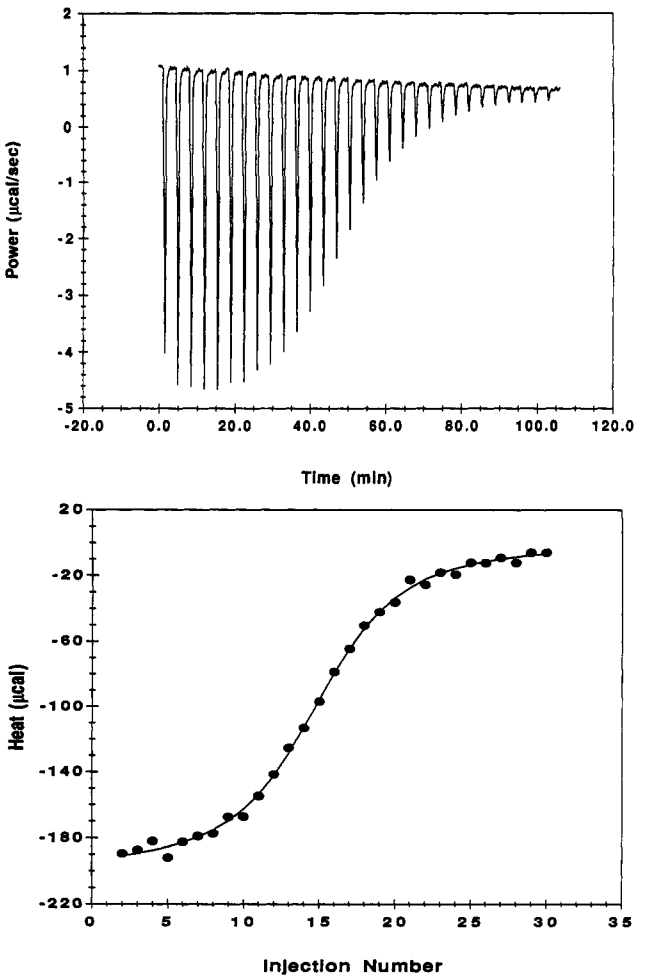

FIGURE 1: Microcalorimetric titration of TrpR with L-Trp. Data were obtained at 4.7 °C from an automated sequence of 30 injections of 10.0 mM L-Trp from a 50-μL syringe into the reaction cell containing 113 μM ApoR dimer. Each injection was 2.0 μL and injections were made at 3.5-min intervals. (**a**, top) Raw data from the titration. Each peak corresponds to one injection. (**b**, bottom) The peaks in Figure 1a were integrated using the ORIGIN software supplied with the instrument of MicroCal, Inc., and the values are plotted *vs* injection number. Each point corresponds to heat in μcal generated by the reaction upon each injection. The solid line is the curve obtained by nonlinear least-squares fitting carried out by the ORIGIN program. This fit yields values for n, K_a, and $\Delta H°$, given in Table I.

entropy. The contribution of the enthalpy change to $\Delta G°$ becomes more favorable as the temperature is raised from 5 to 25 °C, decreasing from −10.1 to −14.7 kcal/mol. The contribution of the entropy change (expressed as $T\Delta S°$) becomes more unfavorable over this interval, decreasing from −3.66 kcal/mol to −8.67 kcal/mol. Thus, entropy–enthalpy compensation occurs with the result that $\Delta G°$ is less temperature-dependent than either $\Delta H°$ or $T\Delta S°$.

The temperature dependence of $\Delta H°$ yields the heat capacity change for the reaction, $\Delta C_p°$. The heat capacity change was assumed to be constant within the temperature range studied, which is a reasonable approximation based on observations by Privalov and Gill (1988). Thus, values of $\Delta H°$ at different temperatures were fit to the straight line $\Delta H°(T) = 54.6 - 0.232(273 + T)$ by linear least-squares fitting ($R = 0.99$), the slope of which yields the value of the standard heat capacity change accompanying the binding of 1 mol of L-Trp to 1 mol of ApoR dimer, −0.23 ± 0.04 kcal/(mol·K). Table I also lists the experimentally derived values of n, the number of binding sites for L-Trp per repressor dimer. The results show that there are two binding sites per dimer, as is of course expected

