Probing Hydration Contributions to the Thermodynamics of Ligand Binding by Proteins. Enthalpy and Heat Capacity Changes of Tacrolimus and Rapamycin Binding to FK506 Binding Protein in D_2O and H_2O

Patrick R. Connelly,* John A. Thomson, Matthew J. Fitzgibbon, and Frank J. Bruzzese

Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge, Massachusetts 02139

Received December 18, 1992; Revised Manuscript Received March 5, 1993

ABSTRACT: The stabilities of native proteins and protein–ligand complexes result from differential interactions among numerous polar and nonpolar atoms within the proteins and ligands and of these atoms with water. Delineation of the various energetic contributions of the stabilities of proteins or protein–ligand complexes in aqueous solution, and an evaluation of their structural basis, requires a direct account of the changes, in the interactions of the protein with the solvent, that accompany the folding or binding reactions. Two largely nonpolar, structurally related macrolide ligands, tacrolimus (also known as FK506) and rapamycin, each bind with high affinity to a common site on a small FK506 binding protein (FKBP-12) and inhibit its peptidylprolyl *cis–trans*-isomerase activity. In an effort to elucidate the influence of water on the thermodynamics of their binding reactions, we have measured the enthalpies of tacrolimus and rapamycin binding to FKBP-12, in buffered solutions of H_2O (at pH 7.0) or D_2O (at pD 7.0), by high-precision titration calorimetry in the temperature range 5–30 °C. For both tacrolimus and rapamycin binding, a large enthalpic destabilization of binding is observed in D_2O relative to H_2O, in the temperature range examined. Additionally, large negative constant pressure heat capacity changes are observed for the binding of the ligands in both H_2O and D_2O. A thermodynamic analysis is presented to identify the structural determinants of the differences in the energetics of binding in light and heavy water. The analysis suggests that a chief contributor to the observed enthalpic destabilization is the differential hydration, of protein and ligand atoms, by light and heavy water.

A number of high-resolution X-ray and neutron diffraction experiments have revealed details on the location and organization of water molecules bound to the surfaces of proteins in their native states (Edsall & MacKenzie, 1983; Teeter, 1991). Individual water molecules and networks of waters have been shown to be hydrogen-bonded to polar atoms of amino acids on the surfaces of proteins, or clustered in pentagonal rings and arrays of rings around nonpolar side chains. A significant feature of the processes of protein folding, and the binding of ligands to proteins, is that a large number of atoms are removed from water and become buried in the interior of proteins or protein–ligand complexes. Despite such advances in our understanding of some of the structural features of the interaction of water with proteins, the contribution of hydration to the energetics of these reactions involving proteins has not been fully elucidated.

Currently, differing views are held on the relative energetic importance of hydrogen bonds, and interactions involving nonpolar groups, to the overall thermodynamic properties of reactions involving proteins (Murphy et al., 1990; Lee, 1991; Sharp et al., 1991; Yang et al., 1992; Pace, 1992). Part of the difficulty in dissecting the relative energetic contributions derives from the fact that hydrophobic interactions and hydrogen bond formation both involve components that reflect the interaction of water with proteins. More specifically, hydrophobic interactions, as referred to here, are differential interactions among nonpolar moieties buried in the protein interior or at protein–ligand interfaces (packing interactions), and of these nonpolar groups with water (hydration interactions).[1] Similarly, folding or binding reactions of proteins, which lead to the formation of new hydrogen bonds involving atoms of reaction products, are often accompanied by the release of water molecules that were hydrogen-bonded to these atoms prior to reaction. Models have been presented arguing that the overall enthalpic contribution of hydrophobic interactions to the process of protein folding at 25 °C is unfavorable (Murphy et al., 1990; Murphy & Gill, 1991), favorable (Yang et al., 1992), or essentially zero (Baldwin, 1986). Arguments have also been advanced stating that the contribution of hydrogen bond formation to the enthalpy of protein folding (or ligand–protein binding) is favorable (Schellman, 1955; Murphy & Gill, 1991; Scholtz et al., 1991; Creighton, 1984), negligible (Fersht, 1987), or possibly unfavorable (Ben-Naim, 1991; Yang et al., 1992). Estimates of the energies of hydrophobic interactions and hydrogen-bonded interactions in specific regions of known protein structures, and an evaluation of their structural basis, are essential for obtaining structure-based predictions of the free energies of binding for the purpose of drug design.

Insights into the hydration components of hydrophobic interactions and hydrogen-bonded interactions have come from comparative studies of simple dissolution processes carried out in light and heavy water (Ben-Naim et al., 1973; Kresheck et al., 1965; Dahlberg, 1972). For example, differences in the thermodynamics of dissolution of nonpolar gases in D_2O and H_2O have revealed a preferential enthalpy of hydration in D_2O (Scharlin & Battino, 1992). A theory of solvation thermodynamics introduced by Ben-Naim and Marcus (1984) provides a framework for analyzing the data from isotope effects on the aqueous solvation of small-molecule solutes, and allows one to probe the energetic and structural properties

* To whom correspondence should be addressed.

[1] The definition of hydrophobicity has been the subject of much recent discussion (Dill, 1990; Hertzfeld, 1991; Sharp, 1991).

0006-2960/93/0432-5583$04.00/0

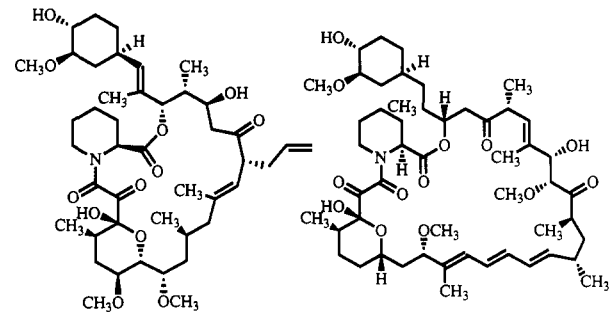
of the liquid state (Marcus & Ben-Naim, 1985). These investigations provide an important basis for understanding hydration in more complex solution processes, and lead one to explore the utility of parallel thermodynamic studies of biopolymers in H_2O and D_2O for probing specific changes in hydration contributions to their thermodynamic reaction properties (Kresheck et al., 1965; Ben-Naim & Marcus, 1984; Ben-Naim, 1991; Schowen & Schowen, 1982).

