Enthalpy of hydrogen bond formation in a protein–ligand binding reaction

(FK506 binding protein/tacrolimus/calorimetry/drug design)

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ABSTRACT Parallel measurements of the thermodynamics (free-energy, enthalpy, entropy, and heat-capacity changes) of ligand binding to FK506 binding protein (FKBP-12) in H2O and D2O have been performed in an effort to probe the energetic contributions of single protein–ligand hydrogen bonds formed in the binding reactions. Changing tryptophan-82 to phenylalanine in FKBP-12 abolishes protein–ligand hydrogen bond interactions in the FKBP-12 complexes with tacrolimus or rapamycin and leads to a large apparent enthalpic stabilization of binding in both H2O and D2O. High-resolution crystallographic analysis reveals that two water molecules bound to the tryptophan-82 hydroxyl group in unliganded FKBP-12 are displaced upon formation of the protein–ligand complexes. A thermodynamic analysis is presented that suggests that the removal of polar atoms from water contributes a highly unfavorable enthalpy change to the formation of C==O·HO hydrogen bonds as they occur in the processes of protein folding and ligand binding. Despite the less favorable enthalpy change, the entropic advantage of displacing two water molecules upon binding leads to a slightly more favorable free-energy change of binding in the reactions with wild-type FKBP-12.

First suggested >70 years ago (1, 2) and particularly emphasized by Pauling et al. (3, 4), the hydrogen bond is now recognized as an interaction of fundamental importance in determining the structures of proteins and their complexes with ligands (5). Nevertheless, conflicting views are still held on the relative energetic contributions of hydrogen bonds and interactions involving nonpolar groups to the thermodynamics of protein folding and ligand binding (6–8). Models have been presented arguing that the overall enthalpic contribution of interactions involving nonpolar moieties (at 25°C) to the process of protein folding is highly unfavorable (7, 9), highly favorable (8), or essentially zero (10, 11). Arguments have also been advanced stating that the overall contribution of amide hydrogen-bond formation to the enthalpy of protein folding (or ligand–protein binding) at 25°C is favorable (7), negligible (12) and possibly unfavorable (8, 13). Clarification of the present controversy requires estimates of the enthalpies of formation of specific hydrogen bonds in particular regions of known protein structures. Such measurements are essential for understanding the energetic basis of protein folding and for obtaining structure-based predictions of the energies of ligand binding for the purpose of drug design.

Part of the difficulty in dissecting the relative energetic contribution of hydrogen bonds to folding or binding reactions of proteins is that the formation of hydrogen bonds between atoms in reaction products is often accompanied by the release of water molecules that were hydrogen-bonded to these atoms prior to reaction. Tacrolimus (also known as FK506) and rapamycin are large macrocyclic compounds each of which binds with high affinity to a common cytosolic protein of 12 kDa known as FK506 binding protein (FKBP-12). Recently, the structures of FKBP-12 in solution and in the crystalline state have been determined (14, 15), as have the crystal structures of the tacrolimus–FKBP-12 and rapamycin–FKBP-12 complexes (16–18). One of the structural features common to both protein–ligand complexes is a hydrogen bond between the Tyr-82 hydroxyl hydrogen (donor) and the amide carbonyl oxygen (acceptor) of either rapamycin or tacrolimus, buried in the hydrophilic interface (Fig. 1). As determined by x-ray crystallography, an important feature of the unliganded protein structure is the well-defined hydration of this tyrosine hydroxyl group. Furthermore, the NMR structure of tacrolimus in chloroform has been reported by Karuso et al. (19); the low solubility of tacrolimus prevents the determination of its structure in water. However, the interaction of unbound tacrolimus with water has been investigated through molecular dynamics simulation (20). The simulation reveals that the amide carbonyl oxygen of tacrolimus is exposed to solvent and makes an average of 1.6 hydrogen bonds with water molecules at 25°C.

