Pressure Perturbation Calorimetry and the Thermodynamics of Noncovalent Interactions in Water: Comparison of Protein−Protein, Protein−Ligand, and Cyclodextrin−Adamantane Complexes†

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Pressure perturbation calorimetry measurements on a range of cyclodextrin−adamantane, protein−ligand (lysozyme−$(GlcNac)_3$ and ribonuclease−2′CMP) and protein−protein (cytochrome *c* peroxidase−pseudoazurin) complexes in aqueous solution show consistent reductions in thermal expansibilities compared to the uncomplexed molecules. Thermodynamic data for binding, obtained by titration calorimetry, are also reported. Changes in molar expansibilities can be related to the decrease in solvation during complexation. Although reasonable estimates for numbers of displaced water molecules may be obtained in the case of rigid cyclodextrin−adamantane complexes, protein expansibility data are less easily reconciled. Comparison of data from this wide range of systems indicates that effects are not simply related to changes in solvent-accessible surface area, but may also involve changes in macromolecular dynamics and flexibility. This adds to the growing consensus that understanding thermodynamic parameters associated with noncovalent interactions requires consideration of changes in internal macromolecular fluctuations and dynamics that may not be related to surface area-related solvation effects alone.

Introduction

Changes in solvation play a central role in the thermodynamics of noncovalent interactions in solution, especially in water, yet there are relatively few experimental methods available to probe this directly. Furthermore, it is often difficult to disentangle the effects of solvation from other less-perceptible effects, such as macromolecular conformational flexibility and dynamics, since such processes frequently display potentially similar thermodynamic signatures.[1] Pressure perturbation calorimetry (PPC) is a technique for measuring the differential heat effects (ΔQ) arising when small pressure pulses (±5 atm) are applied to a sample.[2] For molecules in solution, ΔQ is directly related to the difference in thermal expansivity of the solute molecules compared with the volume of solvent that they have displaced. This is proving of particular relevance to biomolecular interactions in solution, where such volumetric properties and associated thermodynamic quantities may be related at least in part to solvation effects that can be difficult to determine by other techniques. Consequently, PPC, taken together with other volumetric and thermodynamic measurements, presents an opportunity to address the solvation/hydration issue from a different perspective, especially since recent advances in microcalorimetry now allow this technique to be applied to the study of relatively dilute solutions.

Previous work in this area has focused on the use of PPC to probe the volumetric changes associated with conformational transitions in biological macromolecules, including DNA helix−coil transitions and denaturant and cosolvent effects in protein folding.[2−8] By integration of thermal expansivity data, these experiments have shown that protein unfolding in aqueous solution and cosolvent mixtures is accompanied by unpredictable volumetric changes involving either increases or decreases in partial molar volumes, depending on the specific protein and experimental conditions. This variability reflects the balance of a multiplicity of large, often opposing, volumetric effects arising from changes in solvation and macromolecular conformational packing and dynamics during the protein folding/unfolding process. This can be difficult to resolve unambiguously in such complicated systems. Recent work has shown how absolute values of thermal expansivity, as determined by PPC of model peptides, can be related to polypeptide conformation and the nature of the exposed amino acids,[5] although accounting for intrinsic volumetric properties remains problematic.[6]

We are interested in the potentially more tractable problem of solvation/hydration changes during protein−ligand or protein−protein interactions, but even here, the interpretation is not necessarily straightforward. Earlier experiments[1,9] using PPC to explore volumetric changes upon binding of simple inhibitors at enzyme active sites, using both lysozyme/trisaccharide and ribonuclease/2′-CMP as representative models, have shown significant changes in thermal expansivity upon complexation. Similar effects were seen in preliminary experiments with a pseudoazurin/cytochrome *c* peroxidase protein−protein interaction system.[9] In all cases, the thermal expansion coefficient (α) for the enzyme−inhibitor or protein−protein complex

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is less than the sum of the thermal expansion coefficients for the separate components alone.