We have engaged in parallel thermodynamic investigations of the binding of small molecules to proteins in D_2O and H_2O with the overall aim of understanding the energetic consequences of hydration on binding phenomena. In this context, it is desirable to obtain thermodynamic data on protein reactions for which structural information is available for both reactants and products. Presented here is an investigation of the binding of two macrocyclic ligands, tacrolimus and rapamycin, to a small cytosolic protein called FK506 binding protein (FKBP-12).[2] Tacrolimus and rapamycin (Figure 1) are potent immunosuppressive agents that have demonstrated potential for preventing organ transplant rejection (Thomson, 1989). The mechanisms of action of these agents are believed to involve binding to FKBP-12 as a first step (Harding et al., 1989; Schreiber, 1991). Structures of FKBP-12 in solution and in the crystalline state have been determined (Moore et al., 1991; M. Navia, personal communication), as have the crystal structures of the tacrolimus–FKBP-12 and rapamycin–FKBP-12 complexes (Van Duyne et al., 1991a,b; M. Navia, personal communication). Additionally, solution structures of unbound tacrolimus and rapamycin have been solved in organic solvents (Karuso et al., 1990). We have recently reported enthalpy and heat capacity changes of binding of these ligands to bovine FKBP-12 in H_2O, and have analyzed the thermodynamics of the reactions in terms of information derived from the structures and the dissolution of hydrophobic substances into water (Connelly, 1991; Connelly & Thomson, 1992). This report details measurements of the enthalpies of binding of tacrolimus and rapamycin binding to human FKBP-12 in both H_2O (at pH 7.0) and D_2O (at pD 7.0) over the temperature range of 5–30 °C.

MATERIALS AND METHODS

Protein Expression. High-level expression of human recombinant FKBP-12 in *Escherichia coli* was achieved by construction of vectors containing the complete sequence for the gene, subcloned behind a phage T7 RNA polymerase promoter vector (Hopp et al., 1989). FKBP-12 production was initiated by the addition of isopropyl β-D-thiogalactopyranoside (IPTG), to a final concentration of 1 mM. This induces a chromosomal copy of T7 RNA polymerase (behind the *lac* UV promoter) in the host JM109/DE3, which then initiates transcription of the protein gene (Studier & Moffat, 1986). With this promoter system, following induction, FKBP-12 represents 20–30% of the total expressed cellular protein. The typical yield from a single culture in a 10-L fermentor (Biostat ED, B. Braun) was about 250 g of wet cell paste. The cell paste was stored frozen at −70 °C prior to protein purification.

Protein Purification. The methods used to purify FKBP-12 for the present study were essentially very similar to the procedures used by Park et al. (1992). Virtually all of the modifications used herein were for the purpose of increasing the scale and yield of the procedure. Typically, 500 g of frozen cell paste was thawed and slurried in 4 additional volumes of cold buffer [50 mM potassium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride, pH (4 °C) 7.0]. The slurry was briefly homogenized by mechanical disruption in a Polytron blender (PT-6000, with large-scale generator), and then the cells were fully lysed by three serial passages through a high-pressure lab homogenizer (Rannie, Mini-lab 8.30H) at 9000 psi. After each passage, the lysed cell suspension was collected on ice. All subsequent chromatographic and concentration procedures used were essentially larger scale variations of the methods used by Park et al. (1992). The only substantive differences were the use of larger sized ion-exchange and hydrophobic interaction chromatography columns and the substitution of a Pharmacia Sephacryl S-100 HR column (10 × 110 cm), used in the present study, for the Sephadex G-50 size-exclusion column, used by previous authors. From the 500 g of cell paste, about 3.5 g of FKBP-12 was obtained. The protein was typically better than 99% pure FKBP-12, as judged by SDS–PAGE and reversed-phase HPLC. Purity and enzymatic integrity were further confirmed by N-terminal sequence analysis, by analysis of [^{3}H]dihydrotacrolimus binding activity [essentially as described by Park et al. (1992)], and by enzymatic analysis of the peptidylprolyl isomerase activity (Harrison & Stein, 1990). The specific peptidylprolyl isomerase and FK506 binding activities were equal to or greater than those previously reported (Siekierka et al., 1989; Harding et al., 1989; Park et al., 1992), and all of the peptidylprolyl *cis–trans*-isomerase activity was inhibitable by tacrolimus and was unaffected by cyclosporin A. Full details of the protein expression, purification, and biochemical characterization will be published elsewhere (S. P. Chambers and J. A. Thomson, personal communication). The protein was stored in 10 mM sodium cacodylate (pH 6.5) at −70 °C until needed. All protein concentrations were determined using a molar absorption coefficient of 9500 cm^{-1} M^{-1}.



Tacrolimus (FK506) Rapamycin

FIGURE 1: Immunosuppressive ligands of FKBP-12: tacrolimus and rapamycin.

Titration Calorimetry. Solution conditions employed for calorimetric experiments were 50 mM sodium phosphate/50 mM sodium chloride at pH 7.0 in H_2O and at pD 7.0 in D_2O. Human recombinant FKBP-12 was used for all calorimetric studies. Enthalpies were determined by titrating 25–40 μM tacrolimus or rapamycin with 1–3 mM FKBP-12 in an Omega titration calorimeter (MicroCal, Northhampton, MA). Rapamycin and tacrolimus solutions were made by addition of

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; FKBP-12, FK506 binding protein of M_r 12 000; FK506, tacrolimus; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

5–10 μL of drug stock solutions in dimethyl sulfoxide to 10–15 mL of the appropriate buffer, followed by brief sonication. Owing to the tight binding affinities, and high concentrations of compounds in the reaction cell, the enthalpies could be determined from the average heats of six to eight single injections. Injection volumes were 5 or 20 μL, and 4.5 min of equilibration time was allowed between each injection. Dilutions of protein into buffer, and buffer into solutions of the compounds, were performed at each temperature. The protein–drug titration heats were adjusted by these small contributions, which were on the order of the heats produced upon titrating buffer with buffer.

D_2O used for these studies was obtained from Merck (99.9%). All buffer exchanges were done by successive dilution and concentration, in a stirred ultrafiltration cell (M_r 5000 cut-off membrane) under N_2 pressure at 5 °C. Samples in D_2O were allowed to exchange for several hours at room temperature, and then for 1 week at 5 °C (pD 7.0), before experiments were performed. Calorimetric experiments performed after 6 weeks of exchange gave the same enthalpy of binding of tacrolimus as previous experiments performed in the period following 1 week of exchange.

Surface Area Calculations. The solvent-accessible cavity surface areas have been calculated with a program written by Scott R. Presnell (University of California, San Francisco) as described previously (Connelly & Thomson, 1992). The program utilizes the Lee and Richards (1971) algorithm for computing accessible surface. Coordinates of the crystal structures of FKBP-12 and the FKBP–tacrolimus complex were provided by Dr. Manuel Navia, and the coordinates of the FKBP–rapamycin complex were obtained from the Brookhaven Protein Data Bank (Van Duyne et al., 1991b). Coordinates of unbound tacrolimus determined by nuclear magnetic resonance and molecular dynamics were kindly provided by H. Kessler (Institut for Organische Chemie, Technische Universitat, Munich).