In an attempt to dissect the thermodynamics of formation of this hydrogen bond from the overall binding process, we have changed the Tyr-82 residue of FKBP-12 to a phenylalanine residue through site-directed mutagenesis. In this report, we compare measurements of the free-energy, enthalpy, entropy, and heat-capacity changes for the binding of tacrolimus and rapamycin to both wild type (wt) FKBP-12 and the Tyr-82→Phe mutant (termed “Y82F”) in H2O (at pH = 7.0) and D2O (at pH = 7.0). The results are evaluated by comparison with the x-ray structures of the mutant and wild-type proteins.

MATERIALS AND METHODS

Determination of the Thermodynamics of Ligand Binding. Solution conditions for all experiments were 50 mM sodium phosphate/50 mM sodium chloride, pH 7.0 or pH 7.0. Mutagenesis, expression, and purification of the proteins were carried out as described (21–23). Enthalpy changes were determined calorimetrically at 5°C intervals over the temperature range 5–35°C with a MicroCal Omega calorimeter (23). Free-energy measurements for tacrolimus or rapamycin

Abbreviations: wt, wild type; FKBP-12, FK506 binding protein of 12 kDa; Y82F, mutant FKBP-12 carrying the Tyr-82-Phe change.

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Fig. 1. Formation of a hydrogen bond in the binding of FKBP-12 with tacrolimus or rapamycin takes place with the release of water molecules (only the binding cores of tacrolimus and rapamycin are shown). In the complex with tacrolimus, the hydrogen bond between the Tyr-82 hydroxyl and the amide carbonyl oxygen is 2.7 Å long (oxygen–oxygen distance) with a C=O–O angle of 119.7°. The corresponding hydrogen bond in the rapamycin–FKBP-12 complex has a length of 2.76 Å and a C=O–O angle of 122.5°. This geometry is similar to the average geometry observed for hydrogen bonds in which a tyrosine hydroxyl is a hydrogen-bond donor: the average O–O distance is 2.8 ± 0.26 Å and the average angle is 121 ± 16° about the phenolic oxygen (5). Two water molecules, termed A and B, with respective crystallographic temperature factors of 5.71 Å² and 28.3 Å², form hydrogen bonds to the tyrosine hydroxyl group. The hydrogen bond lengths (O–O distance) and angles (C=O–O angle) are 3.01 Å, 130.4° and 2.77 Å, 109.3° for waters A and B, respectively. These parameters compare to the average distance of 2.9 ± 0.33 Å and average angle of 122 ± 15° for waters bound to tyrosine hydroxyl groups observed crystallographically (5).

binding to Y82F were determined by titration calorimetry and fluorescence titration. Fluorometric titrations were performed by adding 0.5–µl injections of 1 mM rapamycin into a cuvette containing 3.0 ml of 2 µM protein. Fluorescence emission spectra were recorded from 290 to 400 nm upon excitation at 280 nm in an LS-50 luminescence spectrometer (Perkin-Elmer).

Concentrations of the wt FKBP-12 were determined by the absorbance at 280 nm (A280) with a Hewlett-Packard UV/visible radiation spectrometer by using a molar absorptivity of 0.81 cm²mg⁻¹ (24). The concentrations of tacrolimus and rapamycin stock solutions were standardized by analytical fluorometric titrations, performed under tight-binding (saturating) conditions. The extinction coefficient of mutant FKBP-12 (Y82F) was determined in reference to the wt protein by analytical fluorometric or calorimetric titrations of mutant protein solutions of known absorbance with standardized tacrolimus or rapamycin solutions. The average of five determinations was A280 = 0.77 ± 0.02 cm²mg⁻¹.