The ubiquity of this decrease in a range of protein interaction systems suggested to us that it might arise predominantly from the reduction in total amounts of solvated water in the vicinity of the binding site and the ligand upon complex formation. The difficulty with this interpretation is that the components involved, particularly the protein molecules, are inherently flexible structures, and we cannot rule out volumetric changes related to ligand-induced effects on structural rigidity and packing densities within the protein-inhibitor complex.[1] Others have pointed out similar ambiguities in interpreting volumetric effects accompanying conformational transitions in biological macromolecules.[10,11]

Consequently, in an attempt to clarify the origins of thermal expansivity changes associated with noncovalent interactions in water, we here describe results obtained using a combination of PPC, isothermal titration calorimetry (ITC) and standard volumetric (density) techniques applied to the cyclodextrin/adamantane host/guest system.[12] The advantages here are that both the host (cyclodextrin) and the guests (adamantanes) are relatively rigid, small molecules (compared to proteins), whose structures and interaction thermodynamics have been extensively studied.[13] This potentially allows us to compare thermodynamic binding parameters with associated volumetric and solvation changes in the absence of competing effects from conformation and dynamics changes in the participating molecules. We have also taken the opportunity here to expand on our previous measurements[1,9] on protein−ligand and protein−protein interactions, with inclusion of additional volumetric data for comparison. The indications from this more comprehensive study are that solvation effects are only part of the story for protein interactions and that changes in macromolecular flexibility and dynamics may contribute extensively to binding thermodynamics and expansibility changes. This may also force us to readdress conventional interpretations of other thermodynamic forces.

Experimental Methods

Materials. β-Cyclodextrin (β-CD), dimethyl-β-cyclodextrin (β-$CDMe_2$), 1-adamantane carboxylic acid (Ad-COOH), 1-adamantane acetic acid (Ad-CH_2COOH), 1-adamantanamine (Ad-NH_2), 1-adamantanmethylamine (Ad-CH_2NH_2), *tri-N*-acetylglucosamine ($(GlcNAc)_3$, tri-(*N*-acetyl)-chitotriose), 2′-CMP, hen egg white lysozyme (HEWL), and ribonuclease A (RNase) from Sigma-Aldrich (Poole, UK), together with D-glucose (BDH Analar), were used as received, except for cyclodextrins, which were dried in vacuo for three days prior to use. Buffers used were 0.1 M Na phosphate, pH 7.2 (cyclodextrin/adamantanes); 0.1 M Na acetate, pH 5.5 (HEWL, RNase); 10 mM Na cacodylate, 10 mM NaCl, 2 mM $CaCl_2$, pH 6 (CCP/PA).

Cytochrome *c* peroxidase (CCP) was isolated from *Paracoccus pantotrophus* as previously described.[14] The gene for pseudoazurin (PA) was isolated from *P. pantotrophus* and expressed in *Escherichia coli* BL21(DE3) in both the wild-type form and a form in which lysine 109 had been replaced by an alanine by site-directed mutagenesis (Pauleta, unpublished).[15] For calorimetric experiments, each protein was transferred into cacodylate buffer on a Sephadex G25 column. To ensure complete equilibration, each protein then underwent repeated cycles of concentration and dilution using a Vivaspin membrane with a cutoff of 5000. The concentration of CCP was determined using the extinction coefficient of 250 mM^{-1} cm^{-1} at 408 nm; the concentration of PA was determined by measurement of amine content using the trinitrobenzene sulfonate method;[16] pseudoazurin has 11 amine groups. Other protein concentrations were determined by UV absorbance using molar extinction coefficients $\varepsilon_{280} = 37\,900$ (HEWL) and $\varepsilon_{278} = 9800$ (RNase). All other concentrations were determined by weight.

