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Heat capacity changes and hydrophobic interactions in the binding of FK506 and rapamycin to the FK506 binding protein

(immunosuppression/calorimetry/accessible surface/entropy)

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ABSTRACT **Differential interactions among nonpolar moieties at protein/ligand interfaces, and of these nonpolar groups with water, collectively termed hydrophobic interactions, are widely believed to make important energetic contributions to the stability of protein/ligand complexes. Quantitative estimates of hydrophobic interactions, 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. Two largely nonpolar, immunosuppressive agents, FK506 and rapamycin, each bind with high affinity to a common hydrophobic pocket on a small peptidylproline cis–trans isomerase known as FK506 binding protein (FKBP-12) and inhibit its activity. In an effort to elucidate the structural features of these ligands responsible for the observed energetics, we have undertaken an investigation of the thermodynamics of binding of FK506 and rapamycin to FKBP-12. Enthalpies of binding have been determined by high-precision titration calorimetry over a range of temperature, allowing estimates of heat capacity changes. By analyzing the distribution of changes in solvent-accessible surface area upon binding of FK506 to FKBP-12 from crystallographic data, it is found that 99% of the net surface buried upon binding involves nonpolar groups. This leads to a heat capacity change of FK506 binding, normalized to the amount of nonpolar surface, of -0.40 ± 0.02 cal·K^{-1}·mol^{-1}·Å^{-2} (1 cal = 4.18 J), a value similar to that obtained for the aqueous dissolution of hydrophobic substances. Our observations are discussed in view of the general nature of hydrophobic interaction processes.**

FK506 and rapamycin are potent immunosuppressive agents that appear to have great potential in preventing organ transplant rejection and also have some promise in treating various autoimmune disorders (1). Although the precise mode of their action is largely unknown, they have been shown to inhibit *different* biochemical signaling pathways of the immune network operating in T lymphocytes (2). Curiously, however, they both bind with high affinity to a common, cytosolic protein ($M_r \approx 12,000$) known as FK506 binding protein (FKBP-12). Recently, the structures of FKBP-12 in solution (3–5) and in the crystalline state (M. M. Yamashita, M. A. Murko, S. H. Rotstein, J. Boger, J. M. Moore, J. A. Thomson, M. J. Fitzgibbon, and M. A. Navia, personal communication) have been determined as have the crystal structures of the FK506/FKBP-12 and rapamycin/FKBP-12 complexes (6, 7). The structural information reveals that FK506 and rapamycin bind in a shallow cavity lined with a large number of hydrophobic amino acid residues. A significant feature of the binding process, inferred from the structures, is that a large fraction of the nonpolar groups of the ligands and of the protein binding site are removed from contact with solvent and become buried in the interior of the protein/ligand complexes upon binding. This observation suggests that hydrophobic interactions may play a key role in determining the stability of these protein/ligand complexes.

Hydrophobic interactions, believed to be important determinants of the stability of many protein structures, multisubunit protein complexes, and protein/ligand complexes, may be considered to be the result of two component interaction processes: the removal of nonpolar groups from water (*hydration*) and the *packing* of these groups within proteins or protein complexes. Early studies by Kauzmann (8) and Tanford (9) emphasized the similarities of protein unfolding and the transfer of nonpolar substances from water to nonpolar phases. More recently, striking correlations between the thermodynamics of protein folding and the aqueous dissolution of hydrophobic substances have been noted by Sturtevant (10), Baldwin (11), and Murphy *et al.* (12). Among the most intriguing of the observations are the linear relationships observed between (*i*) enthalpy and heat capacity changes and (*ii*) entropy and heat capacity changes, for solutes within a given class (gaseous, solid, or liquid hydrophobic compound or protein). Consideration of these relationships has led to differing theories regarding the contribution of hydrophobic interactions to the stabilization of protein structures (11, 13, 14). A key common feature of the theories is the dissection of the free energy of unfolding into its various contributions, with the heat capacity playing a central role in determining the free energy of hydrophobic interaction.

