Spatial Organization versus Total Surface Area as a Predictor of Protein Hydrophobicity. The Hydrophobicity of the Concanavalin A Binding Site†

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The origin of the driving forces for association in aqueous solution remains largely unknown.[1-9] During a binding event, two solutes come together and in the process incur both favorable and unfavorable interactions. Simultaneously, a portion of each solute is desolvated, and the water of solvation is returned to bulk solution. The contribution of desolvation to the energetics of ligand binding depends on the extent of polar and nonpolar surface area desolvated. *We show here that the contribution is also determined by the spatial arrangement of atoms on the desolvated surface.*

Thermodynamic solvent isotope effects (TSIE), or differential enthalpies of binding in light versus heavy water, are now a proven method for probing the contribution of changes in solvation to the enthalpy of ligand binding.[10,11] It has long been recognized that the enthalpy of transfer of a solute from H_2O to D_2O is a function of the nature and extent of surface area being transferred. The pioneering works of Scheraga, Arnett, Ben-Naim, Dahlberg, and others demonstrated that the enthalpy of transfer from H_2O to D_2O of a nonpolar solute is exothermic and linearly related to the extent of nonpolar surface area transferred.[12-15] Alternatively, the enthalpy of transfer of a polar species is positive and is related to both the extent and nature of polar surface area being transferred.

In 1993, Connelly and co-workers extended these early experiments to probe the role of hydration in ligand binding.[10] These researchers recognized that the differential enthalpy of ligand binding in light versus heavy water is identical to the enthalpy of transfer, from H_2O to D_2O, of that portion of the ligand and binding site that is desolvated during binding. We later extended these findings to explore the fraction of binding enthalpy assignable to desolvation of the ligand and binding site.[11] The central conclusion of our work was that the differential enthalpy of binding in light versus heavy water is linearly related to the enthalpy of binding arising from desolvation. *The enthalpy of transfer of a species from light to heavy water is thus a measure of the binding enthalpy available through desolvation of that species.*

Here, we utilize solvent isotope effects to probe the hydrophobicity of a protein binding site. Because the differential enthalpy of binding is the enthalpy of transfer from light to heavy water of the total desolvated surface, subtraction of the enthalpy of transfer of the fraction of the *ligand* desolvated during binding from the total TSIE provides the enthalpy of transfer, from H_2O to D_2O, of the *protein binding site*. This technique provides a direct measure of the hydrophobicity of a protein binding site.

We demonstrate the method here for the binding site of concanavalin A, the lectin from the legume *Canavalia ensiformis*. High-resolution crystallographic data is available for both the apoprotein[16] and the complex with methyl α-D-mannopyranoside,[17] allowing accurate measurement of the surface area of both ligand and protein desolvated during binding.

The enthalpy of transfer of methyl α-D-mannopyranoside from H_2O to D_2O was measured using the Microcal Omega titration microcalorimeter. Details of the instrument design and data reduction procedures appear elsewhere.[18] In a typical experiment, methyl α-D-mannopyranoside at concentrations ranging from 0.125 to 1.0 M in H_2O was titrated into D_2O in a series of 20 2.0 μL injections. To eliminate dilution effects, the D_2O contained an identical concentration of methyl α-D-mannopyranoside. The H_2O into D_2O heat of mixing was subtracted based on the concentration of H_2O in the syringe, which was in turn determined from the density of the saccharide solution. The entire experiment was repeated in the opposite sense, *i.e.*, transfer from D_2O to H_2O.

The enthalpy of transfer any species with exchangeable protons includes an enthalpy for chemical exchange, that is,

$$\mathrm{ROH} + \mathrm{D_2O} \rightarrow \mathrm{ROD} + \mathrm{HOD}$$

Clearly for carbohydrates, this term is significant and must be subtracted from the overall enthalpy of transfer, to yield the purely physical component of the enthalpy of transfer. Exchange enthalpies for methanol and ethanol have been reported.[19] Enthalpies of transfer for a variety of primary and secondary alkanols vary linearly with the surface area of the apolar moiety being transferred, suggesting that the enthalpy of proton transfer is no more than weakly a function of the R group.[15]

Using the Chand and Fenby values for exchange enthalpies,[19] enthalpies of transfer for methyl α-D-mannopyranoside from H_2O to D_2O and from D_2O to H_2O were determined. The polar and nonpolar solvent-accessible surface areas of methyl α-D-mannopyranoside were determined using the hard-sphere exo-anomeric calculation routine contained in the program GEGOP.[20] The rotomeric equilibrium about the C1−O1 bond was determined, and solvent-accessible surface area was calculated from the population-weighted average structure.

The total solvent-accessible surface area of methyl α-D-mannopyranoside by this method is 360 Å^2, of which 178 Å^2 is polar and 189 Å^2 is apolar. Using the value of −1.89 cal mol^{-1} Å^{-2} for the transfer of nonpolar surface area,[10] the specific enthalpy of transfer of polar surface area from light to heavy water of mannose is calculated to be +4.5 cal mol^{-1} Å^{-2}. To determine the generality of this value, we measured the enthalpies of transfer from light to heavy water of methyl α-D-

† This paper is dedicated to Professor R. U. Lemieux on the occasion of his 75th birthday.