Volumetric Measurements. Partial molar volumes in aqueous solution were obtained from density measurements ($\pm 1 \times 10^{-5}$ g cm^{-3}) using a vibrating tube density meter (KEM DA-510, Kyoto, Japan) calibrated with dry air and degassed deionized water. Molal volumes of solutions (V cm^3 kg^{-1}) were calculated using $V = (1000 + mM_r)/d_s$, where M_r is the molecular mass of the solute (g mol^{-1}), m is the molality (mol kg^{-1}) of the solute, and d_s is the density of the solution (g cm^{-3}). For a mixture comprising n_w moles of water (or buffer) and n_s moles of solute, this is related to partial molar volumes by

$$V = n_w V_w^0 + n_s V_s^0$$

where V^0 (cm^3 kg^{-1}) is the partial molar volume of solvent or solute, as appropriate. V_s^0 values were determined from linear regression slopes of experimental data over a range of solute concentrations (molalities, m).

Isothermal Titration Calorimetry. Thermodynamic parameters for protein−protein, protein−ligand, or cylodextrin−adamantane complexation in solution in the 15−55 °C temperature range were measured by standard isothermal titration calorimetry procedures using a MicroCal VP-ITC (1.4 mL working volume, 320 rpm stirring), and all solutions were degassed briefly before use.[17,18] For cyclodextrin complexes, a typical experiment involved 25 sequential 10 μL injections of adamantane solution (3.0 mM) into the ITC cell containing β-cyclodextrin (0.15 mM). Control experiments involved identical injections into buffer alone (for dilution heats) or into equivalent concentrations of glucose (to check for heats arising from nonspecific interactions). Protein−ligand and protein−protein titrations followed a similar protocol, with ITC cell/syringe concentration ranges as follows: HEWL 36 μM/$(GlcNac)_3$ 450 μM; RNase 36 μM/2′-CMP 450 μM; CCP 35 μM/PA 690 μM (wild-type) or 790 μM (K109A mutant). Titration data were corrected for dilution heats (measured separately by injection of ligand or protein into appropriate buffer) and analyzed using a single-set-of-sites equilibrium binding model (MicroCal Origin) to give the apparent binding stoichiometry (N), association/dissociation constants ($K_A = 1/K_D$) and enthalpy of binding (ΔH^0). Other thermodynamic quantities were calculated using standard expressions: $\Delta G^0 = -RT \ln(K_A) = \Delta H^0 - T\Delta S^0$; $\Delta C_p = d\Delta H^0/dT$; 1 cal = 1.484 J.

Pressure Perturbation Calorimetry. Thermal expansivity (expansibility) data ($E^0 = \partial V^0/\partial T = \alpha^0 V^0$) for proteins, ligands, cyclodextrins, adamantanes, and their complexes in solution at 25 °C were obtained using a MicroCal VP differential scanning calorimeter equipped with a PPC accessory.[2] Differential heat effects (ΔQ) in response to pressure pulses (ΔP, ± 5 atm) were determined with the sample cell containing appropriate solutions of host, guest, or equimolar mixtures of the two, as well as for the buffer alone with respect to water or buffer in the reference cell. Each series of experiments comprised typically 20−50 compression/decompression cycles for each sample. However, for the more demanding CCP/PA experiments, sample solutions were removed and reloaded afresh after each 20 cycles, repeated a number of times to give accumulated data on up to 200 cycles. Integrated heat effects (ΔQ) were subsequently averaged for analysis using the expression

$$\alpha_S^0 = \alpha_0 - \Delta Q/(T\Delta P g_S V_S^0)$$

where α_S^0 and α_0 are the thermal expansion coefficients of the solute and pure solvent, respectively; g_S is the mass of the solute; V_S^0 is the partial specific volume of the solute; and T is the absolute temperature. α_0 values for pure solvent (buffer) were obtained in separate PPC experiments with pure water as reference, as described elsewhere.[2] Molar expansivities of solutes (E^0) were calculated using $E^0 = \mathrm{MW} \times V_S^0 \times \alpha_S^0$.