Structure Determination. Crystals (space group P4₁2₁2₁) of human recombinant wt FKBP-12 and the mutant Y82F, each complexed with FK506, were obtained by using reported conditions (16). Complete diffraction data to 1.6 Å were collected for both complexes on a Siemens area detector. Coordinates for the FKBP-12–FK506 complex (ref. 16; Brookhaven Protein Data Bank) were used as starting coordinates for the refinement of the wt and Y82F complexes. Simulated annealing followed by cycles of positional refinement and rebuilding resulted in models for both complexes with good stereochemistry. R factors of 16.6% and 15.4% were obtained for wt and Y82F complexes, respectively. Crystals (space group C2) of the uncomplexed Y82F (human recombinant) were obtained by using the hanging-drop method under different crystallization conditions: 47 mg of protein per ml of 5 mM Tris-HCl/1 mM dithiothreitol/0.1% N-heptylglucose pyranoside (N-pg), pH 8.5 was mixed in equal volume (4 µl/4 µl) with reservoir (55% saturated ammonium sulfate/100 mM potassium phosphate/0.1% N-pg, pH 6.0) and suspended over the reservoir. Crystals grew within 3 weeks to dimensions 1.5 mm × 1.0 mm × 1.0 mm. Data to 1.4-Å resolution were collected on an RaxisIIC image plate system. The Y82F structure was solved by molecular replacement using coordinates of the wt FKBP-12–FK506 complex (16) but with the drug removed. Simulated annealing followed by rebuilding and positional refinement resulted in a model with an R-factor of 20.0% and good stereochemistry (rms deviation from ideal bond lengths and angles of 0.017 Å and 2.9°, respectively).

RESULTS AND DISCUSSION

Mutation Thermodynamic Difference Properties. Enthalpy and heat capacity changes for the binding reactions of the Y82F and wt proteins with tacrolimus or rapamycin, measured in H₂O or D₂O, are given in Table 1. From the measured quantities, we calculated the differences in the thermodynamic properties of binding to the Y82F relative to wt FKBP-12. We refer to these quantities as the “mutation thermodynamic difference properties” (Tables 1 and 2). For the purpose of interpreting these data, it is useful to introduce a pair of identities that relate the mutation thermodynamic difference properties to the thermodynamics of replacing the tyrosine residue by phenylalanine in the complexed and unliganded forms of protein:

\[ \Delta L_1 A_1 J = \Delta L_1 J' - \Delta L_1 J = \Delta L_1 J^{MX} - \Delta L_1 J^{M'} \]

and

\[ \Delta D_1 D_1 J = \Delta D_1 J' - \Delta D_1 J = \Delta D_1 J^{MX} - \Delta D_1 J^{M'} \]

where J can be the enthalpy (H), entropy (S), free-energy (G), or heat-capacity (C) function. The subscript L in Eq. 1 refers to reaction processes in light water, while the subscript D in Eq. 2 refers to reaction processes in D₂O. The prime (') designation refers to the mutant protein, so that \( \Delta J' \) refers to the binding of a ligand X to wt macromolecule M in H₂O, and \( \Delta J'' \) refers to the binding of a ligand X to mutant macro-molecule M' in H₂O.

Perhaps the most striking observation reported here is that, in both light and heavy water, the enthalpies of binding of tacrolimus and rapamycin to Y82F are significantly more exothermic than in their binding to wt protein (i.e., for tacrolimus and rapamycin \( \Delta_1 \Delta_1 H < 0 \) and \( \Delta_1 \Delta_1 D < 0 \), Table 1).

Table 1. Enthalpy changes at 25°C (kJ/mol) and heat-capacity changes (kJ/(mol·K)) for the binding of tacrolimus and rapamycin to wt FKBP-12 (wt) and the Y82F mutant in light and heavy water