Fourier Transform Infrared Spectroscopy. Infrared spectra of bovine FKBP-12 in 50 mM HEPES/50 mM NaCl were recorded at 2-cm^{-1} resolution with a Bruker IFS-66 FT-IR spectrometer equipped with a DTGS detector that contains a KBr window. The sample chamber and spectrometer were continually purged with dry nitrogen. The protein solutions were placed at ambient temperature in the Axiom tunnel cell, an attenuated total reflectance cell (Axiom Analytical Inc., Irvine, CA) with a ZnSe crystal. The volume of the cell used was 40 μL. Spectra of the H_2O and D_2O buffer solutions were also measured, using identical scanning parameters as the protein solutions. All spectra were corrected for the presence of water vapor. Protein spectra were obtained by digital subtraction of the buffer spectrum from the corresponding spectrum of the protein solution.

RESULTS AND DISCUSSION

The enthalpy changes for the reactions of human recombinant FKBP-12 with tacrolimus and rapamycin in the temperature range from 5 to 30 °C are reported in Table I. The enthalpy changes for the reactions depend strongly on temperature, indicating the presence of large negative heat capacity changes accompanying binding. The temperature dependence of the enthalpy change was described well, in all cases, by linear regression models assuming a constant heat capacity change in the temperature range of the experiments (Figure 2). The values of the enthalpy changes at 25 °C and the heat capacity changes obtained in the fitting analysis are given in Table II.

Table I: Enthalpy Changes for the Binding of Tacrolimus and Rapamycin to FKBP-12 Determined by Isothermal Titration Calorimetry in Light and Heavy Water

H_2O, pH 7.0		D_2O, pD 7.0	
T, °C	$\Delta_L H$, kJ/mol	T, °C	$\Delta_D H$, kJ/mol
	Tacrolimus		
5.0	−47.3 ± 0.6	5.0	−40.3 ± 0.8
10.0	−53.1 ± 0.3	9.6	−45.6 ± 0.5
15.1	−59.4 ± 0.5	14.7	−52.7 ± 0.4
20.5	−65.3 ± 0.8	19.6	−56.3 ± 0.6
24.8	−72.0 ± 1.4	25.3	−64.6 ± 1.2
30.2	−77.8 ± 0.5	29.7	−69.5 ± 1.2
	Rapamycin		
5.3	−50.6 ± 1.2	5.3	−36.5 ± 0.5
10.2	−57.7 ± 1.0	11.4	−44.0 ± 1.1
15.0	−68.6 ± 1.2	14.9	−50.1 ± 0.9
20.5	−74.9 ± 1.6	20.4	−54.2 ± 0.4
25.1	−81.2 ± 1.3	25.2	−59.6 ± 0.6
30.6	−88.3 ± 2.1	30.4	−67.8 ± 1.2

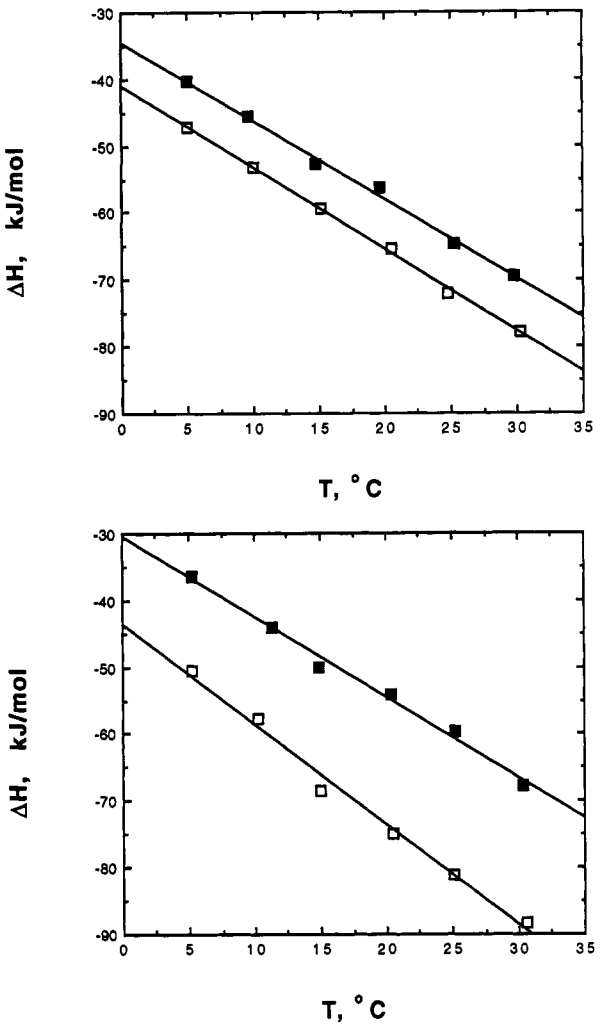


FIGURE 2: Enthalpies of binding of FK506 (top) and rapamycin (bottom) in H_2O and D_2O as a function of temperature. The data were fit to linear equations of the form $\Delta H = \Delta H^* + \Delta C(T - 25)$ where ΔH^* is the enthalpy change at 25 °C and ΔC is the heat capacity change, for a given reaction. The solid lines were calculated from best-fit parameters obtained in the regression analysis. Best-fit parameters are given in Table II.

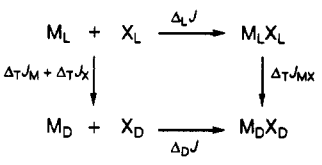
D_2O/H_2O Difference Thermodynamic Properties. It is important to emphasize the relationship of the enthalpy and heat capacity changes to the overall free energy change of binding. For a binding process that takes place with a constant heat capacity change, the free energy change of binding, $\Delta_L G$,

Table II: Enthalpy Changes (at $T^* = 25$ °C) and Heat Capacity Changes for Tacrolimus and Rapamycin Binding to FKBP-12 Determined from Regression Analysis of the Data in Table I and the H_2O/D_2O Difference Thermodynamic Properties[a]

parameter	tacrolimus	rapamycin
$\Delta_L H^*$	−71.5 ± 0.3	−81.0 ± 0.9
$\Delta_D H^*$	−63.9 ± 0.4	−60.5 ± 0.6
$\Delta_D\Delta_L H^*$	7.6	20.5
$\Delta_L C$	−1.22 ± 0.03	−1.50 ± 0.08
$\Delta_D C$	−1.18 ± 0.04	−1.20 ± 0.06
$\Delta_D\Delta_L C$	0.04	0.30

[a] Units of reported enthalpy changes at 25 °C ($\Delta_L H^*$, $\Delta_D H^*$, $\Delta_D\Delta_L H^*$) are kJ/mol; units of reported heat capacity changes ($\Delta_L C$, $\Delta_D C$, $\Delta_D\Delta_L C$) are kJ/(mol·K). Errors on the parameters were determined from the variance–covariance matrix obtained in the regression analysis (Draper & Smith, 1966).

