A Direct Measure of the Contribution of Solvent Reorganization to the Enthalpy of Ligand Binding[1]

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Abstract: The thermodynamics of association of several binding systems, including protein−carbohydrate, small molecule−small molecule, protein−peptide, and protein−nucleic acid, were evaluated calorimetrically in light and heavy water. In every case, the enthalpy of binding in D_2O was decreased relative to that in H_2O: the differences range from 400 to 1800 cal mol^{-1}. A compensating change in ΔS left the free energy of binding virtually unchanged in each case. A strong correlation between the differential enthalpy of binding and ΔC_p for binding was observed, with a slope of 5 K. An analysis of the observed effect utilizing a Born−Haber thermodynamic cycle shows that the measured decrease in enthalpy represents approximately 10% of the binding enthalpy arising from solvent reorganization. For the range of systems investigated, solvent reorganization provides 25−100% of the observed enthalpy of binding.

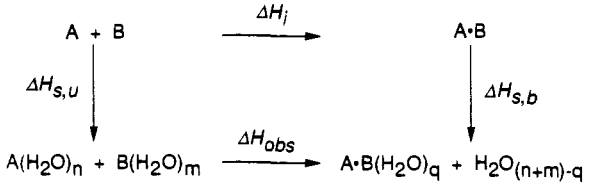
Introduction

At a molecular level, association represents the most important single event in biological macromolecular assembly. The paradigms describing binding affinities are of paramount importance in, for example, the prediction of peptide folding patterns and the creation of structural motifs with designed functionality. It is clear today that the relationships between structure and energetics in aqueous solution are too poorly understood to allow predictions of function from form. Thus, as we move to the 21st century, rational drug design is being supplanted by searches of combinatorial libraries, and protein redesign and *de novo* prediction of peptide secondary structure from primary amino acid sequences rely on empirical rather than theoretical approaches.

At the heart of the puzzle of association in aqueous solution is the debate on the relative importance of solute−solute *versus* solute−solvent interactions.[2-6] A binding event can be decomposed into solute- and solvent-associated processes and represented schematically as a familiar Born−Haber cycle (Scheme 1). During binding, some fraction of the surface of solutes A and B is removed from contact with water, and the water formerly in contact with these surfaces is returned to bulk solvent. The cycle in Scheme 1 provides a conceptual framework for separating the contribution of intrinsic, or solute−solute, interactions from solvation effects in binding phenomena. While studies on protein folding, rational drug design, and small-molecule model studies have traditionally focused on solute−solute interactions as the source of both binding enthalpy and specificity, the role of solvent reorganization in aqueous association phenomena is poorly understood.

In our studies on protein−carbohydrate association, we were struck by the similarities in the thermodynamics of virtually all binding events in aqueous solution.[7] While counter examples exist, associations in water are typified by an enthalpy of binding more negative than the overall binding energy and an unfavorable entropic component. In an attempt to better delineate the origin of the enthalpy of binding in water, we examined the effect of solvent isotopic substitution on the thermodynamics of binding. We report here the results of these studies.

Scheme 1. Born−Haber Cycle Showing Separation of Measured Enthalpy of Binding (ΔH_{obs}) into an Intrinsic (Solute−Solute) Enthalpy (ΔH_i) and Enthalpies of Solution for the Bound ($\Delta H_{s,b}$) and Unbound ($\Delta H_{s,u}$) Systems



Each thermodynamic quantity, ΔG, ΔH, ΔS, or ΔC_p, characterizing an association contains a term for intrinsic, or solute−solute, interactions and a term representing the contribution of solvent reorganization. Thus, the enthalpy of binding, ΔH, can be represented as

$$\Delta H = \Delta H_i + \Delta H_s \quad (1)$$

where ΔH_s, the enthalpy arising from solvent reorganization, includes both the loss of solute−solvent interactions and the necessary gain in solvent−solvent interactions. In terms of Scheme 1, ΔH_s will be equal to the difference between the two vertical processes, *i.e.* $\Delta H_{s,u} - \Delta H_{s,b}$.

That ΔH_s is non-zero, *i.e.* contributes to ΔH, arises from differences in the structure and energetics of water near solutes. Muller has developed a mixture formalism that succinctly describes this phenomenon for the solvation of a solute in a bath of N water molecules.[8] To the extent that binding represents a *desolvation*, Muller's enthalpy of solvation is contained in ΔH_s of eq 1. In Muller's description, the enthalpy of solvation for solution of a species in a bath of N solvent

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(1) A preliminary account of a portion of this work was presented at the 48th Annual Calorimetry Conference, Durham, NC, July 18−23, 1993.

(2) Privalov, P. L.; Gill, S. J. *Adv. Protein Chem.* **1988**, *39*, 191.

(3) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219.

(4) Dill, K. A. *Biochemistry* **1990**, *29*, 7133.

(5) Fersht, A. R. *Trends Biochem. Sci.* **1987**, *12*, 301.

(6) Ben-Naim, A. *J. Phys. Chem.* **1991**, *95*, 1437.

(7) Williams, B. A.; Chervenak, M. C.; Toone, E. J. *J. Biol. Chem.* **1992**, *267*, 22907.