Table I: Thermodynamic Parameters for L-Tryptophan Binding to TrpR

	T, °C			
	4.7	12.2	18.2	25.0[a]
[ApoR], mM	0.113	0.113	0.571	0.571
n	1.97 ± 0.01	1.99 ± 0.01	2.01 ± 0.01	2.00 ± 0.01
K_a, $(mM)^{-1}$	111 ± 2	72 ± 2	48 ± 2	27 ± 1
$\Delta H°$, kcal/mol	−10.1 ± 0.1	−11.2 ± 0.1	−12.9 ± 0.1	−14.7 ± 0.1
$\Delta G°$, kcal/mol	−6.41	−6.34	−6.23	−6.04
$T\Delta S°$, kcal/mol	−3.66	−4.86	−6.67	−8.67

[a] At 25 °C, errors derived from three independent experiments were also obtained, and these have the same magnitude as those reported here, which are from curve fitting as described in Materials and Methods.

from the known protein structure. Fitting of the binding data also yields values for the cooperativity parameter, ω. At all temperatures studied, $\omega = 1$, showing that the two structurally equivalent binding sites have the same affinity for L-Trp. Therefore, the two binding events are equivalent and independent, and the sites are noncooperative, as has been reported previously (Arvidson et al., 1986; Lane, 1986; Marmorstein et al., 1986). Thus, the total heat capacity change for binding of 2 mol of L-Trp to 1 mol of repressor dimer is −0.46 ± 0.08 kcal/(mol·K).

The binding constant (K_a) for L-Trp ranges from 111 mM^{-1} at 4.7 °C to 27 mM^{-1} at 25 °C. These values agree well with those obtained by Gunsalus and co-workers using equilibrium dialysis (Arvidson et al., 1986). Their average value for the van't Hoff enthalpy change determined from the temperature dependence of K_a between 6.5 and 40 °C (−10.6 kcal/mol) is within the range of our directly measured enthalpy changes. For comparison, our derived K_a values yield a van't Hoff $\Delta H°$ value of −11.41 kcal/mol, in good agreement with the calorimetrically determined $\Delta H°$ values and with the absence of cooperativity. Our data yields a value of $\Delta C_p° = -0.78$ kcal/(mol·K) by van't Hoff analysis.

DNase I footprinting titration experiments were used to determine the association constants, K_a, for TrpR binding to operator DNA at several temperatures in the range 1.5–49 °C. In keeping with the requirements for quantitative interpretation of footprinting titrations (Brenowitz et al., 1986), the extent of digestion at each temperature was kept constant at less than 10% of the total DNA by adjusting the incubation time at constant enzyme concentration. Values of K_a determined from the titration midpoints as described in Materials and Methods are listed in Table II and plotted as a function of temperature in Figure 2. The best fit to these data (solid line in Figure 2) yields values of $\Delta H°$, $T\Delta S°$, and $\Delta G°$ at each temperature, assuming that $\Delta C_p°$ is temperature-independent. Derived thermodynamic parameters for DNA binding are listed in Table II. The Gibbs free energy change for the DNA-binding reaction is favorable at all temperatures in the range studied and is only weakly temperature-dependent, while the enthalpy and entropy changes are strongly temperature-dependent. $\Delta H°$ is positive at lower temperature, passes through zero at ~20 °C, and becomes negative at higher temperature. The second derivative of $\ln K_a$ *vs* T yields the heat capacity change for the DNA-binding reaction, although with less confidence than direct measurement by titration microcalorimetry, which could not be done for TrpR–DNA binding due to the magnitude of K_a (see Materials and Methods). The value obtained from such an analysis of the data in Figure 2 is $\Delta C_p° = -0.54 \pm 0.10$ kcal/(mol·K).