Scheme I



can be broken up into a sum of three terms:

$$\Delta_L G = \Delta_L H^* - T\Delta_L S^* + \Delta_L C[(T - T^*) - T\ln(T/T^*)] \quad (1)$$

The first two terms on the right-hand side of eq 1 are measurable constants for a given reaction, defined at a particular temperature T^*: the enthalpy at T^*, $\Delta_L H^*$, and the entropy change at T^*, $\Delta_L S^*$. The third term contains a temperature function (in brackets) and a constant multiplier of this function, the heat capacity change on binding ($\Delta_L C$). We use the subscript L on the Δ to refer to processes carried out in H_2O. Using the subscript D, we also write the corresponding expression for a reaction carried out in D_2O:

$$\Delta_D G = \Delta_D H^* - T\Delta_D S^* + \Delta_D C[(T - T^*) - T\ln(T/T^*)] \quad (2)$$

Equations 1 and 2 allow one to define the difference thermodynamic properties for reactions carried out in D_2O relative to H_2O through the relation:

$$\Delta_D\Delta_L G = \Delta_D\Delta_L H^* - T\Delta_D\Delta_L S^* + \Delta_D\Delta_L C[(T - T^*) - T\ln(T/T^*)] \quad (3)$$

where the double Δ operators $\Delta_D\Delta_L$ for a given thermodynamic property J (for purposes of this paper, J may be H, G, S, or C) are defined through the relation:

$$\Delta_D\Delta_L J = \Delta_D J - \Delta_L J \quad (4)$$

To facilitate the interpretation of the quantities defined by eq 4, we make use of the thermodynamic cycle shown in Scheme I, as it relates the difference thermodynamic properties, $\Delta_D\Delta_H J$, to the processes of transferring reactants (unbound protein and ligand) and product (ligand–protein complex) from H_2O to D_2O. In Scheme I, M_L, X_L, and M_LX_L represent the protein, the ligand, and the protein–ligand complex, respectively, in light water; M_D, X_D, and M_DX_D represent the same species, respectively, in heavy water. The reactions appearing horizontal in the cycle are identified with the binding reaction carried out in light water (top) and heavy water (bottom). The reactions depicted in the vertical direction in the cycle correspond to the processes of transferring unreacted protein and ligand (left) and protein–ligand complex (right) from H_2O to D_2O. The changes in the thermodynamic functions for the transfer (Δ_T) from H_2O to D_2O are given by $\Delta_T J_{MX}$, $\Delta_T J_M$, and $\Delta_T J_X$ for protein–ligand complex, unbound protein, and unbound ligand, respectively. A fundamental relation that follows from the thermodynamic cycle is

$$\Delta_D\Delta_L J = \Delta_T J_{MX} - \Delta_T J_M - \Delta_T J_X \quad (5)$$

so that the differences in binding observed in D_2O relative to H_2O are equal to the differences in the thermodynamics of transfer of product and reactants from light to heavy water.

Structural Determinants of the Difference Thermodynamic Properties. The thermodynamics of transfer of a protein or ligand species from H_2O to D_2O may be broken up into four terms which correspond to (1) hydrogen exchange (replacement of exchangeable hydrogen atoms by deterium atoms), (2) changes in the state of protonation of the protein and ligand due to differences in the affinity of ionizable groups for H^+ and D^+ (this allows for differences in the pK's of groups in light and heavy water), (3) differences in the light and heavy water hydration properties of polar and nonpolar groups,[3] and (4) other changes in the structures of the protein and ligand that occur as a consequence of (1–3).

It is unlikely that differences in the thermodynamics of transfer between H_2O and D_2O, due to exchange, will contribute significantly to the differences in the thermodynamics reported here. There are over 200 exchangeable hydrogens in FKBP-12 and only a few for tacrolimus or rapamycin. Although the enthalpy of exchange may be significant for transfer of FKBP-12 or FKBP-12–tacrolimus, the difference in the heat of exchange between bound and free FKBP-12 is not likely to be large, since it involves essentially the same number of exchangeable sites.[4] Also, there is a very small number of protons linked to the binding reaction at pH 7.0 as evidenced by the independence of binding constant with pH in the pH range 5–8 (Park et al., 1992; D. Livingston, personal communication). Therefore, it is unlikely that differences in the number of protons liberated in the reaction or heats of ionization would contribute significantly to the $\Delta\Delta J$'s. This latter assumption would not be true for a reaction with a large pH dependence, such as the folding of a protein.

There is little reason to believe that large differences in the structures of a hydrogenated and a deuterated protein exist (Finer-Moore et al., 1992). In fact, neutron diffraction studies of protein crystals in H_2O and D_2O have been used to determine the orientations of the water molecules in proteins (Finer-Moore et al., 1992; Kossiakoff et al., 1992). Nevertheless, differences in the vibrational properties of proteins in light and heavy water are evidenced by the differences in the infrared spectra of proteins in the two solvents (Susi & Byler, 1986). Figure 3 shows the Fourier transform infrared spectra of bovine

[3] Hydration is referred to here as the hypothetical process of transferring a solute species (such as a protein) from the dissolved state in water (wet state) to a state in which no water is present (dry state), and in which no change in the internal structure of the solute takes place. The hydration process is thus exclusive of hydrogen exchange, so that hydration by D_2O refers to the transfer of deuterium-exchanged protein (in the hypothetical dry state) to deuterated solvent (D_2O). The concept of the static solvent-accessible surface area (Lee & Richards, 1971) thus provides a crude measure of the extent of the hydration process referred to here. The definition of hydration follows that given by Ooi et al. (1991).

[4] The heats of the reaction ROH + DO → ROD + HOD for methanol (R = CH_3) and ethanol (R = CH_3CH_2) have been determined to be −0.32 kJ/mol (Chand & Fenby, 1978). For the binding reactions of FKBP-12, it is true that the numbers of protons that can exchange will be essentially the same for products and reactants. However, many of the protons will be in different local environments in the reactant and product species. It is possible that this situation may lead to some differences in the heats of hydrogen exchange in liganded vs unliganded protein.