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molecules arising from changes in water−water interactions, ΔH_w, is given by

$$\Delta H_w = 3N/2[(1 - f_b)\Delta H_b - (1 - f_{hs})\Delta H_{hs}] \quad (2)$$

where $3N/2$ is a statistical term related to the maximum possible number of hydrogen bonds, f_b is the fraction of broken hydrogen bonds in bulk solution, ΔH_b is the enthalpy of hydrogen bond formation in bulk solution, and f_{hs} and ΔH_{hs} are the corresponding quantities for water in a hydration shell around a solute. Muller has suggested that both f_{hs} and ΔH_{hs} are larger than the corresponding terms in bulk solution; *i.e.* while individual hydrogen bonds in hydration shells are stronger than those in bulk solution, their proximity to a non-hydrogen bonding surface results in a greater fraction of broken hydrogen bonds. This construction accounts for both the enthalpy and heat capacity change that characterize non-ionic dissolutions. Although Muller has made estimates of both f_{hs} and ΔH_{hs}, the values of both must depend critically on the precise topographical nature of the solute surface in contact with solvent.

Isotopic substitution of deuterium for protium in H_2O has differential effects on f_b *versus* f_{hs} and ΔH_b *versus* ΔH_{hs}.[9] The perturbation from solvent isotopic substitution arises from differences in the A···H *versus* A···D interaction, where A represents any hydrogen bond acceptor. The lower zero-point energy of deuterium relative to protium results in both stronger and more localized deuterium bonds. The restricted rotational motions of water, revealed as librational frequencies, arise from hydrogen bonding interactions. Raman studies of the librational frequencies of H_2O and D_2O show a difference in protium *versus* deuterium interaction energies of approximately 10%.[10] By different means, Scheraga[11] and Ben-Naim[12] both deduced that the enthalpy of an intermolecular hydrogen bond in D_2O is close to 10% greater than that in H_2O: this value now seems to be generally accepted.[9] The enhanced enthalpic interaction is offset by a nearly equal decrease in bond entropy, leading to little or no change in the free energy of the O···H *versus* O···D interaction.[13] As a result, enthalpies of solution are more negative (favorable) in D_2O than in H_2O, but equilibrium distributions between the two solvents are close to unity. Several studies on the thermodynamics of transfer of a variety of solutes from light to heavy water show that such transfers are favored enthalpically and opposed entropically, with a free energy of transfer close to zero.[14-16] Again, through the analogy of binding to a desolvation process, the ΔH_s values in Scheme 1 and eq 1 will be affected by solvent isotopic substitution.

Alternatively, the enthalpy of association from solute−solute interactions (ΔH_i) for associations in heavy *versus* light water will be unaffected by solvent isotopic substitution, providing the same number of hydrogen bonds exist in both solvents. From Scheme 1, this caveat requires that the structure of A, B, and AB be identical in H_2O and D_2O. The conclusion that ΔH_i is unaffected by isotopic substitution holds even though hydrogen bond donor sites on the solutes will exchange for deuterium in D_2O, since the increase in the enthalpy of deuterium···*solute* acceptor site interactions will be offset by an increase in the deuterium···*solvent* acceptor site interactions prior to association. This offsetting behavior is an unavoidable consequence of the nature of aqueous association: solute molecules enthalpically stabilized by interactions with solvent *exchange* these interactions with solvent for new interactions with other solutes. It occurred to us then that the critical separation of the enthalpy of binding into ΔH_i and ΔH_s could be effected by comparing the enthalpy of binding in light *versus* heavy water. *We present results here suggesting that 25−100% of the net measured enthalpy of binding is accounted for by solvent reorganization.*

Results and Discussion

Calorimetric studies in light and heavy water were carried out for the association of the lectin from *Canavalia ensiformis* (concanavalin A) to methyl α-D-mannopyranoside, methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, and methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, for the lectin from *Dioclea grandiflora* to methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside, for vancomycin to the tripeptide α,ε-diacetyl-L-lysine-D-alanine-D-alanine, and for ribonuclease A (RNAse) to 2′-cytidine monophosphate (CMP). The results of these studies are shown in Table 1. Included in Table 1 are the results of Connelly and co-workers for the association of FK506 to FK-binding protein (FKBP).[17] The systems investigated thus include protein−carbohydrate, protein−nucleotide, protein−peptide, and small-molecule−small-molecule systems. In each case, ligand and receptor were dissolved in identical buffer solutions. Deuterated samples were lyophilized and redissolved in an appropriate volume of >99% D_2O. Allowing solutions to stand for up to 1 week in D_2O to ensure complete deuteration of exchangeable protons did not alter binding enthalpies.

Calorimetric measurements were made using the Microcal Omega titration microcalorimeter: details of the instrument design and data analysis are given elsewhere.[19] To a solution of receptor (30−300 μM) was added ligand (5−20 mM) in 2 to 5 μL injections. Typically 20−40 injections, 4−10 s in duration were made with 3 min intervals between injections.

The addition of a ligand (L) to a receptor (R) to produce a complex (LR) is governed by the equilibrium expression:

$$K_{eq} = \frac{[LR]}{[L][R]} \quad (3)$$

where

$$[L]_{total} = [L] + [R] \quad (4)$$

and

$$[R]_{total} = [R] + [LR] = [LR] + [LR]/K_{eq}[L] \quad (5)$$

These expressions can be rearranged to a quadratic and solved to give the concentration of complex in terms of the known