An early analysis by Sturtevant (10) indicated that heat capacity changes for protein unfolding are determined largely by the exposure of nonpolar groups to water but that one must also consider contributions due to the changes in the numbers of easily excitable vibrational modes upon unfolding. The concept of solvent-accessible surface area introduced by Lee and Richards (15) provided an important structural parameter with which to describe the interaction of water with proteins. Recently, several reports have focused on the relationship between heat capacity changes and changes in solvent-accessible surface areas accompanying protein unfolding. Spolar *et al.* (16) and Livingstone *et al.* (17) analyzed the heat capacity changes for the unfolding of proteins for which high-resolution x-ray crystallographic information was available. Their analyses indicated that the heat capacity change for unfolding proteins is proportional to the difference in solvent-accessible nonpolar surface area between native and denatured states. Another investigation on the binding of various S peptides to S protein casts doubt on the generalization of the relationship between nonpolar surface and heat capacity (18, 19).

To clarify the structural determinants for heat capacities and their relationship to the free energy of hydrophobic interaction, it is important to obtain high-precision measurements of heat capacity changes, in addition to other thermo-

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dynamic properties, for reactions in which there is high-resolution structural information available for *both* the reactants and the products. Additionally, probing the nature and magnitudes of hydrophobic interactions is fundamental to the design of molecules that bind to specific biological target proteins and inhibit their function in disease processes. Several laboratories have focused on the design of compounds that bind to FKBP-12, and some have proceeded to obtain crystallographic and solution structural information for the protein/ligand complexes (refs. 6 and 7; Yamashita *et al.*, personal communication). Recently, we have discussed the relationship between the thermodynamics of immunosuppressant binding and other processes in which nonpolar groups are removed from water (20). In this study, we provide a detailed report on the thermodynamics of binding of FK506 and rapamycin to FKBP-12.

MATERIALS AND METHODS

Materials. FKBP-12 used in these studies was isolated from bovine thymus by successive size fractionation and hydrophobic interaction chromatography. These methods require no thermal precipitation procedures to remove contaminating proteins. Details of the purification procedure will be presented elsewhere (J.A.T., M. J. Fitzgibbon, J. R. Black, and J. Boger, unpublished).

Titration Calorimetry. Enthalpies were determined by titrating 25–40 μM FK506 or rapamycin with 1–3 mM FKBP-12 in an Omega titration calorimeter (MicroCal, Northhampton, MA) (21). Rapamycin and FK506 solutions were made by addition of 5 μl of drug stock solutions in dimethyl sulfoxide to 3 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 multiple single injections. Injection volumes were 5 μl 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.

Surface Area Calculations. The solvent-accessible cavity surface areas of bovine FKBP-12, FK506, and the human FKBP-12/FK506 complex have been calculated with a C program written by Scott R. Presnell (University of California, San Diego) that uses the Lee and Richards algorithm (15). A slice width of 0.01 Å was adopted and all calculations were performed on a Silicon Graphics 480 computer. The atomic radii used were essentially those derived from the packing observed between nonhydrogen atoms in amino acid crystals (22): nitrogen, 1.65 Å; oxygen, 1.4 Å; sulfur, 1.85 Å; α and β carbons, 1.87 Å; carbonyl carbons, 1.76 Å; and all other carbons, 1.83 Å. The water probe sphere was taken as 1.4 Å. Coordinates of the cis and trans configurations of FK506 were taken from solution NMR structures kindly provided by H. Kessler (Institut für Organische Chemie, Technische Universität, Munich). The coordinates of the FKBP-12/FK506 complex were obtained from the Brookhaven Protein Data Bank, and the crystallographically determined coordinates of uncomplexed FKBP-12 were obtained from M. Yamashita (personal communication).