Table II: Thermodynamic Parameters for Operator DNA Binding to TrpR

	T, (°C)				
	1.5	8.0	23.0	37.0	49.0
K_a, $(nM)^{-1}$	4.3 ± 0.4	5.3 ± 0.3	6.7 ± 0.9	4.8 ± 0.2	2.2 ± 0.1
$\Delta G°$, kcal/mol	−12.1 ± 0.1	−12.5 ± 0.1	−13.3 ± 0.1	−13.7 ± 0.1	−13.8 ± 0.1
$\Delta H°$, kcal/mol	10.0	6.51	−1.55	−9.07	−15.5
$T\Delta S°$, kcal/mol	22.1	19.0	11.8	4.67	−1.75

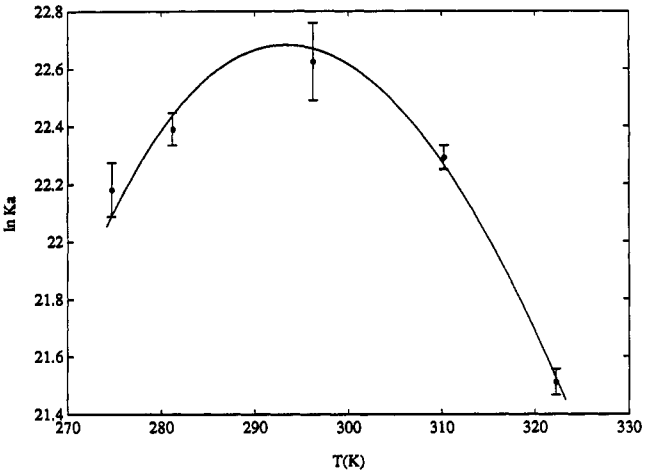

FIGURE 2: Van't Hoff plot for operator DNA binding to HoloR. The points are experimental data with errors from three determinations, and the solid line is calculated from the expression $\ln K_a = -\Delta H°/RT + \Delta S°/R = (\Delta C_p°/R)(T_H/T - \ln(T_S/T) - 1)$, with nonlinear least-squares fit (R = 0.99) values of $\Delta H°$ and $T\Delta S°$ given in Table II; $\Delta C_p°$ = −0.54 ± 0.10 kcal/(mol·K); T_H = 293.3 K; T_S = 319.3 K.

Water-accessible polar and nonpolar surface areas (A_p, A_{np}) of the various molecular species participating in the binding equilibria were calculated from X-ray structures using the ACCESS program of Lee and Richards (1971). The results are given in Table III. To calculate the surface area of free DNA, the coordinates of TrpR were deleted from the crystal structure of the protein–DNA complex. The ACCESS program was modified to include atom types unique to DNA (phosphorus and phosphate oxygen) by using experimental van der Waals radii from Alden and Kim (1979). In addition to calculations based on the X-ray structures, we also modeled ApoR and HoloR to more closely approximate the solution structures determined by NMR (Arrowsmith et al., 1990, 1991a,b; Zhao et al., 1993). These approximations included treating helices D and/or E as having β-extended conformations, allowing us to calculate an upper limit for their surface areas. The rationale for this treatment is explained in the Discussion.

Surface area data were used to calculate the changes in water-accessible polar and nonpolar surface areas (ΔA_p and ΔA_{np}) upon binding of L-Trp or DNA to TrpR. For binding of 2 mol of L-Trp to 1 mol of ApoR dimer to form HoloR, ΔA_{np} = 5645 − (5622 + 518) = −495 Å² and ΔA_p = 5594 − (5406 + 222) = −34 Å², when calculated directly from the crystal structures of ApoR, HoloR, and L-Trp. When we treat helix D as fully extended in both ApoR and HoloR and helix E as extended in ApoR but in helical conformation in HoloR, we find ΔA_{np} = (5645 − 469 + 1414) − (5622 − 1600 + 4138) − 518 = −2088 Å² and ΔA_p = (5594 − 806 + 1414) − (5406 − 1473 + 3156) − 222 = −1109 Å². For binding of 1 mol of DNA to 1 mol of HoloR dimer, ΔA_{np} = 7004 − 5161 − 3314 = −1471 Å² and ΔA_p = 6272 − 4097 − 4059 = −1884 Å², when calculated directly from the X-ray crystal structures. When we treat helix D as fully extended in the HoloR but in helical conformation in the complex with DNA, we find ΔA_{np} = 7004 − (5161 − 469 + 1414) − 3314 = −2416 and ΔA_p = 6272 − (4097 − 806 + 1414) − 4059 = −2492.