(8) Muller, N. *Acc. Chem. Res.* **1990**, *23*, 23.
(9) Muller, N. *J. Solution Chem.* **1991**, *20*, 669.
(10) Walrafen, G. E. In *Water: A Comprehensive Treatise*; Franks, F., Ed.; Plenum: New York, 1972; Vol. 1, p 151.
(11) Némethy, G.; Scheraga, H. A. *J. Chem. Phys.* **1964**, *41*, 680.
(12) Marcus, Y.; Ben-Naim, A. *J. Chem. Phys.* **1985**, *83*, 4744.
(13) Jansco, G.; VanHook, W. A. *Chem. Rev.* **1974**, *74*, 689.
(14) Arnett, E. M.; McKelvey, D. R. In *Solute−Solvent Interactions*; Coetzee, J. F., Fitchie, C. D., Eds.; Dekker: New York, 1969; pp 343−397 and references therein.
(15) Ben-Naim, A.; Wilf, J.; Yaacobi, M. *J. Phys. Chem.* **1973**, *77*, 95.
(16) Kresheck, G. C.; Schneider, H.; Scheraga, H. A. *J. Phys. Chem.* **1965**, *69*, 3132.
(17) Connelly, P. R.; Thomson, J. A.; Fitzgibbon, M. J.; Bruzzese, F. J. *Biochemistry* **1993**, *32*, 5583. In the same work, Connelly and co-workers report the thermodynamics of binding of rapamycin to FKBP. This binding shows a larger ΔC_p than any of those in Table 1 (425 eu), and a larger $\Delta\Delta H$ at 25 °C than any in Table 1 (4.9 kcal mol^{-1}): this value is roughly twice the $\Delta\Delta H$ expected from Figure 2. These workers observed a change of ~20% in ΔC_p between H_2O and D_2O for rapamycin binding. We note that qualitatively the data are faithful to our trend of increased $\Delta\Delta H$ with increased ΔC_p.
(18) Connelly, P. R.; Thomson, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4781.
(19) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131.

Table 1. Thermodynamic Solvent Isotope Effects

system[a]	$\Delta G^{b,c}$		ΔH^{b}		$\Delta C_p{}^{d}$		
	H_2O	D_2O	H_2O	D_2O	H_2O	D_2O	$10(\Delta\Delta H)^{b}$
Dioclea–Trimann	−8.2 ± 0.03	−8.2 ± 0.05	−13.0 ± 0.05	−12.6 ± 0.03	−96 ± 9	−96 ± 9	−4
concanavalin A–MeMan	−5.0 ± 0.02	−5.3 ± 0.01	−7.1 ± 0.02	−6.6 ± 0.01	−48 ± 8	−53 ± 9	−5
concanavalin A–1,6-DiMan	−5.3 ± 0.05	−5.3 ± 0.03	−7.0 ± 0.05	−6.1 ± 0.03	−40 ± 7	−40 ± 3	−9
concanavalin A–Trimann	−7.5 ± 0.04	−7.7 ± 0.06	−10.7 ± 0.04	−10.2 ± 0.06	−63 ± 10	−63 ± 4	−5
vancomycin–peptide	−7.2 ± 0.04	−7.1 ± 0.02	−11.5 ± 0.04	−10.8 ± 0.02	−143 ± 7	−143 ± 6	−7
RNAse–CMP	−7.5 ± 0.08	−7.5 ± 0.09	−14.1 ± 0.03	−12.7 ± 0.04	−210 ± 9	−208 ± 7	−14
FK506–FKBP	−12.3[e]	NR[g]	−17.2[f]	−15.4[f]	291[f]	291[f]	−18

[a] MeMan = methyl α-D-mannopyranoside; Triman = methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; 1,6-DiMan = methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; peptide = α,ε-di-*N*-acetyl-Lys-D-Ala-D-Ala; CMP = cytidine 2′-monophosphate. [b] kcal mol^{-1}, 25 °C. [c] Free energies were calculated using the expression $\Delta G = RT \ln K_{eq}$ with K_{eq} expressed in molar units. [d] cal mol^{-1} deg^{-1}. [e] From ref 18. [f] From ref 17. [g] NR = not reported.

quantities $[L]_{total}$, $[R]_{total}$, and K_{eq}. Differentiation of this expression with respect to $[L]_{total}$ provides an expression for complex concentration as a function of total ligand concentration. This term can then be substituted into the expression for the incremental heat, dq, arising from complex formation, *i.e.*:

$$dq = d[LR]\cdot\Delta H\cdot V_0$$

where ΔH is the molar enthalpy of binding and V_0 is the cell volume. The complete expression for the measured enthalpy during calorimetric titration in terms of $[R]_{total}$, $[L]_{total}$, and K_{eq} is therefore:

$$\frac{1}{V_0}\frac{dq}{d[L]_{total}} = \Delta H\left[\frac{1}{2} + \frac{1 - (1 + r)/2 - L_r/2}{(L_r^2 - 2L_r(1 - r) + (1 + r)^2)^{1/2}}\right] \quad (6)$$

where $1/r = [R]_{total} K_{eq}$ and $L_r = [L]_{total}/[R]_{total}$. Data from calorimetric titrations were fit to eq 6 using a nonlinear least-squares fit that continually varies ΔH and K_{eq}. A more complete description of the data reduction procedure can be found in ref 19. Free energies of binding were determined from binding constants in the usual way. In all cases, the concentration of binding sites was such that the product of the binding constant K and the concentration of binding sites was in the range of 10−100, and in all cases the final ligand concentration in the cell was at least $10K_D$. Heat capacities of binding were obtained from least-squares linear fits to plots of enthalpy *versus* temperature over the range of 4−40 °C.

Fits of individual data sets to eq 6 also provide a stoichiometry of binding, n. In all cases, $0.95 < n < 1.05$: this criteria provides an independent test of the validity of both ligand and receptor concentrations. Furthermore, fits of the data using the methodology of Bundle, which does not require knowledge of the receptor concentration, provided identical results.[20]

A typical set of raw and integrated data is shown in Figure 1 for the titration of vancomycin with α,ε-diacetyl-L-lysine-D-alanine-D-alanine: experimental conditions are provided in the figure legend. A corresponding plot of enthalpy *versus* temperature for associations in light and heavy water is shown in Figure 2 for vancomycin/tripeptide binding. Differences in enthalpies of binding in H_2O and D_2O, $\Delta\Delta H$, were determined from the vertical distances between linear fits to enthalpy *versus* temperature plots.