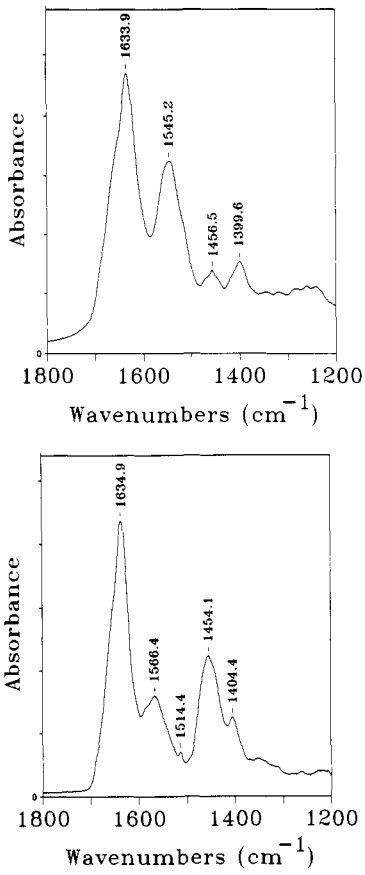


FIGURE 3: Fourier transform infrared spectra of recombinant bovine FKBP-12 in H_2O (top) and D_2O (bottom) in 50 mM HEPES/50 mM NaCl at 20 °C. Expression and purification of the bovine recombinant FKBP-12 were the same as those used for the human recombinant protein (see Materials and Methods).

FKBP-12 in H_2O and D_2O. In H_2O buffer, two main bands are seen in the spectral region between 1800 and 1400 cm^{-1}: first, the amide I band centered around 1644 cm^{-1}, which primarily reflects the C=O stretching vibration of the amide portion of the protein backbone (Krimm & Bandekar, 1986); second, the amide II band centered around 1550 cm^{-1}, which primarily reflects the N–H bending vibration strongly coupled to C–N stretching. The features of the amide II band change most upon exchange into D_2O, as has been observed previously (Susi & Byler, 1986). [The sharp band that is resolved at 1515 cm^{-1} is due to tyrosine side chain absorption, as has been shown for other proteins (Fabian et al., 1992).] The energetic consequences of these observed changes are uncertain, although many of the differences seen in the vibrational properties of the unliganded protein are also present in the protein–ligand complex (unpublished observations). A more detailed analysis of the infrared spectra of FKBP and its complexes with tacrolimus and rapamycin, in H_2O and D_2O, is underway.

The hydration contributions to the difference thermodynamic properties of eq 4 may be divided into changes in hydration of the various polar and nonpolar atoms that occur upon binding. One measure of the changes in hydration of the protein in a reaction comes through an analysis of the changes in the solvent-accessible surface area that accompany binding (Lee & Richards, 1971). The top and bottom panels of Figure 4 detail these changes for tacrolimus and rapamycin, respectively. Upon tacrolimus binding, there is a net burial of 650 Å^2 of nonpolar groups and a net exposure of 7 Å^2 of polar atoms. For rapamycin binding, 554 Å^2 of nonpolar surface is buried, and 263 Å^2 of polar surface is buried. Upon inspection of the structures of FKBP-12 and its complexes

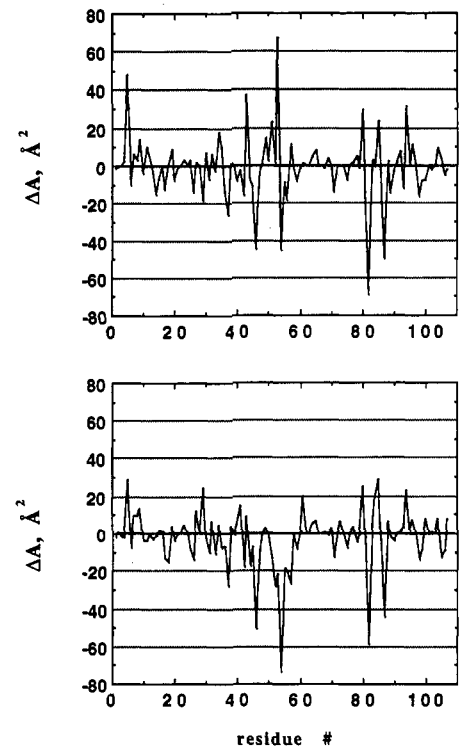


FIGURE 4: Differential surface area spectra for tacrolimus (top) and rapamycin (bottom) binding to FKBP-12. The difference solvent-accessible surface area of binding is defined $\Delta A = A_{MX} - A_M - A_X$ where A_{MX}, A_M, and A_X represent the surface areas of the protein–ligand complex, unliganded protein; and free ligand, respectively.

with tacrolimus and rapamycin, it is evident that the largest area changes per residue (differential surface area residue density) are due to those groups which undergo changes in hydrogen bonding upon complexation. The largest negative peaks in Figure 4 occur at residues Asp-37, Glu-54, Ile-56, and Tyr-82. These residues participate in four of the five hydrogen-bonding interactions between tacrolimus and protein, and rapamycin and protein. The fifth residue that is involved in a hydrogen bond with tacrolimus (Gln-53) gives rise to a large positive peak in the difference surface area spectrum. However, this hydrogen bond, from the Gln-53 CO to the C-24 hydroxyl of tacrolimus, is mediated by a water molecule. In the FKBP-12–rapamycin complex, the main chain carbonyl of the Gln-53 residue becomes highly buried as it forms a hydrogen bond with the C-40 hydroxyl of rapamycin (Van Duyne et al., 1991b).

Figure 5 details, separately, the changes in solvent exposure of polar atoms and nonpolar atoms upon binding. The burial of nonpolar surface tends to be fairly evenly distributed on a per residue basis with the exception of the aromatic residues Tyr 82 and Phe-46 and the heterocyclic His-87 side chain, all of which form direct van der Waals interactions with the tacrolimus molecule. Several polar residues increase their exposure upon binding of tacrolimus and rapamycin: Glu-5, Asn-43, Lys-52, Thr-85, and Glu-107. Inspection of the structures indicates that these residues undergo changes in hydrogen bonding with other residues, or with water, upon ligand binding. For instance, one of the ϵ-oxygens of Glu-5 forms a hydrogen bond with a water molecule in the FKBP–tacrolimus complex. The water molecule is not seen in the unliganded structure. (However, there is a difference in the resolution of the two protein structures.) Similarly, the backbone carbonyl of Lys-52 and the ζ-nitrogen form hydrogen bonds to water molecules in the FKBP–tacrolimus complex which are not seen in the unliganded protein.