Table III: Total Water-Accessible Polar and Nonpolar Surface Areas, A_p and A_{np}, for Various Species Calculated from Structural Data

species	A_p (Å²)	A_{np} (Å²)
ApoR[a]	5406	5622
HoloR[b]	5594	5645
2(L-Trp)[c]	222	518
2(αDE)[d]	1473	1600
2(βDE)[e]	3156	4138
2(αD)[f]	806	469
2(βD)[g]	1414	1414
ApoR(βDE)[h]	7089	8160
HoloR(βD)[i]	6202	6590
HoloR*[j]	4097	5161
DNA[k]	4059	3314
complex[l]	6272	7004

[a] ApoR dimer (residues 8–108) from the original PDB file 3WRP modified for comparison with file 2WRP by the following deletions: Ser-8, atom Oγ; Met-11, atoms Cβ, Cε, Cγ, and Sδ; Glu-74, atoms Cγ, Oε1, Cδ, and Oε2. [b] HoloR dimer (residues 8–108) from PDB file 2WRP modified for comparison with file 3WRP by the following deletion: Asp-108, atom OXT. [c] Two molecules of free L-tryptophan. [d] Two helix–turn–helix regions of ApoR (residues 68–92) from PDB file 3WRP. [e] Two helix–turn–helix regions (residues 68–92) in fully extended β conformation. [f] Two helix D regions of ApoR (residues 68–75) from PDB file 3WRP. [g] Two helix D regions (residues 68–75) in fully extended β conformation. [h] ApoR dimer [modified as in (a)] with residues 68–92 of each subunit in fully extended β conformation. [i] HoloR dimer [modified as in (b)] with helix D of each subunit in fully extended β conformation. [j] HoloR dimer (residues 14–105) from PDB file 2WRP, modified for comparison with file 0TRO by the following deletions: reduction of the disordered side chain of Gln-14, Arg-15, Gln-17, Glu-65, Glu-70, Glu-74, Gln-98, and Glu-102 to methyl groups; reduction of the side chain of Arg-63 to that of Lys. [k] Free DNA derived by deleting the coordinates of HoloR from the HoloR/DNA complex of file 0TRO. [l] HoloR/DNA complex of file 0TRO.

DISCUSSION

L-Trp binding to TrpR appears to be an ideal case for analysis by titration microcalorimetry because of the magnitude of $\Delta H°$ and K_a and the ease of producing large quantities of TrpR protein (~1 g; Paluh & Yanofsky, 1986). Values for the binding constant, K_a, the number of binding sites, n, the cooperativity, ω, and the enthalpy change, $\Delta H°$, were obtained from titration experiments at four different temperatures between ~5 and 25 °C. From these values one can calculate $\Delta G°$, $T\Delta S°$, and $\Delta C_p°$ with high accuracy and precision, permitting simultaneous determination of all thermodynamic parameters in a single experiment. The measured and extracted thermodynamic parameters characterizing the binding of L-Trp to TrpR confirm that two molecules of L-Trp bind noncooperatively to the TrpR dimer with a free energy per L-Trp of about −6 kcal/mol. The reaction is accompanied by a favorable net change in enthalpy and an unfavorable net change in entropy at all temperatures studied, although both $\Delta H°$ and $T\Delta S°$ depend strongly on temperature. The temperature dependences of the enthalpic and entropic contributions to $\Delta G°$ are offsetting, with the result that the

binding free energy is much less temperature-dependent than either $\Delta H°$ or $T\Delta S°$. Enthalpy–entropy compensation (Lumry & Rajender, 1970) is a common phenomenon in biopolymer processes, especially those occurring in aqueous solvents (Breslauer et al., 1987).