Errors in individual enthalpy determinations were determined from the quality of the fit of eq 6 to the data, and typically ranged from 0.1 to 1% of the measured enthalpy. For the data shown in Figure 1, the enthalpy was calculated as −10785 ± 34.4 cal mol^{-1}. For each system, multiple (five to ten) independent runs were used to calculate standard deviations: such deviations were typically on the order of 2% of the reported enthalpy. Those points falling outside the 90% confidence level were discarded. Errors in ΔC_p were determined from linear regression analysis of ΔH *versus* T plots. Errors in $\Delta\Delta H$ were determined from errors in predicted values of ΔH, which follow from the standard deviation in the regression analysis of ΔH *versus* T. At the midpoint of the plot, the error in a predicted value of ΔH, $s_{\Delta H}$, is given by $s(1/n)^{1/2}$, where s is the standard deviation in the slope and n is the number of points on the plot.[21] Errors in vertical distances were obtained by propagating the errors in each predicted ΔH value in the usual way. In all cases, errors in $\Delta\Delta H$ are <5% of the measured values. Where errors are larger than the point sizes, they are indicated by error bars.

It is well-known that enthalpies of association can be strongly influenced by pH. For this work, it is clearly imperative that protonation states of the interacting species are identical in light and heavy water. Because glass electrodes measure ion activities rather than actual concentrations, it has long been recognized that at equal concentrations of H^+ and D^+ a glass electrode pH meter reading will be 0.4 unit lower in D_2O. Because solvent isotopic substitution also affects ionization constants or pK_a values of ionizable groups, a second variable is introduced to the problem of how to achieve identical ionization states in the two solvents. Empirically, it has been noted that the difference in pK values for deuterated acids *versus* the corresponding protonated derivative is $0.4 + 0.02pK_H$. Thus, under no pH/pD regime will identical protonation states of ionizable groups result in light and heavy water. Wüthrich and co-workers have shown by NMR titration that changes in pK_a values for ionizable groups in proteins are almost exactly offset by differences in proton *versus* deuteron activities.[22] Thus for globular proteins at pH = pD, as measured by a glass electrode, identical protonation states result: consequently this condition was used for all titrations reported in Table 1. Furthermore, of the systems studied here, only for RNAse/2′-CMP binding is the enthalpy of association a function of pH over the range ±1 pH unit from the pH at which the measurements were made.

For each binding investigated, solvent isotopic substitution resulted in a less negative enthalpy of binding. An offsetting change in entropy leads to unaltered Gibbs binding energies: such enthalpy–entropy compensations are the hallmark of associations in aqueous solution.[23,24] There is a strong linear

(20) Sigurskjold, B. W.; Altman, E.; Bundle, D. R. *Eur. J. Biochem.* **1991**, *197*, 239.

(21) Hecht, H. G. *Mathematics in Chemistry: An Introduction to Modern Methods*; Prentice Hall: New Jersey, 1990; p 271.

(22) Bundi, A.; Wüthrich, K. *Biopolymers* **1979**, *18*, 285.

(23) Lumry, R.; Rajender, S. *Biopolymers* **1970**, *9*, 1125.

(24) The danger of self-correlation as the cause of enthalpy–entropy compensation must be recognized when ΔG and ΔH are obtained from the same measurement. Ideally, an independent estimate of ΔG by a non-calorimetric technique is required to unambiguously identify enthalpy–entropy compensation. Non-calorimetrically-determined association constants are available for the vancomycin/tripeptide system[25] (affinity electrophoresis), concanavalin A binding to α-methyl mannose[26] (UV/vis),

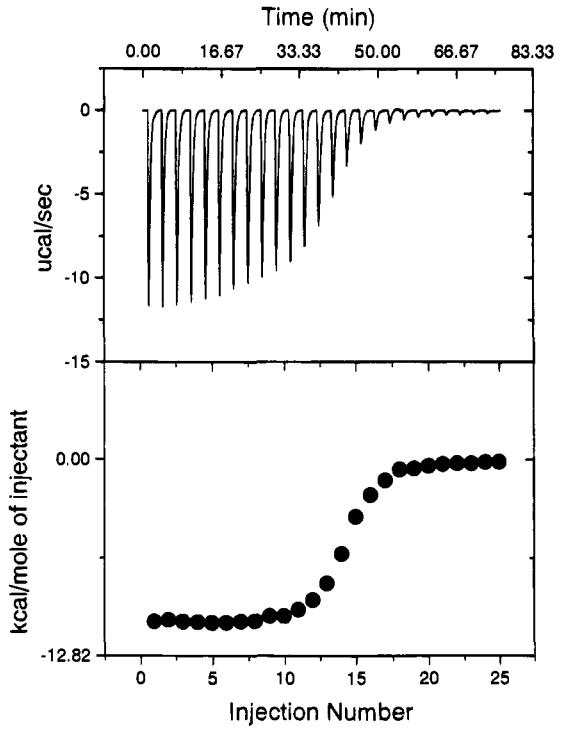

Figure 1. Raw (top) and integrated (bottom) data for binding of α,ε-di-Ac-Lys-D-Ala-D-Ala to vancomycin. The vancomycin concentration (cell) was 28 μM and the peptide concentration (syringe) was 15 mM. Both solutions were in potassium phosphate buffer, pH 7.0. The titration was carried out with 25 4.4 μL injections of 4.4 s duration with 3 min intervals between injections. Fitting of the integrated data to eq 6 yielded a binding constant of $4.44 \pm 0.19 \times 10^5\ M^{-1}$ and an enthalpy of binding of -10.8 ± 0.03 kcal mol^{-1}.