<table>
<thead>
<tr>
<th>System (protein/solvent)</th>
<th>Parameter</th>
<th>Tacrolimus</th>
<th>Rapamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/H₂O</td>
<td>ΔL₁H</td>
<td>−71.5 ± 0.3</td>
<td>−81.0 ± 0.9</td>
</tr>
<tr>
<td>wt/D₂O</td>
<td>ΔP₁H</td>
<td>−63.9 ± 0.4</td>
<td>−60.5 ± 0.6</td>
</tr>
<tr>
<td>wt/H₂O</td>
<td>ΔC₁C</td>
<td>−1.22 ± 0.03</td>
<td>−1.50 ± 0.08</td>
</tr>
<tr>
<td>wt/D₂O</td>
<td>ΔC₁C</td>
<td>−1.17 ± 0.04</td>
<td>−1.20 ± 0.06</td>
</tr>
<tr>
<td>Y82F/H₂O</td>
<td>ΔL₁H'</td>
<td>−89.1 ± 0.4</td>
<td>−93.7 ± 1.3</td>
</tr>
<tr>
<td>Y82F/D₂O</td>
<td>ΔP₁H'</td>
<td>−71.1 ± 1.3</td>
<td>−81.6 ± 0.5</td>
</tr>
<tr>
<td>Y82F/H₂O</td>
<td>ΔL₁C'</td>
<td>−1.59 ± 0.04</td>
<td>−1.92 ± 0.13</td>
</tr>
<tr>
<td>Y82F/D₂O</td>
<td>ΔC₁C'</td>
<td>−1.34 ± 0.10</td>
<td>−1.97 ± 0.08</td>
</tr>
<tr>
<td>(Y82F – wt)/H₂O</td>
<td>ΔL₁H''</td>
<td>−17.6</td>
<td>−12.7</td>
</tr>
<tr>
<td>(Y82F – wt)/D₂O</td>
<td>ΔP₁H''</td>
<td>−7.2</td>
<td>−21.1</td>
</tr>
<tr>
<td>(Y82F – wt)/H₂O</td>
<td>ΔL₁C''</td>
<td>−0.37</td>
<td>−0.42</td>
</tr>
<tr>
<td>(Y82F – wt)/D₂O</td>
<td>ΔC₁C''</td>
<td>−0.37</td>
<td>−0.77</td>
</tr>
</tbody>
</table>

Enthalpy changes were determined at 5°C intervals over the temperature range 5–35°C, as described by Connolly et al. (23). Parameters were obtained by fitting the data to linear regression models of the form \( \Delta H = \Delta H^* + AC(T - T^*) \), assuming constant heat-capacity changes, where \( T^* = 25°C \). Differences in the enthalpy and heat-capacity changes of binding to Y82F relative to wt FKBP-12 are given by the appropriate thermodynamic difference mutation parameters \( \Delta(L_1 A_1 H, \Delta P_1 H, \Delta L_1 C, \Delta C_1 C) \), defined as follows: for a given thermodynamic property \( J \), \( \Delta J' = \Delta J - \Delta J \), and \( \Delta D_1 J' = \Delta D_1 J - \Delta D_1 J \).
Table 2. Free-energy and entropy differences for binding of tacrolimus and rapamycin to wt and mutant FKBP-12 at 25°C

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\Delta_{1}G^{\circ}$</th>
<th>$\Delta_{p}G^{\circ}$</th>
<th>$\Delta_{1}S^{\circ}$</th>
<th>$\Delta_{p}S^{\circ}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kJ/mol</td>
<td>kJ/(mol K)</td>
<td>kJ/mol</td>
<td>kJ/(mol K)</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>2.5$^t$</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>3.3$^t$</td>
<td>14.7$^t$</td>
<td>--</td>
<td>54$^t$</td>
</tr>
<tr>
<td></td>
<td>5.4$^t$</td>
<td>--</td>
<td>-120$^t$</td>
<td>61$^t$</td>
</tr>
</tbody>
</table>