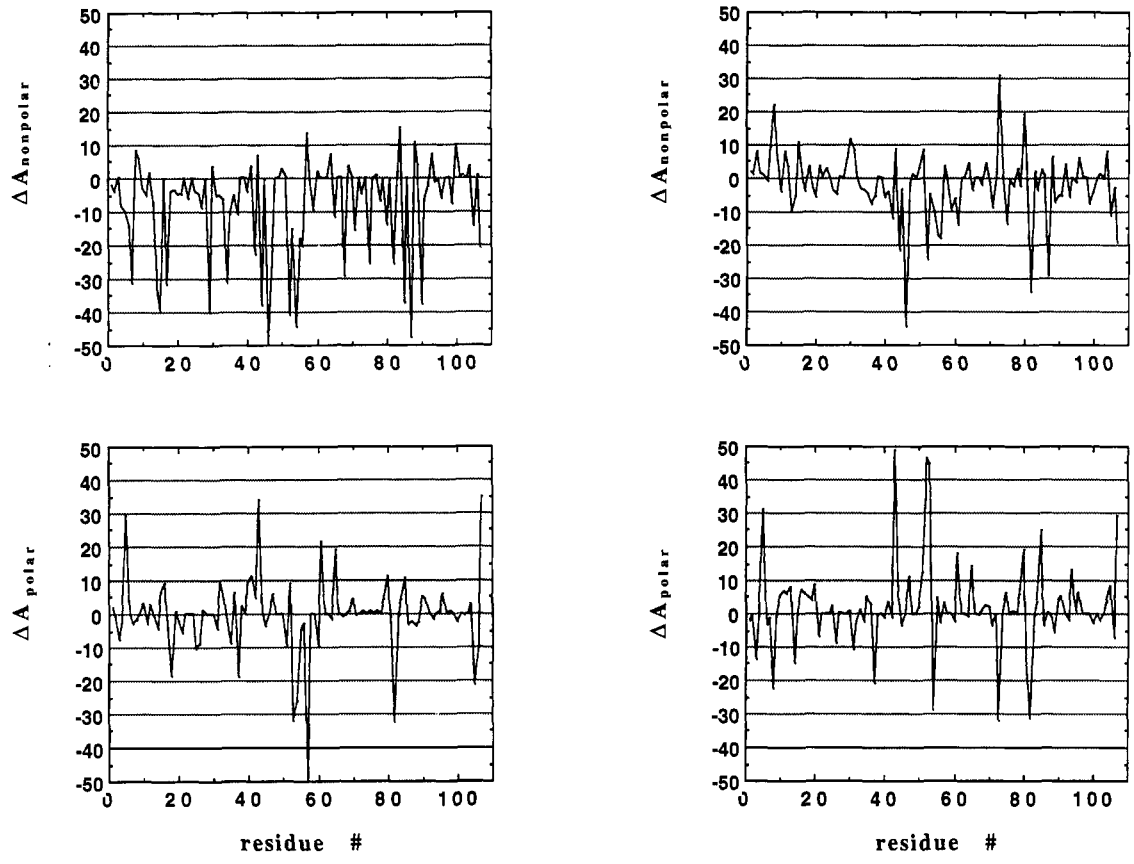


FIGURE 5: Nonpolar (top) and polar (bottom) differential surface area spectra for tacrolimus (left) and rapamycin (right) binding to FKBP-12.

Enthalpic Destabilization of Tacrolimus and Rapamycin Binding to FKBP-12 in D_2O Relative to H_2O. The data in Figure 2 reveal that the enthalpy of binding of rapamycin and tacrolimus to FKBP-12 is less negative in D_2O over the range of temperatures examined. Such an observation indicates that D_2O either stabilizes the unassociated reactants or destabilizes the protein–ligand complexes, relative to H_2O. One factor that could contribute to the observed enthalpic destabilization is that the enthalpy of dehydrating the various nonpolar moieties of the unassociated ligand and protein in D_2O requires more energy than the dehydration of H_2O from these groups. The data in Figure 4 clearly demonstrate that binding is accompanied by a large net loss in the amount of surface area that is accessible to water. In this regard, it is useful to compare the results reported here with the enthalpies of transfer of pure gaseous and liquid substances between H_2O and D_2O.

Enthalpies of transfer from H_2O to D_2O are negative for nonpolar compounds (Scharlin & Battino, 1992; Hallen et al., 1986). For example, calorimetric data on the heats of transfer of alkan-1-ols of increasing chain length, from H_2O to D_2O, reveal that the enthalpy of transfer of a CH_2 group is approximately –0.23 kJ/mol at 25 °C (Hallen et al., 1986). Since a CH_2 group has an accessible surface area of approximately 29 Å^2, the enthalpy of transfer of a CH_2 group from H_2O to D_2O, normalized to the amount of solvent-accessible surface area, is –7.9 J(mol·Å^2) at 25 °C. Muller (1991) has presented a modified hydration-shell hydrogen bond model for estimating the difference in the enthalpy of transfer of nonpolar solutes from light to heavy water. On the basis of his model, the enthalpy of transfer of a nonpolar solute with 16.9 molecules of water in its first hydration shell, from H_2O to D_2O, is equal to $-1.339(N/16.9)$, where N is the number of water molecules in the first hydration shell. Assuming that a water molecule occupies 9 Å^2 of the accessible surface of nonpolar solutes (Herman, 1972), one obtains a value for the enthalpy of transfer of nonpolar surface of $-1.339/(16.9)(9)$, or –8.8 kJ/(mol·Å^2) at 25 °C. This is very similar to our –7.9 kJ/(mol·Å^2) estimate based on the heats of transfer of alkan-1-ols.

These estimates compare with the H_2O/D_2O difference enthalpies of binding of tacrolimus to FKBP-12: 650 Å^2 of nonpolar surface is buried upon binding of tacrolimus to FKBP-12. The enthalpy associated with transferring 650 Å^2 of nonpolar surface from H_2O to D_2O is –5.1 kJ/mol, based on the analysis of alcohol dissolution presented above, or –5.7 kJ/mol, based on the model given by Muller (1991). The experimental difference ($-\Delta_D\Delta_L H^*$, from Table II) in the *dissociation* of tacrolimus from FKBP-12 in D_2O relative to H_2O at 25 °C is –7.6 kJ/mol. The similarity of the experimental and derived values suggests that a chief contribution to the enthalpic destabilization of tacrolimus binding is due to differential hydration of nonpolar atoms by D_2O relative to H_2O. It is also of interest to note that the temperature dependence of the enthalpy of dissolution of alkan-1-ols in H_2O and D_2O reveals no appreciable difference in the heat capacity change for transfer from light and heavy water (Hallen et al., 1986). The model presented by Muller (1991) indicates that the heat capacity change for the transfer of nonpolar molecules from D_2O is approximately 10–15% greater than in H_2O. We find that the value of $\Delta_D\Delta_L C$ for tacrolimus binding is zero, within experimental error (Table II).