Solute concentrations for PPC measurements on separate components and their equimolar mixtures were as follows: 4 mM (cyclodextrin/adamantanes); 1−2 mM (HEWL); 0.1−0.2 mM (RNase); 0.15 mM (CCP/PA). Except for the CCP/PA system, these concentrations ensured complexation greater than 90−95% in experiments on equimolar mixtures. For the weaker binding CCP/PA, sample availability and solubility limitations did not allow use of higher concentrations, and complexation is estimated to be ~72% in these experiments (using binding data determined here by ITC). This was taken into account in subsequent analysis of PPC data.

Accessible surface area data for protein−ligand and protein−protein complexes were estimated from available crystallographic structures using EBI-PISA software (protein interfaces, surfaces and assemblies service at the European Bioinformatics Institute[19]) and protein structure database codes: 1rob (RNase[20]); 1hew and 1zb (HEWL[21,22]); 2c1u (CCP[23]) and 3erx (PA[24]), together with published modeling/docking predictions for the CCP−PA case.[15]

Results

Cyclodextrin/Adamantane Complexes. We used a combination of ITC, densitometry and PPC techniques to determine the binding affinities ($K_A = 1/K_D$), thermodynamics (ΔH^0, ΔS^0, ΔG^0, ΔC_p), and volumetric properties of a range of β-cyclodextrin/adamantane host−guest complexes in aqueous solution under similar conditions. To determine the generality of any observed effects, experiments were performed with a range of adamantanes with different substituent groups (positively or negatively charged under experimental pH conditions) and, in one instance, with a modified cyclodextrin (β-CDMe$_2$). In each case, we have measured separately the partial molar volumes (V^0) and expansion coefficients (α_S^0) for each component and for complex mixtures. Using K_D values to calculate the proportions of bound and free species in each case, we can extract thermal expansivities (E^0) for each species and, thereby, changes in expansivity during complexation.

ITC experiments show titration behavior typical of simple 1:1 binding of adamantanes to β-CD or β-CDMe$_2$ in solution. Thermodynamic data for binding are given in Table 1 and are comparable with previous observations on similar systems.[12,13] Binding is uniformly exothermic for all combinations under these conditions, with entropy changes that are thermodynamically favorable (ΔS^0 positive) at low temperatures but decreasing with increasing temperature and becoming negative at higher temperatures. This is accompanied by similar changes in heats of binding (ΔH^0), as reflected in the significant heat capacity changes (ΔC_p) and enthalpy/entropy compensation effects that are now recognized as the norm in such systems.[1,13,25]

Volumetric and thermal expansion data at 25 °C for cyclodextrin/adamantane complexes are shown in Table 2, together with values for the separate compounds in solution under the same conditions. Comparable literature data for these systems are sparse, but partial molar volume values determined here for β-CD and glucose (Table 2) are in agreement with published data.[26,27]

Examples of PPC heat pulses are shown in Figure 1. Integration of these responses, after appropriate baseline interpolation, gives the observed ΔQ values from which molar expansivities are derived. Baseline interpolation remains the most significant source of experimental error in these measurements, especially in experiments in which the calorimetric responses show transient positive- and negative-going signals, which are thought to arise from instrumental effects related to pressure-induced oscillations. Consequently, data were obtained by averaging over a large number of compression/decompression cycles. These experiments show consistently that molar expansivities of β-CD/adamantane complexes are significantly less than the sums of molar expansivities of the separate components under the same conditions (Table 2).

Consistently similar values for ΔE^0 were observed at 5 and 45 °C, with no significant temperature variation apparent within current levels of experimental accuracy. Control experiments on comparable mixtures of adamantane with D-glucose (to which adamantanes do not bind) show no ΔE^0 effect, indicating that the negative ΔE^0 seen with cyclodextrins is, indeed, related to formation of the host−guest complex. For β-CD/Ad-COO−, the observed ΔE^0 (-0.126 cm^3 mol^{-1} K^{-1}) is in agreement with previous PPC observations[12] and with the value ($\Delta E^0 = -0.12 \pm 0.01$ cm^3 mol^{-1} K^{-1}) determined by Taulier and Chalikian[28] by direct densitometry. Observed ΔE^0 values for different adamantanes are the same within experimental uncertainty, indicating that the effect arises predominantly from interactions between β-CD and the adamantyl group, with little interference from substituents. The decrease in expansivity for Ad-COO− binding to dimethyl-β-CD is slightly smaller than observed with unmodified cyclodextrin.