Differences thermodynamic parameters are defined in the legend to Table 1. The free energy of rapamycin binding to Y82F in D$_2$O was obtained by calorimetric titration at 25°C ($\Delta_{p}G^{\circ} (25) = -32.6$ kJ/mol). The free energy of binding of rapamycin to wt FKBP-12 in D$_2$O was determined by fluorometric titration at 35°C ($\Delta_{p}G^{\circ} (35) = -46.9$ ± 0.9 kJ/mol) and corrected to 25°C by the Gibbs–Helmholtz equation using the enthalpy and heat-capacity changes reported in Table 1, giving $\Delta_{p}G^{\circ} (25) = -47.3$ kJ/mol. The association constant was too high to accurately determine the free energy of rapamycin binding to wt FKBP-12 at a lower temperature. Similarly, the large association constants of tacrolimus and rapamycin binding to wt FKBP-12 in H$_2$O prevented their determination by the fluorescence or calorimetric methods. However, enzyme inhibition constants at 15°C and pH = 8.0 for tacrolimus and rapamycin binding to wt and Y82F in H$_2$O have been measured in H$_2$O (22). Since the binding constants exhibit no appreciable pH dependence, we used the reported inhibition constants and the enthalpy and heat-capacity changes in Table 1 to estimate free-energy changes of binding at 25°C through the Gibbs–Helmholtz equation. The resulting values were $-49.8$ kJ/mol and $-49.0$ for rapamycin and tacrolimus binding to Y82F in H$_2$O, respectively; and $-53.1$ kJ/mol and $-51.1$ kJ/mol for rapamycin and tacrolimus binding to wt FKBP-12 in H$_2$O, respectively. We also were able to determine the free energy of rapamycin binding to Y82F in H$_2$O by calorimetric titration to be $-47.7$ kJ/mol at 25°C, consistent with the extrapolated value obtained from the inhibition constant. Determination of the binding constant of tacrolimus to Y82F in D$_2$O was performed by both calorimetric and fluorometric titrations. The calorimetric experiment allows one to determine the free energy of binding and the enthalpy of binding at a single temperature; the fluorometric titrations provide measures of the binding constants at three different temperatures and a measure of the enthalpy of binding through the van’t Hoff relation. Global analysis of the four data sets yielded $\Delta_{p}H^{\circ} = -69.9$ kJ/mol and $\Delta_{p}S^{\circ} = -39.9$ kJ/mol, where the asterisk indicates 25°C.

$^t$Thermodynamic difference parameters corresponding to the free-energy changes calculated from temperature-corrected enzyme inhibition data (22).

$^t$Thermodynamic difference parameters corresponding to the free-energy changes obtained from fluorometric and/or calorimetric measurements reported here.

1. Since a protein–ligand hydrogen bond is effectively abolished upon replacement of Tyr-82 by phenylalanine, one immediately wonders why the binding to the mutant protein is enthalpically stabilized rather than destabilized. A first clue to understanding the observation came through an examination of the structure of the unliganded wt protein. Two well-ordered water molecules, hydrogen-bonded to the tyrosine hydroxyl group, were present in the structure (Fig. 1). This led us to develop a thermodynamic argument to more clearly define the structural basis for the enthalpic stabilization of binding effected by the replacement of Tyr-82 by phenylalanine.

For the purpose of interpreting the observed differences in the thermodynamic binding properties of Y82F relative to wt FKBP-12, it is convenient to divide the quantities, defined on the right hand side of Eqs. 1 and 2 into component terms (25). The quantity represented by $\Delta_{1}J_M'$ in Eq. 1 corresponds to the hypothetical process of replacing Tyr-82 by phenylalanine in the unliganded form of the protein in H$_2$O; the quantity represented by $\Delta_{1}J_M^\alpha$ corresponds to the process of replacing Tyr-82 by phenylalanine in the liganded form of the protein. From a thermodynamic viewpoint, the process of changing tyrosine to phenylalanine in the liganded structure may be considered to involve three component processes (we indicate the name and symbol for the thermodynamic parameter corresponding to these component processes parenthetically following their description): (i) formation of a cavity at the protein–ligand interface corresponding to the size of an oxygen atom and any conformational change of the protein, global or local, that accompanies the replacement of Tyr-82 by phenylalanine in the unliganded protein (reorganization term: $\Delta_{1}J_R$); (ii) removal of interactions between the tyrosyl hydroxyl group and the two water molecules that are bound to it in the unliganded wt conformation (water interaction term: $\Delta_{1}J_I$); and (iii) breaking of the tyrosyl hydroxyl oxygen–hydrogen and oxygen–carbon bonds and formation of the carbon–hydrogen bond to convert residue 82 to phenylalanine (covalent bond term: $\Delta_{1}J_C$). Similarly, the process of changing tyrosine to phenylalanine in the liganded structure may be considered to involve three steps: (i) the change in conformation of the wt protein complex that occurs upon mutation (this includes both protein and ligand and is termed the complex reorganization term: $\Delta_{1}J_{R,M}$); (ii) the removal of interactions of the tyrosyl hydroxyl group in the wt complex (ligand interaction term: $\Delta_{1}J_{I,M}$); and (iii) breakage and formation of the covalent chemical bonds to convert residue 82 to phenylalanine (covalent bond term: $\Delta_{1}J_C$). Therefore, for reactions in H$_2$O, we may write