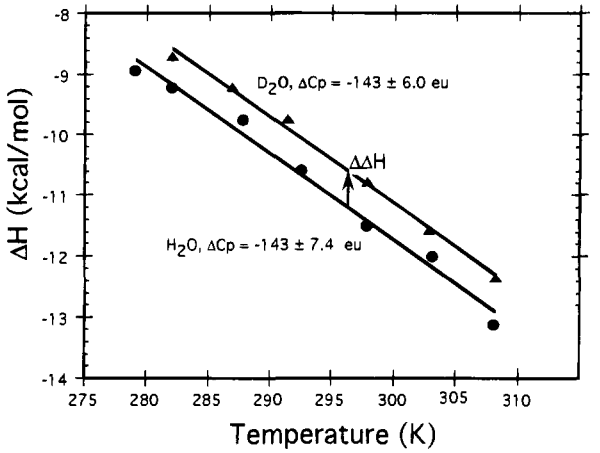

Figure 2. Enthalpy of binding of α,ε-di-Ac-Lys-D-Ala-D-Ala to vancomycin in H_2O (circles) and D_2O (triangles) as a function of temperature. The error in each point is <2%; the error in ΔC_p (from regression analysis) is ~2%. Molar heat capacity changes were measured by evaluating ΔH as a function of temperature. Over small temperature ranges (25−30 K) ΔC_p is expected to be constant, and straight lines were fit to each data set.

correlation ($R = 0.92$) between ΔC_p and $\Delta\Delta H$ (Figure 3): the slope of the plot is approximately 5 K. No interpretable correlations were observed in plots of $\Delta\Delta H$ against either ΔG (Figure 4, $R = 0.65$) or ΔH ($R = 0.66$, plot not shown).

and RNAse binding to 2′-CMP[27] (UV/vis). In all three cases the association constants agree to within experimental error with the values measured here.

(25) Chu, Y.-H.; Whitesides, G. M. *J. Org. Chem.* **1992**, *57*, 3524.

(26) Portez, R. D.; Goldstein, I. J. *Biochemistry* **1970**, *9*, 2890.

(27) Anderson, D. G.; Hammes, G. G.; Walz, F. G. *Biochemistry* **1968**, *7*, 1637.

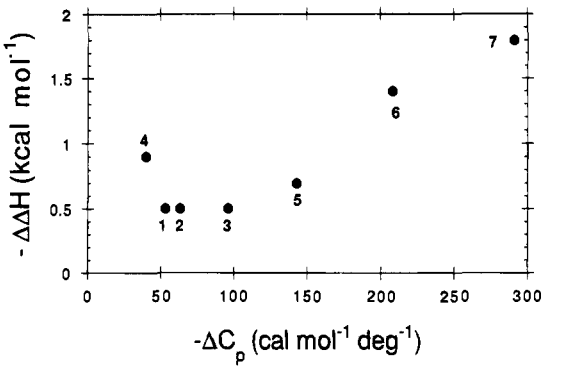

Figure 3. Differential enthalpy of binding in D_2O relative to H_2O ($\Delta\Delta H$, $\Delta H_H - \Delta H_D$) as a function of ΔC_p. The slope of the plot is 4.8 K, with a correlation coefficient of 0.92. The error in ΔC_p in each case is ~2%; the error in $\Delta\Delta H$ is <5% in all cases: where error bars are not shown the error is estimated at less than the diameter of the points. **1**, concanavalin A/methyl α-D-mannopyranoside; **2**, concanavalin A/methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; **3**, *Dioclea*/methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; **4**, concanavalin A/methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; **5**, vancomycin/α,ε-diacetyl-L-lysine-D-alanine-D-alanine; **6**, ribonuclease A/2′-CMP; **7**, FK506/FK-binding protein.

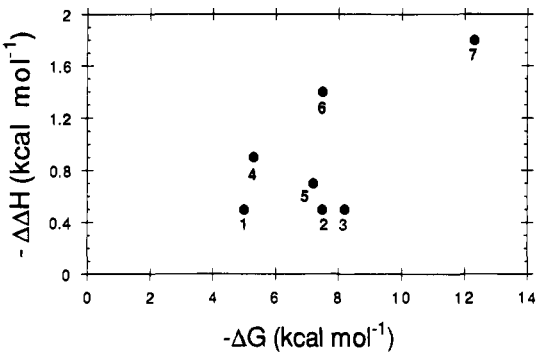

Figure 4. Differential enthalpy of binding in D_2O relative to H_2O ($\Delta\Delta H$, $\Delta H_H - \Delta H_D$) as a function of free energy of binding. The correlation coefficient for the best fit to these data is 0.65. **1**, concanavalin A/methyl α-D-mannopyranoside; **2**, concanavalin A/methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; **3**, *Dioclea*/methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; **4**, concanavalin A/methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside; **5**, vancomycin/α,ε-diacetyl-L-lysine-D-alanine-D-alanine; **6**, ribonuclease A/2′-CMP; **7**, FK506/FK-binding protein.

We interpret the thermodynamic solvent isotope effect in terms of solvent reorganization that accompanies binding. The observed effect can be explained entirely by changes in ΔH_s arising from the differential thermodynamics of O···H *versus* O···D interaction. As described above, solvent isotopic substitution will affect only the portion of the enthalpy of binding contained in ΔH_s, assuming the structure of the ligand, the receptor, and the complex are identical in both solvents. This assertion is supported by considerable biophysical data, and we are unaware of any evidence to the contrary.[28] The decreased enthalpy of binding in D_2O relative to H_2O reflects the expected stabilization of the unbound state relative to the bound state. Also as predicted from known thermodynamics of hydrogen bonding, an offsetting change in the entropy of association leads to little or no change in ΔG.