Protein/Ligand Interactions. ITC-derived thermodynamic data for the binding of the trisaccharide inhibitor $(GlcNAc)_3$ to hen egg white lysozyme and 2′-CMP to ribonuclease are given in Table 3. These are both well-characterized systems, and the data derived here to complement PPC measurements under the same conditions are consistent with previously published data.[1,17,29] Both systems show typical temperature dependence in both enthalpy and entropy of complexation, with a negative heat capacity change (ΔC_p) and significant entropy/enthalpy compensation effects that are again characteristic of macromolecular complexes in solution.

Volumetric parameters for the individual protein and ligands and for their complexes, determined by PPC and densitometry under the same conditions, are given in Table 4. Again, the most interesting observation here is that binding of specific inhibitors to enzyme active sites results in a decease in overall molar expansivity.

Protein−Protein Interactions. The interaction between wild-type pseudoazurin and cytochrome *c* peroxidase was examined by ITC and PPC under identical conditions, together with similar experiments with a more weakly interacting PA mutant as a negative control. In contrast to the other systems examined here, ITC experiments show that binding is endothermic (positive ΔH^0) and entropy-driven (positive ΔS^0) in both cases (Table 5). The binding affinity for WT-PA/CCP, corresponding to a K_D of ~16 μM, confirms previous observations[15] and implies that >70% of the protein molecules were complexed in the equimolar mixtures used for PPC. This was taken into account during analysis of PPC data (Table 6), which shows again a reduction in molar expansibility for the complex. Binding of

TABLE 1: Thermodynamic Data for Binding of Adamantane Derivatives to β-Cyclodextrins in Aqueous Solution at pH 7.2, Determined by Isothermal Titration Calorimetry

host	guest	T/°C	N	$K_A/10^3$ M^{-1}	ΔH^0/kJ mol^{-1}	ΔS^0/J mol^{-1} K^{-1}	ΔG^0/kJ mol^{-1}	ΔC_p/kJ mol^{-1} K^{-1}
β-CD	Ad-COO$^-$	15	1.00	54.3 ± 1.0	−18.43 ± 0.09	26.70	−26.10	
		25	1.00	39.0 ± 1.1	−22.13 ± 0.19	13.67	−26.19	
		35	0.96	28.5 ± 0.8	−25.73 ± 0.26	1.78	−26.27	−0.32 ± 0.02
		40	1.00	24.7 ± 0.7	−27.09 ± 0.29	−2.41	−26.32	
		45	1.04	21.6 ± 1.3	−27.49 ± 0.70	−3.44	−26.39	
		55	1.01	14.3 ± 0.4	−31.43 ± 0.46	−16.25	−26.09	
β-CD	Ad-CH$_2$COO$^-$	15	0.97	145.9 ± 7.5	−21.48 ± 0.17	24.31	−28.48	
		25	0.98	116.0 ± 3.2	−25.07 ± 0.11	12.88	−28.91	−0.36 ± 0.03
		35	0.93	81.2 ± 2.8	−29.65 ± 0.23	−2.18	−28.98	
		45	0.97	62.2 ± 2.9	−32.02 ± 0.34	−8.87	−29.20	
β-CD	Ad-NH$_3^+$	15	0.98	9.6 ± 0.6	−18.49 ± 0.81	12.11	−21.98	
		20	1.01	8.9 ± 0.3	−19.99 ± 0.44	7.36	−22.15	
		25	1.01	7.8 ± 0.2	−20.62 ± 0.39	5.33	−22.21	−0.28 ± 0.02
		30	0.99	6.5 ± 0.1	−23.25 ± 0.62	−3.71	−22.12	
		35	0.98	6.9 ± 0.4	−23.45 ± 1.23	−2.66	−22.63	
		40	1.01	4.8 ± 0.2	−25.54 ± 1.64	−11.05	−22.08	
β-CD	Ad-CH$_2$NH$_3^+$	15	0.99	49.4 ± 1.4	−22.13 ± 0.16	13.08	−25.88	
		25	1.02	36.6 ± 0.5	−25.05 ± 0.10	3.35	−26.05	−0.30 ± 0.01
		35	1.02	25.3 ± 0.9	−28.42 ± 0.37	−7.93	−25.98	
		45	1.01	16.5 ± 0.4	−30.95 ± 0.50	−16.55	−25.68	
β-CD-Me$_2$	Ad-COO$^-$	15	1.00	20.83 ± 0.5	−14.55 ± 0.16	32.18	−23.82	
		25	0.98	16.22 ± 0.4	−18.69 ± 0.22	17.92	−24.03	−0.30 ± 0.02
		35	0.98	15.45 ± 0.7	−20.61 ± 0.62	13.23	−24.71	
		45	1.05	9.38 ± 0.6	−24.22 ± 1.32	−0.08	−24.19	
		55	1.01	7.10 ± 0.5	−26.97 ± 1.88	−8.46	−24.20	