RESULTS AND DISCUSSION

Titration calorimetric experiments were performed under two sets of solution conditions. These conditions represent the native state solution conditions under which the enzyme inhibition constants are measured and also under conditions for which we have characterized the thermal stability of the FKBP-12. The buffers employed (potassium phosphate and sodium acetate) have low enthalpies of ionization, so that the enthalpies of binding reported do not reflect any contributions due to buffer protonation. The enthalpies of reactions with FK506 and rapamycin depend strongly on temperature, indicating the presence of large negative heat capacity changes accompanying binding. The temperature dependence of the enthalpy was described well, in all cases, by a linear regression model assuming a constant heat capacity change in the temperature range of the experiments (Table 1).

Heat Capacity Changes and Solvent-Accessible Surface Area. Edsall (23) was among those who first drew attention to the large changes in the constant pressure heat capacity that accompanied the interaction of nonpolar groups with water. The heat capacity changes for the aqueous dissolution of a number of nonpolar gaseous and liquid substances have since been shown to be proportional to their solvent-accessible surface areas (24, 25). This observation has been taken to indicate that the heat capacity increment observed in these processes is determined entirely by the hydration of these substances, and suggested a possible origin of the large heat capacity changes observed in the unfolding of proteins. Using the heat capacity data compiled by Privalov and Gill (25) and the changes in water-accessible surface area given by Livingstone *et al.* (17), one calculates a value of $\Delta C_p = 0.41 \pm 0.02$ cal·K^{-1}·mol^{-1}·Å^{-2} for the dissolution of five nonpolar gases (cyclopropane, propane, isobutane, *n*-butane, and 1-butene). Similarly, for liquid hydrocarbon dissolution, one obtains a

Table 1. Enthalpies of reaction of FKBP-12 with FK506 and rapamycin determined by isothermal titration calorimetry

50 mM potassium phosphate, pH 7.00				50 mM NaCl/50 mM sodium acetate, pH 4.01			
FK506		Rapamycin		FK506		Rapamycin	
t, °C	ΔH	t, °C	ΔH	t, °C	ΔH	t, °C	ΔH
5.3	−9.2 ± 0.2	5.3	−10.0 ± 0.3	5.6	−5.3 ± 0.2	5.2	−7.6 ± 0.1
9.8	−10.9 ± 0.1	11.3	−12.5 ± 0.1	9.7	−8.0 ± 0.2	10.3	−9.5 ± 0.1
12.1	−11.4 ± 0.2	15.3	−13.7 ± 0.2	14.3	−8.7 ± 0.1	15.2	−12.8 ± 0.5
15.7	−12.2 ± 0.2	19.9	−14.8 ± 0.3	21.1	−9.7 ± 0.5	19.8	−14.6 ± 0.5
19.6	−13.4 ± 0.1	24.1	−16.1 ± 0.4	25.4	−11.1 ± 0.1	24.9	−15.0 ± 0.2
24.1	−14.2 ± 0.1	24.6	−16.4 ± 0.2				
29.6	−15.7 ± 0.2	29.8	−17.4 ± 0.3				

Enthalpies (ΔH) were averaged from three to five individual determinations and are reported in kcal·mol^{-1} (1 cal = 4.18 J). Errors are given as standard deviations. Each of the four data sets was fit with a linear regression model assuming a constant heat capacity change (ΔC_p) over the experimental temperature range and a reference enthalpy at 15°C of ΔH(15°C). The fitting function employed was $\Delta H = \Delta H(15°C) + \Delta C_p(t - 15°C)$. For FK506, ΔH(15°C) = −12.0 ± 0.1 kcal·mol^{-1} and ΔC_p = −0.26 ± 0.01 kcal·K^{-1}·mol^{-1} at pH 7; at pH 4, ΔH(15°C) = −8.5 ± 0.3 kcal·mol^{-1} and ΔC_p = 0.26 ± 0.05 kcal·K^{-1}·mol^{-1}. For rapamycin, ΔH(15°C) = −13.3 ± 0.1 kcal·mol^{-1} and ΔC_p = −0.30 ± 0.01 kcal·K^{-1}·mol^{-1} at pH 7; at pH 4, ΔH(15°C) = −11.9 ± 0.4 kcal·mol^{-1} and ΔC_p = 0.41 ± 0.06 kcal·K^{-1}·mol^{-1}.