The enthalpy of transfer of polar atoms from H_2O to D_2O is more difficult to ascertain from model compound dissolution data, for purposes of applying them to reactions involving proteins. The values for the enthalpies of transfer depend critically upon the atoms to which they are attached (Dahlberg,

1972; Jancso & Van Hook, 1974). Reported enthalpies of transfer of several *n*-alkan-1-ols (after correcting for hydrogen exchange) are less exothermic than the corresponding alkane (Hallen et al., 1986), whereas the enthalpy of transfer of carbon dioxide is more exothermic than methane (Ben-Naim & Marcus, 1984). Rapamycin binding is accompanied by the burial of a larger amount of polar surface, and a smaller amount of nonpolar surface, than for tacrolimus binding, yet a much larger enthalpic destabilization by D_2O (relative to H_2O) is observed for rapamycin binding at 25 °C (see Table II). If differential hydration is a dominant contributor to the thermodynamic difference properties of rapamycin binding, it is possible that the polar atoms of the protein have a significant preferential hydration by D_2O relative to H_2O at 25 °C. This would account for the large $\Delta_D\Delta_L H$ observed for rapamycin. It should also be pointed out that the heat capacity change in D_2O is significantly less negative than in H_2O. This may be due to the differences in heat capacities of hydration, by the isotopic waters, of polar moieties of the protein. Also, one must bear in mind that the solvent-accessible surface is only a crude description of the protein–water interface. It is useful as a simple one-parameter representation of the interface, based on a hard-sphere model of the protein structure. However, it does not reflect the details of the local interaction of water with specific regions of protein surface (Varadarajan et al., 1990). In order to more clearly delineate the contributions that changes in exposure of various atoms to solvent have on the difference thermodynamic properties, it would be useful to investigate the differences in the thermodynamics of binding of tacrolimus and rapamycin in H_2O and D_2O to several site-specific mutants of FKBP in which various polar and nonpolar atoms have been selectively deleted. Unfortunately, the solubilities of tacrolimus and rapamycin are too low to allow direct titration calorimetric measurement of the enthalpies of transfer from H_2O to D_2O.

Heat Capacity Changes, Hydration, and Solvent-Accessible Surface Area. Over fifty years ago, Edsall (1935) drew attention to the fact that the constant pressure heat capacity change that accompanied the interaction of nonpolar groups with water was quite large. Upon obtaining evidence of a positive heat capacity change upon protein unfolding, Brandts (1964) suggested that the heat capacity change could be due in part to the exposure of nonpolar groups to water upon unfolding. An analysis by Sturtevant (1977) is consistent with this suggestion, and argues that the negative heat capacity changes for ligand binding are largely due to the burial of nonpolar groups from water. Since then, the heat capacity changes for the aqueous dissolution of a number of nonpolar gases and liquids have been shown to be proportional to their solvent-accessible cavity surface areas (Herman, 1972; Privalov & Gill, 1988). These observations have been taken to indicate that the heat capacity increments observed in these processes are determined entirely by the hydration of the substances. Recently, several reports have focused on correlations between heat capacity changes and changes in solvent-accessible surface areas accompanying protein unfolding (Livingstone et al., 1990; Spolar et al., 1992; Privalov & Makhatadze, 1990; Murphy & Gill, 1991). The analyses indicate that the heat capacity change for unfolding proteins can be attributed almost entirely to the differences in solvent-accessible nonpolar and polar surface areas between native and denatured states. An important assumption in all of the models, which relate heat capacity changes of unfolding to structural features of the protein, is that the structure of the denatured state is an extended, fully solvent-exposed form. This is a questionable assumption that we do not need to make in our analysis.

Tacrolimus binding is accompanied by a net burial of surface area that is almost entirely nonpolar. Previously, we have shown that the heat capacity change for tacrolimus binding to FKBP-12 can be accounted for simply by the amount of this nonpolar surface area buried from water. In particular, we divided the contribution of nonpolar group burial to the heat capacity change, into terms that reflect aromatic and nonaromatic group burial. The total heat capacity change of a given reaction will most likely contain terms due to the changes in the exposure of polar atoms to solvent upon binding, in addition to any nonhydration contributions to the heat capacity change [such as changes in the number of vibrational modes, as pointed out by Sturtevant (1977) (see also, Varadarajan et al., 1992)]. The results of Spolar et al. (1992), Murphy and Gill (1991), and Privalov and Makhatadze (1990) indicate that the heat capacity changes due to the burial of polar groups of proteins are positive, opposite to the effect of burying nonpolar surface. Since rapamycin binding leads to a significant burial of polar surface, and less nonpolar surface burial than with tacrolimus binding, then one might expect the heat capacity change for the binding reaction to be less negative than for tacrolimus (if changes in hydration are the only contributors to heat capacity changes). The results in Table II reveal that the heat capacity change for rapamycin binding is *more* negative. Additionally, we have performed surface area calculations on other proteins for which heat capacity changes for binding as well as high-resolution structures are known, and we find no simple correlation between amounts of polar and nonpolar surface area burial and the heat capacity changes (unpublished data). Possible contributions to heat capacity changes, other than those related to hydration, include (1) the existence of a temperature-dependent conformational equilibrium in the unliganded protein and (2) changes in the intramolecular vibrational properties of the protein or ligand upon binding (Sturtevant, 1977). For FKBP-12, there is no evidence for any such conformational equilibrium. However, tacrolimus is known to exist as an equilibrium mixture of cis and trans isomers (about the prolyl amide bond) in chloroform solution. It is not known what the equilibrium constant for this interconversion is in water. Further analysis and high-precision measurements of heat capacity changes of reactions, for which high-resolution structures of both bound and unliganded proteins are known, are necessary to clarify the general connection between heat capacity changes and the hydration properties of proteins.

Conclusion. In the present investigation, we have made use of the advances in high-precision titration calorimetry (Wiseman et al., 1989) to measure enthalpy and heat capacity changes of binding of two ligands to FKBP-12 in light and heavy water. A thermodynamic cycle is presented which relates the differences in the thermodynamics of binding in H_2O and D_2O to the differences in the thermodynamics of transfer of the various protein and ligand species from H_2O to D_2O. Comparison of the enthalpies and heat capacities of transfer of alkan-1-ols, for which accurate calorimetric measurements are known (Hallen et al., 1977), with the D_2O/H_2O difference enthalpy of binding changes reported here suggests that the dehydration of protein and ligand represents an important contribution to the observed enthalpic destabilization by D_2O. In view of the availability of structural data for the FKBP-12 system, together with the ability to mutate amino residues in FKBP-12 in a site-specific manner (Aldape et al., 1992), and the recent availability of high-

precision thermodynamic measurements for simple dissolution processes in D_2O and H_2O, a systematic investigation of the solvent isotope effects, in the binding reactions of FKBP-12 and its mutants, may allow for a more detailed analysis of the hydration processes involving proteins.

ACKNOWLEDGMENT

We are very grateful to Dr. Stephen Chambers for his work on the construction of the protein expression system and for supplying the recombinant cell paste. We also thank Dr. Mark Murko for insightful discussions on the hydration of proteins and for his critical reading of the manuscript.