The $\Delta\Delta H/\Delta C_p$ plot provides additional evidence that the origin of the thermodynamic solvent isotope effect originates in differential O···H *versus* O···D hydrogen bond strengths.

(28) Finer-Moore, J. S.; Kossiakoff, A. A.; Hurley, J. H.; Earnest, T.; Stroud, R. *Proteins: Struct., Funct., Genet.* **1992**, *12*, 203.

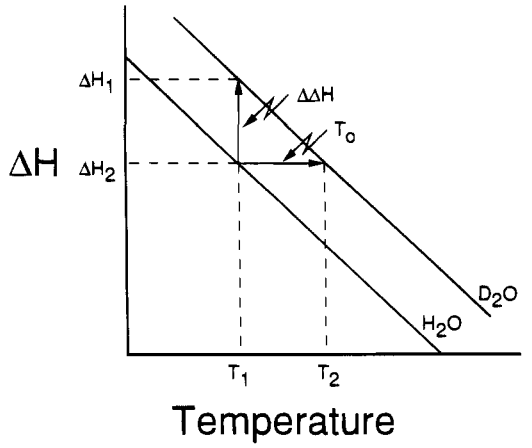

Figure 5. Generalized enthalpy *versus* temperature plot for binding in light and heavy water. The offset temperature, T_o, is the temperature difference required to give identical enthalpies of binding in light and heavy water.

Figure 5 shows a generalized ΔH *versus* temperature plot for association in light and heavy water (*cf.* Figure 3). ΔC_p is rigorously defined as the partial derivative of ΔH with respect to temperature at constant pressure, *i.e.* $\Delta C_p = (\partial H/\partial T)_p$. Over short temperature ranges, ΔC_p is predicted to be linear and can be approximated as $\Delta(\Delta H)/\Delta T$ or $(\Delta H_1 - \Delta H_2)/(T_1 - T_2)$ from Figure 5. The slope of the $\Delta\Delta H$ *versus* ΔC_p plot is a temperature (cal mol^{-1}/cal mol^{-1} deg^{-1}) which we define as the *offset temperature*, T_o, or the temperature difference required to yield identical enthalpies of binding in light and heavy water. That this slope is indeed the offset temperature is apparent from Figure 5. In Figure 5 the thermodynamic solvent isotope effect, $\Delta\Delta H$, is defined as $\Delta H_2 - \Delta H_1$, and the offset temperature is $(T_2 - T_1)$. If one considers the slope of the ΔH *versus* temperature plot over the range T_1 to T_2, then ΔC_p is given by $(\Delta H_2 - \Delta H_1)/(T_2 - T_1)$, or $\Delta\Delta H/T_o$. A plot of $\Delta\Delta H$ *versus* ΔC_p thus yields a line with a slope of T_o, or the offset temperature. The 5 K offset temperature observed here is reminiscent of other offsets between H_2O and D_2O attributed to different O···H *versus* O···D hydrogen bond strengths, including melting points (4 K), triple points (4 K), temperatures of maximum density (8 K) and critical points (2 K).[9,29]

The $\Delta\Delta H$ versus ΔC_p plot also demonstrates that the primary contributor to ΔC_p in aqueous association processes is solvent reorganization. The factors contributing to heat capacity changes during binding have long been a source of discussion, beginning with the work of Sturtevant.[30] Briefly, although changes in solvent structure, hydrogen bonding, electrostatic interactions, and low-frequency protein vibrations can in principle affect ΔC_p, Sturtevant concluded that the loss of water of solvation and changes in low-frequency protein oscillations were the major contributors to ΔC_p. The relative magnitude of each contribution has long been a source of debate. The correlation between $\Delta\Delta H$ and ΔC_p argues strongly that ΔC_p is primarily a measure of solvent reorganization and that protein stiffening contributes a minor amount to this term. Although solvent isotopic substitution will alter several protein vibrational modes, the changes will occur in both the free and bound forms of the protein: thus, ΔC_p arising from loss of protein vibrational modes will be identical in both light and heavy water. To the extent this latter mechanism contributes to ΔC_p, correlation between $\Delta\Delta H$, which arises exclusively from solvent properties, and ΔC_p should be weak. The strong linear correlation thus belies a significant contribution of non-solvent-associated phenomena to ΔC_p.

Most significantly, the magnitude and sign of $\Delta\Delta H$ show that solvent reorganization provides a large favorable contribution to the net enthalpy of binding. From eq 1 the enthalpy of reaction is given by

$$\Delta H = \Delta H_i + \Delta H_s \tag{1}$$

and $\Delta\Delta H$ is therefore

$$\Delta\Delta H = (\Delta H_{i,H} + \Delta H_{s,H}) - (\Delta H_{i,D} + \Delta H_{s,D}) \tag{7}$$

or

$$\Delta\Delta H = (\Delta H_{i,H} - \Delta H_{i,D}) + (\Delta H_{s,H} - \Delta H_{s,D}) \tag{8}$$

where the subscripts H and D indicate light and heavy water processes, respectively. Since isotopic substitution will not change the intrinsic enthalpy of binding, the quantity $\Delta\Delta H$ is a measure of the term $\Delta H_{s,H} - \Delta H_{s,D}$. In all cases, $\Delta\Delta H$ is negative: because the absolute value of $\Delta H_{s,H}$ is larger than $\Delta H_{s,D}$ (*vide supra*), it follows that $\Delta H_{s,H}$ must be negative. The actual magnitude of $\Delta H_{s,H}$ can be estimated from $\Delta\Delta H$ using the known difference in H···O *versus* D···O interaction enthalpies. *To a first approximation, $\Delta\Delta H$ represents 10% of the contribution of changes in solvation to the enthalpy of binding.* From Table 1 it is clear that this effect is large, contributing from 4 to 18 kcal mol^{-1} of binding enthalpy.