value of $\Delta C_p = 0.34 \pm 0.09$ cal·K^{-1}·mol^{-1}·Å^{-2} when considering cyclohexane, *n*-pentane, *n*-hexane, benzene, tolulene, ethylbenzene, and propylbenzene. However, the aromatics in this series give systematically lower normalized heat capacities than the aliphatics, and there is a trend within the aromatic hydrocarbons indicating a lower area-normalized heat capacity for the aromatic ring. (For benzene, $\Delta C_p = 0.25$ cal·K^{-1}·mol^{-1}·Å^{-2} compared to an average of the three alkanes of 0.37 ± 0.01 cal·K^{-1}·mol^{-1}·Å^{-2}.) This compares favorably with the hydration heat capacities for aromatic and aliphatic portions of amino acids derived from partial molar heat capacity measurements of amino acids in water: 0.29 ± 0.06 cal·K^{-1}·mol^{-1}·Å^{-2} for aromatic portions, 0.5 ± 0.1 cal·K^{-1}·mol^{-1}·Å^{-2} for aliphatic portions (26). From measurements on the dissolution of solid cyclic dipeptides (13), one estimates a value of $\Delta C_p = 0.44 \pm 0.02$ cal·K^{-1}·mol^{-1}·Å^{-2} for the nonaromatic nonpolar portions of the molecules; the aromatic groups of this series again give a smaller value (K. Murphy, personal communication).

Averaging of the area-normalized heat capacities for the transfer of nonaromatic nonpolar surface from various initial phases, weighted by the respective errors of the measurements, yields 0.40 ± 0.02 cal·K^{-1}·mol^{-1}·Å^{-2}. The area-normalized heat capacity for aromatic portions is taken as 0.27 cal·K^{-1}·mol^{-1}·Å^{-2} by averaging the value for benzene and the value reported for aromatic portions of amino acids. These averages can be used to predict the heat capacity change for the binding of ligands to proteins due to the change in the amount of nonpolar surface area:

$$\Delta C_{p,\,\mathrm{nonpolar}} = 0.27\ \Delta A_{\mathrm{aromatic}} + 0.40\ \Delta A_{\mathrm{nonaromatic}}, \quad [1]$$

where the ΔA values indicate the amount of surface area exposed upon binding. From the data listed in the legend to Fig. 1 one can calculate that 650 Å^2 of nonpolar surface is buried upon binding of FK506 to FKBP-12 (558 Å^2 are contributed from nonaromatic groups and 92 Å^2 are contributed from aromatic groups). This leads to an estimate of the heat capacity change due to the burial of nonpolar groups of -248 cal·K^{-1}·mol^{-1}. Comparison with the measured total heat capacity change of -260 ± 10 cal·K^{-1}·mol^{-1} indicates that the heat capacity change can be accounted for simply on the basis of the change in solvent exposure of nonpolar groups in FK506 and FKBP-12.

This conclusion is somewhat surprising since there are clearly other factors that could contribute to the heat capacity function. Changes in the number of easily excitable internal vibrational modes upon ligand binding are also thought to contribute to the heat capacity changes (10). Since binding would be expected to decrease the overall flexibility of the protein and ligand structures, one would anticipate that any vibrational effects would further decrease the heat capacity change. On the other hand, binding interactions that involve polar groups could contribute to a more positive heat capacity change. There are five hydrogen bonds formed upon the association of FK506 and FKBP-12. Although few heat capacity data exist for the formation of hydrogen bonds in aqueous solution, the available data indicate that there is a *positive* heat capacity for their formation. Heat capacity measurements for the dissolution of cyclic dipeptides indicate a value of 14.3 cal·K^{-1}·mol^{-1} for the formation of amide hydrogen bonds and a value of 10.8 cal·K^{-1}·mol^{-1} for the formation of hydrogen bonds involving a hydroxyl group (13). Privalov and Makhatadze (26) report values for the heat capacity of dehydration of the polar portions of amino acids in the range 0–0.9 cal·K^{-1}·mol^{-1}·Å^{-2} at 25°C.