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Table 1. Enthalpies of Transfer of Carbohydrates from H_2O to D_2O

carbohydrate	ΔH_{trans}[a] (cal mol^{-1})	polar surf. area (Å^2)[b]	nonpolar surf. area (Å^2)[b]	specific polar ΔH_{trans} (cal mol^{-1} Å^{-2})
methyl α-D-mannopyranoside	+451 ± 18	178	189	+4.5 ± 0.1
methyl α-D-glucopyranoside	+465 ± 64	178	189	+4.6 ± 0.3
methyl α-D-galactopyranoside	+443 ± 49	177	189	+4.5 ± 0.3
methyl β-D-galactopyranoside	+446 ± 43	179	192	+4.5 ± 0.2
sucrose	+898 ± 26	304	197	+4.2 ± 0.1
α,α-trehalose	+965 ± 68	305	199	+4.4 ± 0.2

[a] Enthalpies of transfer have been corrected to remove exchange enthalpies. [b] Population-weighted average solution structures.

glucopyranoside, methyl α-D-galactopyranoside, methyl β-D-galactopyranoside, sucrose, and α,α-trehalose (Table 1). Each saccharide gives a remarkably constant value of +4.4 ± 0.2 cal mol^{-1} Å^{-2} for the enthalpy of transfer of polar surface area from light to heavy water.

Together with crystallographic data, these values for the enthalpy of transfer of polar and nonpolar surface area allow prediction of the TSIE for concanavalin A methyl α-D-mannopyranoside binding. The total reduction in carbohydrate solvent-accessible surface area on binding is 261 Å^2, of which 138 Å^2 is polar and 123 Å^2 is nonpolar. The protein solvent accessible surface area is reduced by 215 Å^2 on binding, including 26 Å^2 of polar and 189 Å^2 of nonpolar surface area.[21] Using −1.89 cal mol^{-1} Å^{-2} for the enthalpy of transfer of nonpolar surface area and +4.4 cal mol^{-1} Å^{-2} for the enthalpy of transfer of polar surface area, the predicted differential enthalpy of binding is thus +132 ± 32 cal mol^{-1}. This value differs markedly from the measured TSIE of −500 ± 23 cal mol^{-1}.[11] Thus, by the criteria of enthalpy of transfer from light to heavy water, *the binding site of concanavalin A is considerably more hydrophobic than is predicted based on enthalpies of transfer of model compounds.*

In our calculation of the enthalpy of transfer of the concanavalin A binding site, we have used the enthalpy of transfer of polar surface area calculated from both alkanol and carbohydrate transfer. The validity of the extension of this value to those polar groups contained on the surface of the protein binding sites is of concern, since there is evidence that the enthalpy of polar transfer is dependent both on the total surface area transferred and on the nature of the polar groups being transferred.[15] This concern is ameliorated here on two counts. First, the enthalpy of transfer of alkanols is a *conservative* estimate of the enthalpy of transfer for other polar groups: enthalpies of transfer for charged residues are significantly more endothermic.[15] Our estimate of the thermodynamic solvent isotope effect based on alcohol transfer is therefore a low estimate: inclusion of more endothermic values for the enthalpy of transfer of, for example, carboxylates would exacerbate the discrepancy between the estimated and the measured TSIE. Second, concanavalin A buries only a small amount of polar surface area, and errors from incorrect values of transfer enthalpies are thus minimized.

Our results suggest that the enthalpy of desolvation depends exquisitely on the *arrangement* of atoms in the binding site, and not simply on the number and type. This conclusion simply requires that the structure of water is strongly influenced by the precise structure of the surface it solvates. This conclusion was previously reached by Kauzmann in a study of volume changes accompanying protonations of carboxylic acids,[22] and Wolfenden in a study of the hydration of aldehydes to *gem*-diols.[23]

In their studies of FK506 and rapamycin binding to FK-binding protein, Connelly and co-workers observed an effect similar to that discussed here.[10] These investigators noted that the measured thermodynamic solvent isotope effect was significantly more negative than predicted based *only* on desolvation of apolar surface area. Since enthalpies of transfer of polar surface are endothermic, inclusion of polar area in the calculation would widen the gap between predicted and measured TSIE. Furthermore, the discrepancy is greatest for systems that bury a significant amount of polar surface area.

Together, these results imply that *the hydrophobicity of a binding site is determined not only by the nature of the atoms in the site but also by their spatial arrangement.* The results are thus an experimental demonstration of the effect predicted by Lemieux and co-workers based on both Monte Carlo simulations of water structure over the combining site of the lectin from *Griffonia simplicifolia*, and binding studies to the same lectin with synthetic ligands.[24,25] In these studies, Lemieux demonstrated that water structure near areas of mixed polar and apolar sites is especially disordered. Water constrained near these sites suffers a large enthalpic destabilization relative to water in bulk solution: the removal of this water from the *Griffonia* binding site is thus predicted to contribute strongly to the enthalpy of binding.

In conclusion, we have shown that the binding site of concanavalin A is considerably more hydrophobic than is predicted from small-molecule model studies, using enthalpy of transfer from H_2O to D_2O as a probe of hydrophobicity. These results suggest that the hydrophobicity of a site is determined both by the nature and by the spatial orientation of atoms on a molecular surface. We are currently studying enthalpies of transfer of a variety of small molecules, to better delineate those motifs that provide large enthalpies of desolvation, and will report our results in due course.

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(21) The structure of concanavalin A outside of the binding site is not perturbed by ligand binding. Examination of high-resolution structures of free and bound concanavalin A show backbone root mean square deviations of less than 0.1 Å. The lack of movement outside of the binding site rules out remote changes in protein solvent-exposed surface area as the source of the discrepancy in the predicted and measured thermodynamic solvent isotope effects.

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