Our analysis further explains the lack of correlation between $\Delta\Delta H$ and ΔH or ΔG: in both cases terms unrelated to solvation contribute. $\Delta\Delta H$ is related only to the ΔH_s term of ΔH, and clearly will not correlate with ΔH_i. The intrinsic enthalpy is also contained in ΔG, although the Gibbs free energy of binding also includes an entropic term. We note that higher order trends may emerge when systems with a larger range of ΔG values are explored (Figure 4).

Although our experiments clearly demonstrate that a significant fraction of the enthalpy of binding arises from solvent reorganization, the fraction of the free energy of binding that can be accounted for by solvent effects is less clear. Enthalpy–entropy compensation may mask a portion of the enthalpy gain from solvent reorganization, and the lack of correlation of $\Delta\Delta H$ with either ΔH or ΔG suggests ΔH_i contributes significantly to both terms.

For the systems examined here, ΔC_p remained constant, or very nearly constant, in both H_2O and D_2O (Figure 2). The generality of this phenomenon remains to be seen, but it need not be universal.[17] Different ΔC_p values in the two solvents implies that $\Delta\Delta H$, or the contribution to binding enthalpy from solvation, is a function of temperature. This mirrors suggestions by Privalov and Gill that the enthalpy of hydrophobic hydration is a function of temperature.[2]

We return briefly to our assertion that ΔH_i is unaffected by solvent isotopic substitution. A model of binding that rests heavily on solute–solute interactions would require that solute–solute hydrogen bonds be stronger than the corresponding prebinding solute–solvent interactions. It follows then that an increase in the enthalpy of interaction of both interactions by ~10% would lead to a differential enthalpy of binding in light *versus* heavy water. We assert the observed thermodynamic solvent isotope effect arises from solvation effects on the basis of two observations. First, differential solute–solute *versus* solute–solvent hydrogen bonds would produce an effect of *opposite* sign to that observed, *i.e.* ΔH would be *more* negative in heavy water than in light. Such an effect, if operative here,

(29) Kirshenbaum, J. *Physical Properties and Analysis of Heavy Water*; McGraw-Hill: New York, 1951.
(30) Sturtevant, J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 2236.

would mask part of the favorable enthalpy of binding from solvent reorganization and lead us to *underestimate* the contribution of solvent reorganization to the net enthalpy of binding. Second, an origin of $\Delta\Delta H$ in solute−solute interaction belies the correlation of $\Delta\Delta H$ with ΔC_p, a term dominated by solvent effects.

There exists both computational and structural support for the thesis that the removal of enthalpically unfavorable water from apposing surfaces during binding contributes favorably to association energies. High-resolution protein X-ray structures typically identify significant areas of protein surface covered with water sites of partial occupancy, signaling either static or dynamic disorder.[31,32] This weakly bound disordered water is perfectly positioned to contribute favorably to the enthalpy of binding as it returns to bulk solvent. It has previously been suggested that stripping loosely bound water could contribute to the binding energy between protein and substrate.[33] Fitzpatrick and co-workers recently reported a crystallographic study in which lightly cross-linked subtilisin crystals were soaked in acetonitrile.[34] Virtually all tightly associated (*i.e.* visible) water molecules are retained in identical positions even after extensive soaking with a water miscible solvent. Following soaking, 12 bound acetonitrile molecules were observed in the crystals, with a cluster of 4 of the 12 bound in the subtilisin active site. Although the protein structure in the soaked and unsoaked crystals is identical, bound acetonitrile is observed only in areas where no water was observed prior to soaking: clearly acetonitrile is bound to the crystals by displacing weakly bound disordered water. This observation suggests that water in enzyme active sites is weakly associated with the protein and readily displaced by substrate.

Computational studies on the behavior of water near biological surfaces also indicate that removal of water to bulk solvent could drive association processes. The existence of disordered water near protein surfaces suggests that the apposing faces of binding pairs are complementary to each other, but not to any structure that water can reasonably adopt. Lemieux and coworkers have recently used Monte Carlo simulations of water over a "polyamphiphilic" region of a protein binding site to show that dynamic disorder can indeed play a significant role in aqueous association phenomena.[35,36]

We note finally that our analysis includes many molecular processes in a single term that we have described as "solvent reorganization". A more precise description of the molecular events that contribute to ΔH_s awaits additional experimental and computational study. We have presented a molecular origin of an observed phenomenon in terms of a mixture model of water, specifically that developed by Muller, in which water is considered as a mixture of two species with different thermodynamic properties. Models of this type originate with the work of Scheraga.[37] In addition to that of Muller, similar models have recently been proposed by Gill[38,39] and Wadsö.[40] Although these models accurately describe much of the phenomenology of the dissolution of nonpolar compounds and are conceptually straightforward, there are limitations to two-state models: these limitations and alternative models have recently been reviewed.[41] Other models, such as those of Lumry[42] and of Privalov,[2] without invoking mixture models of water, still arrive at the fundamental conclusion that the low solubility of nonpolar compounds in water arises from enthalpic considerations. Thus, while the molecular model used here may be an oversimplification, to the extent that the analogy of binding to a desolvation process is correct, it is inevitable that reorganization of solvent during aqueous association processes will contribute a significant portion of the enthalpy of binding.