Although a significant number of polar groups become buried upon FK506 binding to FKBP-12, the net change in exposure of polar surface area is close to zero. This suggests a reason why there is no apparent polar contribution to the heat capacity change. To investigate the distribution of changes in surface area upon binding of FK506 to FKBP-12, we analyzed the difference in total surface area between the free and complexed protein, residue by residue. The resulting map, or spectrum, of area changes is given in Fig. 1. The negative peaks in the spectrum correspond to residues that become buried upon binding. For example, His-87 interacts with the pyranose methyl group region of FK506, and Phe-46 is a highly conserved residue in the binding pocket. The largest negative peaks occur at residues Asp-37, Glu-54, Ile-56, and Tyr-82. These residues participate in four of the five hydrogen bonding interactions between drug and protein. The fifth residue that is involved in a hydrogen bond with FK506 (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 FK506, is mediated by a water molecule. There are a number of polar amino acid residues, not in the FK506 binding site, that become exposed upon binding (Glu-5, Lys-34, Asn-43, Tyr-80, and Thr-80). This is intriguing in view of the recent report that the FK506/FKBP complex (and not uncomplexed FKBP or FK506) binds to calcineurin and inhibits its phosphatase activity, suggesting a possible step in the mechanism of FK-506's immunosuppressive effect (27). Determination of whether these exposed residues play a role in the interaction of the FKBP/FK506 complex with calcineurin and/or other proteins that may be involved in immunosuppression awaits further investigation.

The FKBP-12/rapamycin complex contains a high degree of structural similarity to the FKBP-12/FK506 complex. Two important differences in the heat capacity changes for the two reactions are evident from the data in Table 1. First, a larger heat capacity reduction is observed for rapamycin binding than for FK506 binding. Second, the heat capacity for

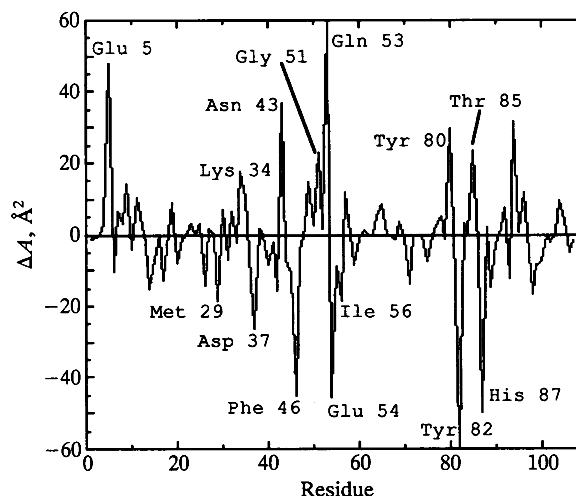

FIG. 1. Difference accessible surface area spectrum revealing the distribution of changes in solvent-accessible surface area per residue upon binding of FK506 to FKBP-12. Solvent-accessible surface areas were computed for polar (A_p) and nonpolar (A_{np}) atoms in the following structures: (*i*) FK506 (cis); A_p = 164 Å^2, A_{np} = 811 Å^2; (*ii*) FK506 (trans); A_p = 131 Å^2, A_{np} = 843 Å^2; (*iii*) FKBP-12 (bovine); A_p = 2393 Å^2, A_{np} = 3193 Å^2; (*iv*) FKBP-12/FK506 complex (human); A_p = 2534 Å^2, A_{np} = 3413 Å^2. Human recombinant FKBP-12 and bovine FKBP-12 differ by three amino acids: Val-49 → Met, Asn-94 → His, and Ile-98 → Val for bovine → human. Considering the accessible surface of FK506 to be an average of the values for cis and trans structures, and subtracting the area changes due to the three amino acids that differ between bovine and human FKBP-12 (43 Å^2 nonpolar and −14 Å^2 polar), leads to the following changes in accessible surface area on binding: ΔA_p = 7 Å^2 and ΔA_{np} = −650 Å^2. The plotted area is the total change: $\Delta A = \Delta A_{np} + \Delta A_p$.