TABLE 2: Volumetric Properties of Cyclodextrins, Adamantanes, Glucose and Their Complexes/Mixtures in pH 7.2 Phosphate Buffer at 25 °C

	partial molar volume/cm^3 mol^{-1}	partial specific volume/cm^3 g^{-1}	expansion coefficient, $\alpha_S^0/10^{-3}$ K^{-1}	molar expansivity, E^0/cm^3 mol^{-1} K^{-1}	ΔE^0/cm^3 mol^{-1} K^{-1}
β-CD	706 ± 1 (lit. 705)[a]	0.62	0.732 ± 0.028	0.517 ± 0.020	
β-CD/Ad-COO$^-$	869 ± 11	0.66	0.565 ± 0.024	0.491 ± 0.021	−0.126 ± 0.031
β-CD/Ad-CH$_2$COO$^-$	877 ± 4	0.66	0.550 ± 0.026	0.482 ± 0.023	−0.153 ± 0.033
β-CD/Ad-NH$_3^+$	825 ± 2	0.64	0.594 ± 0.029	0.490 ± 0.024	−0.141 ± 0.036
β-CD/Ad-CH$_2$NH$_3^+$	839 ± 3	0.65	0.616 ± 0.033	0.517 ± 0.028	−0.162 ± 0.036
β-CD-Me$_2$	991 ± 3	0.74	0.784 ± 0.026	0.777 ± 0.026	
β-CD-Me$_2$/Ad-COO$^-$	1156 ± 8	0.76	0.711 ± 0.028	0.822 ± 0.033	−0.055 ± 0.045
Ad-COO$^-$	160 ± 2	0.89	0.625 ± 0.086	0.100 ± 0.014	
Ad-CH$_2$COO$^-$	174 ± 2	0.90	0.676 ± 0.079	0.118 ± 0.014	
Ad-NH$_3^+$	121 ± 2	0.80	0.946 ± 0.155	0.114 ± 0.019	
Ad-CH$_2$NH$_3^+$	132 ± 1	0.80	1.230 ± 0.098	0.162 ± 0.013	
glucose	114 ± 2 (lit. 112)[a]	0.63	1.071 ± 0.097	0.122 ± 0.011	
glucose/Ad-COO$^-$			0.800 ± 0.046	0.219 ± 0.013	−0.003 ± 0.022
glucose/Ad-CH$_2$COO$^-$			0.832 ± 0.049	0.240 ± 0.014	0.000 ± 0.022