Values of $\Delta H°$ at different temperatures were fit to a straight line, the slope of which yields the standard heat capacity change for the binding of 1 mol of L-Trp per ApoR dimer. Linear treatment of the temperature dependence of $\Delta H°$ is predicated on the assumption that $\Delta C_p°$ is constant within the temperature range studied. The heat capacity change for binding of 2 mol of L-Trp per dimer thus is -0.46 ± 0.08 kcal/(mol·K). It is noteworthy that, although the van't Hoff enthalpy change calculated from our extracted values of K_a is in good agreement with the directly determined values of $\Delta H°$, the values of $\Delta C_p°$ calculated by the two methods are quite different. The discrepancy is presumably due to the fact that L-Trp binding does not show a clear maximum in ln K_a with temperature, suggesting that, in such cases, direct measurement of $\Delta C_p°$ by calorimetry is apparently more accurate than taking a second derivative, as required in van't Hoff analysis.

The experimentally measured value of $\Delta C_p°$ for L-Trp binding was compared with the value predicted from the change in surface area calculated from structural data on ApoR, L-Trp, and HoloR. Polar and nonpolar surface areas were calculated using the ACCESS program of Lee and Richards (1971), and changes in surface area were calculated by subtracting the surface areas of reactants from those of products. To relate surface area changes to $\Delta C_p°$, we used values derived from studies of model compounds and protein folding. Two different approaches are used in these model studies for estimating the contribution of surface area change to $\Delta C_p°$. Spolar et al. (1992) and Murphy and Freire (1992) use average values for polar and for nonpolar groups, whereas Makhatadze and Privalov (1990) treat each chemically distinct functional group of the amino acid residues separately. The average treatment yields predicted values of $\Delta C_p° = -0.15 \pm 0.03$ kcal/(mol·K) using the values of Spolar et al. (1992) and $\Delta C_p° = -0.23 \pm 0.01$ kcal/(mol·K) using the values of Murphy and Freier (1992), while the individual treatment yields -0.22 ± 0.02 kcal/(mol·K). All these values are smaller than the experimentally measured $\Delta C_p°$ value by more than a factor of 2, which is well outside the error limits of the calculations and measurements. These results suggest that a direct relation between crystalline surface area and heat capacity change is an oversimplified model for this ligand-binding reaction.

Comparison of the X-ray structures of ApoR and HoloR shows that L-Trp binding is accompanied by a hinge-bending type motion that alters the position of the helix–turn–helix region (helices D and E) relative to the dimeric core of the protein, with little change in the internal organization of each domain (Zhang et al., 1987). NMR analysis, on the other hand, shows that the helix–turn–helix region is quite disordered in ApoR, with no clear evidence of helical structure from NOE data, fast hydrogen exchange of amide protons (Arrowsmith et al., 1991b), and exchange-limited T_1 relaxation rates of backbone protons (Zhao et al., 1993). Upon L-Trp binding, exchange rates throughout TrpR are slowed by 3- to 10-fold, and helix E, but not helix D, becomes a well-defined helix (Arrowsmith et al., 1991b). Molecular dynamics studies (Komeiji et at., 1991; Guenot & Kollman, 1992; Howard & Kollman, 1992) support this view of TrpR. Because our measurements of $\Delta C_p°$ were made in solution, we also calculated surface area changes on an approximate structure derived from the NMR data. This approximation treated helix D as fully extended (in β conformation) in both ApoR and HoloR, whereas helix E was treated as extended in ApoR and helical in HoloR. The nonpolar surface area change calculated for the resulting reaction is ~4 times larger than that calculated from X-ray data, while the polar surface area change is ~30 times larger. The values of $\Delta C_p°$ calculated for this approximated surface area by using average values for nonpolar and polar groups are -0.5 ± 0.1 kcal/(mol·K) according to the relation of Spolar et al. (1992) and -0.65 ± 0.08 kcal/(mol·K) with the Murphy and Freire (1992) relation, and -0.36 ± 0.03 kcal/(mol·K) by treating chemically distinct groups separately. All these values are much closer to the experimentally determined $\Delta C_p°$ value than are those from X-ray data.