We must further note that myriad experimental details, for example subtle pH effects between light and heavy water, trace impurities in solvents and reagents, etc., can contribute to the relatively small changes in enthalpies observed in these studies. While such effects could conceivably affect enthalpies of interaction in light and heavy water, the striking correlation observed in Figure 3, which includes data from other laboratories, argues strongly for a more fundamental explanation for the observed solvent isotope effects.

In conclusion, we have presented experimental evidence that the reorganization of solvent during binding in aqueous solution contributes a significant portion of the enthalpy of complexation. The implications of this work on other systems, including protein folding, are clear. We are currently evaluating the magnitude of thermodynamic solvent isotope effects on other associations, including those in non-aqueous systems, and will report our results in due course.

Experimental Section

General. Concanavalin A (Type IV, lot No. 81H7020), vancomycin (lot No. 82H0055 >98%), ribonuclease A (from bovine pancreas, Type XII-A, lot No. 73H7012), cytidine 2′-monophosphate (lot No. 124F7110, >98%), and methyl α-D-mannopyranoside (lot No. JF 10312 DW) were purchased from Sigma Chemical Company and used without further purification. Concanavalin A was also prepared by affinity chromatography on Sephadex G75 from commercial jack bean meal (Sigma) according to literature procedures.[26] Ribonuclease A and the lectin from *Dioclea grandiflora* gave single-band Coomassie-stained SDS-PAGE gels. Concanavalin A gave bands at 26 kDa (monomer) as well as at 12 and 14 kDa, representing previously described nicked fragments. The nicked fragments form competent binding dimers, and binding with protein devoid of nicked fragments (by ammonium bicarbonate precipitation) gave identical results.[43] The tripeptide α,ε-diacetyl lysine-D-alanine-D-alanine was purchased from Bachem Feinchemikalien AG (lot No. 119783) with a reported purity of 97.6% and used without further purification. Methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside and methyl 3,6-bis-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside were synthesized by literature methods and were >95% pure by NMR. The lectin from *Dioclea grandiflora* was purified by affinity chromatography on Sephadex G75, according to literature procedures, from seed samples collected in Northeastern Brazil. Protein concentrations in all cases were determined by the method of Edelhoch.[44] Carbohydrate concentrations were determined by

(31) Rupley, J. A.; Careri, G. *Adv. Protein Chem.* **1991**, *41*, 37.
(32) Saenger, W. *Annu. Rev. Biophys. Biophys. Chem.* **1987**, *16*, 93.
(33) Frey, M. In *Water and Biological Macromolecules*; Westoff, E., Ed.; CRC Press: Boca Raton, 1993; p 98.
(34) Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8653.
(35) Lemieux, R. U. *Carbohydrate Antigens*; ACS Symp. Series No. 519; American Chemical Society: Washington, D.C., 1992; pp 6−18.
(36) Bierbeck, H.; Lemieux, R. U. *Can. J. Chem.* **1990**, *68*, 820.
(37) Némethy, G.; Scheraga, H. A. *J. Chem. Phys.* **1962**, *36*, 3382.
(38) Dec, S. F.; Gill, S. J. *J. Solution Chem.* **1985**, *14*, 417.
(39) Dec, S. F.; Gill, S. J. *J. Solution Chem.* **1985**, *14*, 827.
(40) Olofsson, G.; Oshodi, A. A.; Qvarnström, I.; Wadsö, I. *J. Chem. Thermodyn.* **1984**, *16*, 1041.
(41) Blokzijl, W.; Engberts, J. B. F. N. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1545.
(42) Huot, J.-Y.; Battistel, E.; Lumry, R.; Villeneuve, G.; Lavalle, J.-F.; Anusiem, A.; Jolicoeur, C. *J. Solution Chem.* **1988**, *17*, 601.
(43) Cunningham, B. A.; Wang, J. L.; Pflumm, M. N.; Edelman, G. M. *Biochemistry* **1972**, *11*, 3233.
(44) Edelhoch, H. *Biochemistry* **1967**, *6*, 1948.

phenol–sulfuric acid chars according to the method of Dubois.[45] Vancomycin and 2′-CMP concentrations were measured spectrophotometrically.

Water in all cases was purified with a Millipore purification system that involved passage through reverse osmosis, charcoal, and two ion exchange filters. In all cases the water had a resistivity of >10 MΩ cm^{-1}. Heavy water was purchased from Cambridge Isotope Limited and used without further purification. The D_2O showed a minimum resistance of 10 MΩ.

Calorimetry. All titrations were run using the general protocols outlined in the Results and Discussion section.

Titrations using concanavalin A or the lectin from *Dioclea grandiflora* (Table 1, entries 1, 2, and 4) were run at pH 7.2, in 50 mM phosphate buffer, augmented with 250 mM NaCl and 1 mM each $CaCl_2$ and $MnCl_2$.

(45) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350.

Titrations of concanavalin A with methyl 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (Table 1, entry 3) were run at pH 5.5 in 50 mM 3,3-dimethylglutarate buffer, augmented with 250 mM NaCl, 1 mM each $CaCl_2$ and $MnCl_2$.

Titrations of vancomycin with α,ε-diacetyl-L-lysine-D-alanine-D-alanine (Table 1, entry 5) were run at pH 7.0 in 50 mM phosphate buffer augmented with 250 mM NaCl.

Titrations of RNAse with 2′-CMP (Table 1, entry 6) were run at pH 5.5 in 200 mM KOAc buffer.

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