REFERENCES

Aldape, R. A., Futer, O., DeCenzo, M. T., Jarrett, B. P., Murko, M. A., & Livingston, D. J. (1992) *J. Biol. Chem. 267*, 16029–16032.
Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A 83*, 8069–8072.
Ben-Naim, A. (1991) *J. Phys. Chem. 95*, 1437–1444.
Ben-Naim, A., & Marcus, Y. (1984) *J. Chem. Phys. 81*, 2016–2027.
Ben-Naim, A., Wilf, J., & Yaacobi, M. (1973) *J. Phys. Chem. 77*, 95–101.
Brandts, J. (1964) *J. Am. Chem. Soc. 86*, 4302–4314.
Chand, A., & Fenby, D. V. (1978) *J. Chem. Thermodyn. 10*, 997–1010.
Connelly, P. R. (1991) *Transplant. Proc. 23*, 2883–2885.
Connelly, P. R., & Thomson, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4781–4785.
Creighton, T. E. (1984) *Proteins: Structure and Molecular Principles*, W. H. Freeman, New York.
Dahlberg, D. B. (1972) *J. Phys. Chem. 76*, 2045–2050.
Dill, K. A. (1990) *Science 250*, 297–298.
Draper, N. R., & Smith, H. (1966) *Applied Regression Analysis*, John Wiley & Sons, New York.
Edsall, J. T. (1935) *J. Am. Chem. Soc. 57*, 1506–1507.
Edsall, J. T., & Mackenzie, (1983) *Adv. Biophys. 16*, 53–183.
Fabian, H., Naumann, D., Misselwitz, R., Ristau, O., Gerlach, D., & Wefle, H. (1992) *Biochemistry 31*, 6532–6538.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301.
Finer-Moore, J. S., Kossiakoff, A. A., Hurley, J. H., Earnest, T., & Stroud, R. (1992) *Proteins: Struct., Funct., Genet. 12*, 203–222.
Hallen, D., Nilsson, S. O., Rothschild, W., & Wadso, I. (1986) *J. Chem. Thermodyn. 18*, 429–442.
Harding, M. W., Galat, A., Uehling, D. F., & Schreiber, S. L. (1989) *Nature 341*, 758–760.
Harrison, R. K., & Stein, R. L. (1990) *Biochemistry 29*, 3813–3816.
Hermann, R. B. (1972) *J. Phys. Chem. 76*, 2754–2759.
Hertzfeld, J. (1991) *Science 253*, 88–89.
Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L., & Conlon, P. J. (1989) *Bio/Technology 6*, 1204–1210.
Jancso, G., & Van Hook, W. A. (1974) *Chem. Rev. 74*, 689–750.
Karuso, P., Kessler, H., & Mierke, D. F. (1990) *J. Am. Chem. Soc. 112*, 9434–9436.
Kossiakoff, A. A., Sintchak, M. D., Shpungin, J., & Presta, L. G. (1992) *Proteins: Struct., Funct., Genet. 12*, 223–236.
Kresheck, G. C., Schneider, H., & Scheraga, H. A. (1965) *J. Phys. Chem. 69*, 3132–3144.
Krimm, S., & Bandekar, J. (1986) *Adv. Protein Chem. 38*, 181–364.
Lee, B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5154–5158.
Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379–400.
Livingstone, J. R., Spolar, R. S., & Record, T., Jr. (1991) *Biochemistry 30*, 4237–4244.
Marcus, Y., & Ben-Naim, A. (1985) *J. Chem. Phys. 83*, 4744–4759.
Moore, J. M., Peattie, D. A., Fitzgibbon, M. J., & Thomson, J. A. (1991) *Nature 351*, 248–250.
Muller, N. (1991) *J. Solution Chem. 20*, 669–680.
Murphy, K. P., & Gill, S. J. (1991) *J. Mol. Biol. 222*, 699–709.
Murphy, K. P., Privalov, P. L., & Gill, S. J. (1990) *Science 247*, 559–561.
Ooi, T., & Oobatake, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2859–2863.
Pace, C. N. (1992) *J. Mol. Biol. 226*, 29–35.
Park, S. T., Aldape, R. A., Futer, O., DeCenzo, M. T., & Livingston, D. J. (1992) *J. Biol. Chem. 267*, 3316–3324.
Privalov, P. L., & Gill, S. J. (1988) *Adv. Protein Chem. 39*, 191–234.
Privalov, P. L., & Makhatadze, G. I. (1990) *J. Mol. Biol. 213*, 385–391.
Scharlin, P., & Battio, R. (1992) *J. Solution Chem. 21*, 67–91.
Schellman, J. (1955) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 29*, 223–229.
Scholtz, J. M., Marqusee, S., Baldwin, R. L., York, E. J., Stewart, J. M., Santoro, M., & Bolen, D. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2854–2858.
Schowen, K. B. & Schowen, R. L. (1982) *Method Enzymol. 87*, 552–621.
Schreiber, S. L. (1991) *Science 251*, 283–287.
Sharp, K. A. (1991) *Curr. Opin. Struct. Biol. 1*, 171–174.
Sharp, K. A., Nichols, A., Fine, R. F., & Honig, B. (1991) *Science 252*, 106–109.
Siekierka, J. J., Hung, S. H. Y., Poe, M., Lin, C. S., & Sigal, N. H. (1989) *Nature 341*, 755–757.
Spolar, R. S., Ha, J., & Record, M. T., Jr. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8382–8385.
Spolar, R. S., Livingstone, J. R., & Record, M. T., Jr. (1992) *Biochemistry 31*, 3947–3955.
Studier, F. W., & Moffat, B. A. (1986) *J. Mol. Biol. 189*, 113–130.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Susi, H., & Byler, D. M. (1986) *Methods Enzymol. 130*, 290–311.
Teeter, M. M. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 577–600.
Thomson, A. W. (1989) *Immunol. Today 10*, 6–9.
Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., & Clardy, J. (1991a) *Science 252*, 839–842.
Van Duyne, G. D., Standaert, R. F., Schreiber, S. L., & Clardy, J. (1991b) *J. Am. Chem. Soc. 113*, 7433–7434.
Varadarajan, R., Richards, F. M., & Connelly, P. R. (1990) *Curr. Sci. 59*, 819–824.
Varadarajan, R., Connelly, P. R., Sturtevant, J. M., & Richards, F. M. (1992) *Biochemistry 31*, 1421–1426.
Wiseman, T., Williston, S., Brandts, J., & Lin, L. (1989) *Anal. Biochem. 179*, 131–137.
Yang, A., Sharp, K. A., & Honig, B. (1992) *J. Mol. Biol. 227*, 889–900.