The simple relationship between crystalline surface area and heat capacity change holds reasonably well for processes that have very large changes in water-accessible surface area, such as protein folding (Spolar et al., 1992). Even if the surface area of a folded protein is larger in solution than the X-ray structure indicates, the deviation is small relative to the total surface area change between folded and unfolded states. Furthermore, the burial of nonpolar surface dominates protein folding (Chothia, 1976) and makes a larger contribution to heat capacity change per unit surface area change than does polar surface burial, according to all model compound studies. In contrast, ligand-binding reactions that are not coupled to protein folding can have quite small surface area changes, and because these reactions occur largely on the surfaces of proteins, the contribution of polar surface to the total area change is relatively larger. Because ΔA_p contributes to $\Delta C_p°$ with opposite sign from ΔA_{np}, the net $\Delta C_p°$ effect can be quite small even when the total surface area change is quite large. Comparison of measured and predicted heat capacity changes may therefore be useful to detect coupling of protein-folding reactions to ligand-binding equilibria.

In the case of HoloR–DNA binding, the change in polar surface area exceeds the change in nonpolar surface area, and many charged groups on the phosphate backbone must necessarily become buried in the complex. Nevertheless, the measured $\Delta C_p°$ [-0.54 ± 0.10 kcal/(mol·K)] is in reasonable agreement with that predicted from the change in surface area when helix D is treated as unfolded in the free state and helical in the complex: [-0.4 ± 0.2 kcal/(mol·K), using the relation of Spolar et al. (1992), and -0.44 ± 0.12 kcal/(mol·K), using that of Murphy and Freire (1992)]. This result is likely to be fortuitous for the following reasons. First, although the magnitude of the contribution of polar DNA groups to $\Delta C_p°$ is unknown, we have treated them the same as for protein polar groups due to the lack of model compound data. Second, X-ray analysis of the cocrystalline complex reveals a layer of water at the interface between TrpR and DNA (Otwinowski et al., 1988). Since there is no channel leading to this interface, its surface area is considered inaccessible by the Richards algorithm, leading to overestimation of the change in water-accessible surface area for the reaction and, hence, its contribution to the calculated $\Delta C_p°$. Despite these limitations, the TrpR system represents the first opportunity to relate surface areas calculated from known structures of a complex ligand-binding system to experimentally measured values of $\Delta C_p°$, and we hope the TrpR example will stimulate work in this area as the required data become available in other systems.

The functionally important regions of proteins often map to flexible surface sites, and interactions with ligands are often accompanied by changes in flexibility that can be harnessed for allostery. Ha et al. (1989) have proposed that large

conformational changes may be important generally for specific protein–DNA binding. Adaptation of complementary molecular surfaces upon interaction is a common theme in protein–DNA interactions [see, e.g., Ellenberger et al. (1992) and references therein] and is likely to be a feature of small-ligand-binding reactions as well. In contrast to preorganziation [see, e.g., Cram and Trueblood (1985)], structural adaptability permits independent, rather than coordinated, modulation of affinity and specificity. Our results suggest that the conformational changes conferring adaptability can correspond to partial protein-folding reactions and that the resulting burial of surface area can be an important cryptic contributor to the reaction thermodynamics. In addition to effects on free energy and heat capacity due to changes in solvation, disorder-to-order transitions coupled to ligand binding may contribute to the reaction thermodynamics by altering low-frequency vibrational modes of the system (Sturtevant, 1977; Irikura et al., 1985), although at present the frequency range of these modes is outside the realm of experiment. Our results suggest, however, that the contribution of changes in vibrational modes to ΔC_p° is likely to be small.

It is reasonable, and not unexpected, that X-ray structures may underestimate the flexibility of biopolymers in solution. While *B*-factors report on this feature to some degree, they do not represent the extent of chain excursions apparently accessible to parts of the chain in solution. Crystal packing is not thought to affect the overall secondary and tertiary structures of proteins [for recent discussion, see Kossiakoff et al. (1992) and references therein]. Nevertheless, the requirement to fit into the lattice may result in selective crystallization of forms that are more compact than the isolated molecule in solution. The observation that compactness leads to regular secondary structure (Dill, 1990) suggests that X-ray structures of proteins may indicate a higher content of secondary structure than is present in solution, which could obscure disorder-to-order transitions that may accompany ligand binding.

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