rapamycin binding assumes a different value depending on the pH at which data were collected. Exploration of these effects awaits an analysis of the recently solved rapamycin/FKBP-12 structure (7).‡

Thermodynamics of Hydrophobic Interaction. It is important to emphasize that hydrophobic interactions in the binding of FK-506 or rapamycin to FKBP-12 involve (*i*) the removal of nonpolar groups from water (dehydration) and (*ii*) the packing of the nonpolar groups in the complex. As stated above, the observed heat capacity changes for the transfer of nonpolar groups from water to their pure phases (solid, liquid, or gases), or into protein interiors, primarily reflect the desolvation step. The correlation of heat capacity of FK506 binding with the amount of buried nonpolar surface supports the notion that the heat capacity reflects only solvation changes. Applying the above model of the heat capacity change for FK-506 binding, embodied by Eq. **1**, does not commit one to using either solid or liquid hydrophobic substances as a reference state, when modeling hydrophobic interactions as measured by the other thermodynamic functions. Free energies, enthalpies, and entropies of hydrophobic interaction may contain significant contributions due to packing. Arguments have been proposed for using data on the dissolution of solid or liquid hydrophobic substances in developing models for the thermodynamics of hydrophobic effects in the folding of proteins. It remains controversial as to which model is more appropriate.

The free energy change for a binding process is given by the difference between enthalpic and entropic terms as usual, $\Delta G^o = \Delta H - T\Delta S^o$. In the case that the heat capacity is a constant, the enthalpy and entropy changes can be written as linear functions of the heat capacity change as follows,

$$\Delta H = \Delta H^* + \Delta C_p(T - T_a^*) \qquad [2]$$

$$\Delta S^o = \Delta S^* + \Delta C_p \ln(T/T_b^*), \qquad [3]$$

where ΔH^* is the enthalpy change evaluated at an arbitrary reference temperature T_a^*, and ΔS^* is the entropy change evaluated at a different (in general) arbitrary reference temperature T_b^*. This allows one to express the free energy in terms of the heat capacity as

$$\Delta G^o = \Delta H^* - T\Delta S^* + \Delta C_p[(T - T_a^*) - T\ln(T/T_b^*)]. \qquad [4]$$

Free energy changes for the binding of FK506 and rapamycin may be evaluated from the values for their enzymatic inhibition constants: $\Delta G^o = -12.3$ kcal·mol^{-1} for FK506 and $\Delta G^o = -12.7$ kcal·mol^{-1} for rapamycin at 25°C (20). These free energy values, taken together with the enthalpy values reported in Table 1 at 25°C (pH 7), allow for the calculation of the entropies of binding: -7.7 cal·K^{-1}·mol^{-1} and -11.7 cal·K^{-1}·mol^{-1} for FK506 and rapamycin, respectively.