$$\Delta_{1}J_M' = \Delta_{1}J_R + \Delta_{1}J_I + \Delta_{1}J_C$$

and

$$\Delta_{1}J_M^\alpha = \Delta_{1}J_{R,M} + \Delta_{1}J_{I,M} + \Delta_{1}J_C.$$
\[ \Delta_0 \Delta_0 H \] (27). Having stated our assumptions, we find the following expression to follow from Eqs. 1 and 3a:

\[
\Delta_L \Delta_L H = \Delta_L H_{i,M} - \Delta_L H_{i,\text{MX}}, \quad [4]
\]

so that the observed difference in enthalpy of binding of wt and mutant proteins is due to the difference between the enthalpy of interaction of the tyrosyl hydroxyl group with the water molecules in the unliganded state and the enthalpy of interaction of the tyrosyl hydroxyl group in the protein–ligand complex. Although structures of protein in D\(_2\)O have not been determined, the corresponding expression would apply, \( \Delta_0 \Delta_0 H = \Delta_0 H_{i,\text{MX}} - \Delta_0 H_{i,M} \), provided that the assumptions stated above are valid for structures in D\(_2\)O.

Breaking a protein–ligand hydrogen bond interaction in the tacrolimus–FKBP-12 or rapamycin–FKBP-12 complex is an unfavorable energetic process (i.e., turning on the interaction is exothermic, \( \Delta_L H_{i,\text{MX}} < 0 \)). For the observed \( \Delta_1 \Delta_1 H \) to be less than zero, the enthalpy of interaction of the tyrosyl hydroxyl group with the two water molecules (\( \Delta_L H_{i,M} \)) must also be less than zero and of greater magnitude than the enthalpy of interaction of the tyrosyl hydroxyl group with the ligand amide carbonyl oxygen (\( \Delta_L H_{i,\text{MX}} \)). To summarize mathematically, we write: \( 0 > \Delta_L H_{i,\text{MX}} > \Delta_L H_{i,M} \). The net effect we observe is that the overall formation of the hydrogen bond between the tyrosyl hydroxyl group and amide carbonyl oxygen is an endothermic process (\( -\Delta_L \Delta_1 H > 0 \)). Thus, for tacrolimus or rapamycin binding, the data indicate that the water interaction term (solvation term) is far greater in magnitude than the ligand interaction energy in the complex.

\(^{\dagger}\)A crude estimate of \( \Delta_L H_{i,M} \) comes from the difference in the enthalpies of hydration (in H\(_2\)O) of benzene relative to phenol, 25 kJ/mol. Also, the heat capacity change for the hydration of a hydroxyl group, based on experimental data for hydroxyl-substituted aromatic and heterocyclic rings in H\(_2\)O, is \(-32.2 \text{ J/(mol} \cdot \text{K}) \).
at 25°C. This may result from a difference in the actual number and/or differences in the relative strengths of the hydrogen bonds that are exchanged in the overall formation of the ligand-C==O -·HO-Tyr-82 hydrogen bonds. One should bear in mind that the formation of the C==O -·HO hydrogen bond occurs at the expense of breaking maximally of three hydrogen bonds from the two water molecules (A and B in Fig. 1) as well as breaking any hydrogen bonds formed between water molecules and the amide carbonyl-oxygen atom of the ligand. It is important to point out that the water-interaction enthalpy and the ligand-interaction enthalpy terms in Eq. 4 each reflect van der Waals interactions in addition to the electrostatic portion of the hydrogen-bond interactions involving the tyrosine hydroxyl group.

**Entropy and Free-Energy Difference Properties.** From measurements of the free energies of interaction for rapamycin and tacrolimus, we have been able to calculate the respective entropies of interaction (Table 2). The entropic differences in binding of rapamycin and tacrolimus to Y82F relative to wt protein are all negative, indicating that the overall effect of the mutation is entropically destabilizing and that the formations of the ligand-C==O -·HO-Tyr-82 and ligand-C==O -·DO-Tyr-82 hydrogen bonds in the tacrolimus and rapamycin wt complexes are entropically favorable processes. The more favorable entropy of ligand binding to wt FKBP-12 may be due in part to the dissociation of the two water molecules (bound to the Tyr-82 hydroxyl group of unliganded FKBP-12) upon binding of the macrocycles to the wt protein. These waters are not involved in the binding reaction of Y82F, and hence the overall entropy changes of binding to the mutant are less favorable.

The formation of the protein–ligand hydrogen bond is enthalpically unfavorable but entropically favorable. The net consequence for the free energy of binding at 25°C upon replacement of tyrosine by phenylalanine is a small destabilization of rapamycin and tacrolimus binding in H2O. The free-energy differences of ligand binding to Y82F relative to wt FKBP-12, reported in Table 2 (ΔΔG), are similar to what has been observed for the destabilization of proteins by mutations that disrupt hydrogen-bonded residues in the folded states of proteins (29).

**Enthalpy of Hydrogen-Bond Formation in Aqueous Solution.** The results have immediate implications for examining the validity of models that predict the energetics of protein folding and ligand binding. Murphy and Gill (7), arguing on the basis of a comparison of the thermodynamics of protein folding and the dissolusion of solid cyclic dipeptides, find that proteins are stabilized largely through the exothermic enthalpy of formation of amide hydrogen bonds at 25°C and that the enthalpy of formation of nonpolar contacts is unfavorable. Yang et al. (8) present an alternative analysis to account for the stability of proteins, arguing that the formation of nonpolar interactions is essential for protein stabilization. A central feature of their analysis is the indication that the dehydration of polar groups upon protein folding carries a large enthalpic penalty. This is consistent with the analysis of Ben-Naim (13), suggesting that the overall formation of hydrogen bonds in protein folding or ligand binding may be unfavorable, owing to the unfavorable enthalpy of desolvation. A recent analysis of the thermodynamics of hydration in protein folding by Makhatadze and Privalov (30) further supports the hypothesis of an enthalpic destabilization because of the removal of polar groups from water.

The observations reported here provide a direct examination of the thermodynamics of formation of single protein–ligand hydrogen bonds that specifically involves the structurally observable dehydration of the hydrogen-bond donor and acceptor atoms. Our analysis supports the general view that the desolvation of uncharged polar groups of proteins can be a highly unfavorable enthalpic process at 25°C. However, one must bear in mind that two water molecules were dissociated from the tyrosyl hydroxyl group upon formation of the specific hydrogen bond investigated here. In seeking a general response to the question “What are the signs and magnitudes of the enthalpies of hydrogen bonds formed in reactions involving proteins?,” it is clear that one must consider the donor and acceptor atoms of each hydrogen bond, in the context of the specific environments of the protein, ligand, and solvent in which they reside in both the reactants and products. If the hydrogen-bond donor and acceptor atoms are removed from contact with water upon hydrogen-bond formation, one can expect an unfavorable enthalpic component due to the dissociation of water from the ligand or protein or both.

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