Linear relationships between the entropy and the heat capacity changes for protein unfolding and the dissolution of hydrophobic substances have been recently discussed (11–14). A particularly searching analysis of these relationships has been given by Lee (14). Curiously, for all of these processes, plots of the entropy changes versus heat capacity changes at 25°C give nearly identical slopes (12). The values of the slopes for protein unfolding, and for the dissolution of liquid, gaseous, and solid model compounds, are -0.25, -0.24, -0.28, and -0.23, respectively (12). From Eq. **3** the slopes of such plots may be identified with the term $\ln[T/T_b^*]$, with $T_b^* \approx 385$ K. This is the same temperature at which the entropy of transfer of nonpolar liquids to water vanishes and has led to an expression for the entropy of hydration of nonpolar groups in proteins: $\Delta S_{hydration} = \Delta C_{p,\ nonpolar}\ln[T/385]$ (19, 25). For FK506 binding, if one assumes that the hydration entropy accounts for the temperature-dependent component in Eq. **3**, then with $\Delta S^o = -7.7$ cal·K^{-1}·mol^{-1} and $\Delta C_p = -260$ cal·K^{-1}·mol^{-1} (measured for FK506 binding), we calculate $\Delta S^* = -74$ cal·K^{-1}·mol^{-1}. A large part of ΔS^* can be accounted for by the total loss of entropy due to changes in translation and overall rotations that accompany the association of a binary complex. This has been estimated to be approximately -50 cal·K^{-1}·mol^{-1} (28, 29).

The enthalpy changes for the binding of FK506 to FKBP may be considered to arise from hydrophobic interactions and hydrogen bond interactions, $\Delta H = \Delta H_{hydrophobic} + \Delta H_{H\ bond}$. For the present discussion, interactions of nonpolar groups and hydrogen bonding groups, with the solvent and within the protein/ligand complexes, are included in these terms. A model introduced by Baldwin (11) for calculating the enthalpy of hydrophobic interactions is based on the enthalpy of transfer of liquid hydrocarbons into water. It may be expressed in the same form as Eq. **2** with $T_a = 295$ K and $\Delta H^* = 0$, so that the hydrophobic contribution to binding is written as $\Delta H_{hyd} = \Delta C_{p,\ nonpolar}[T - 295]$. At 25°C this hydrophobic enthalpy term is 0.8 kcal·mol^{-1} for FK506 and 0.9 kcal·mol^{-1} for rapamycin. In comparison, the observed total enthalpy values are -14.7 kcal·mol^{-1} and -16.3 kcal·mol^{-1}, respectively. An analysis based on the liquid model leads one to conclude that the enthalpies of binding of these compounds to FKBP-12 at this temperature are due almost entirely to the formation of hydrogen bonds. Another model for hydrophobic interactions formulated by Murphy *et al.* (12) is based on the dissolutions of solid hydrophobic substances into water. They argue that the contribution to the enthalpy from the transfer of nonpolar groups to the interior of proteins is equal to $\Delta C_{p,\ nonpolar}(T - 375)$. Applied to the binding situation discussed here, this term would lead to a large positive enthalpy of binding due to nonpolar groups at 25°C. Again, one is led to conclude, in the case of FK506 and rapamycin binding, that the observed negative enthalpies of binding near 25°C are primarily due to hydrogen bond formation rather than hydrophobic interactions.

The quantitative nature of hydrophobic interaction energetics in processes involving proteins is currently a matter of some controversy. However, it has been widely recognized that the one thermodynamic quantity associated with their presence in a reaction process is the heat capacity change. The existence of high-resolution structural information on uncomplexed and complexed FKBP-12 and its ligands and the availability of high-precision calorimetric instrumentation for the determination of enthalpies and heat capacities have allowed us to explore the structural determinants of heat capacity changes in the binding reactions of FKBP-12. In particular, for FK506 binding, the heat capacity change is accounted for by the change in nonpolar surface exposure. Analysis of the other thermodynamic functions for the binding process suggest that (*i*) hydrogen bonds rather than hydrophobic interactions provide enthalpic stabilization to the complexes at 25°C and (*ii*) the entropy gain due to the removal of water from nonpolar surfaces, upon binding of FK506 to FKBP-12, provides an important source of binding energy opposing the loss of translational and rotational entropy.

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‡During the process of review of this manuscript, coordinates for the complex of FKBP-12 with rapamycin became available from the Brookhaven Protein Data Bank (7) and independently from personal communication by M. A. Navia and M. Yamashita.

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