[a] Literature values for β-CD and glucose.[26,27]

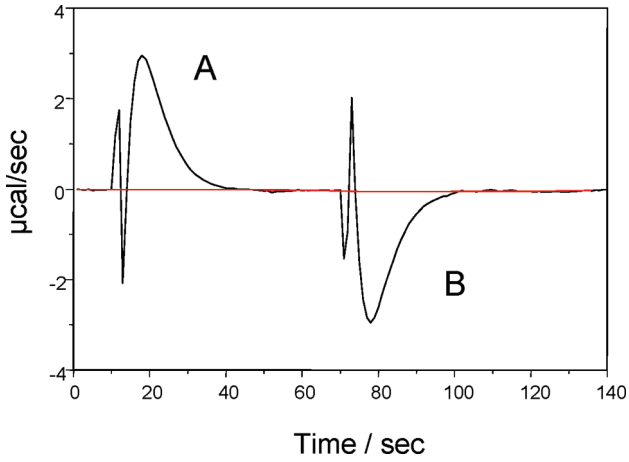

Figure 1. Examples of PPC raw data showing the thermal response obtained upon pressure perturbation of a solution of β-cyclodextrin at 25 °C: (A) decompression $\Delta P = -75$ psi, (B) compression $\Delta P = +75$ psi. Integration with respect to the interpolated baseline (red) gives ΔQ for each pressure pulse. 1 cal = 4.184 J.

the K109A PA mutant was much weaker under these conditions ($K_D \sim 550$ μM; Table 5), and PPC experiments showed no significant difference in measured E^0 compared with what would be expected from the sum of the separate components.

The PPC experiments are particularly challenging in this case because of the relatively low concentrations of proteins used (limited by sample availability and solubility) and the relatively small magnitudes of the effects in proportion to total protein volume. Consequently, to verify the statistical significance of the observed expansibility changes, we have subjected the ΔE^0 data to more rigorous analysis using statistical bootstrapping techniques This involves comparison of the experimental distribution of thermal expansibilities for the mixed solution of the two proteins to a distribution obtained by random pairwise combinations within the range of measurements of individual thermal expansivities for the two proteins. The resulting distributions (Figure 2A), derived by comparing 10^7 random combinations of sums of pairs of E^0 values (120 samples) with actual results for the protein−protein mixtures clearly show that the observed ΔE^0 for the cytochrome c peroxidase interaction

TABLE 3: Thermodynamic Data for Protein−ligand Interactions in 0.1M Na Acetate Buffer, pH 5.5

protein	ligand	T/°C	N[a]	$K_A/10^3$ M^{-1}	ΔH^0/kJ mol^{-1}	ΔS^0/J mol^{-1} K^{-1}	ΔG^0/kJ mol^{-1}	ΔC_p/J mol^{-1} K^{-1}
lysozyme	$(GlcNAc)_3$	15	1.01	236 ± 10	−53.3 ± 0.6	−82.1	−29.62	
		25	0.99	120 ± 5	−58.5 ± 1.0	−99.2	−28.97	−674 ± 134
		35	0.94	64 ± 1	−62.2 ± 1.6	−109.9	−28.33	
		45	0.83	29 ± 2	−74.5 ± 6.3	−148.7	−27.25	
RNase	2′CMP	15	0.92	1949.0 ± 95.3	−58.79 ± 0.30	−83.6	−34.70	
		25	0.89	857.4 ± 40.5	−66.57 ± 0.47	−109.6	−33.09	−609 ± 99
		35	0.90	342.2 ± 14.9	−74.39 ± 0.76	−135.5	−32.65	
		45	0.92	151.7 ± 8.1	−76.48 ± 1.81	−141.2	−31.57	

[a] Error estimates for N returned by MicroCal Origin ITC fitting procedures are typically ±0.01 or lower. However, we believe that these are unrealistically small and do not take into account systematic errors arising from uncertainties in sample concentration and purity; consequently, we have chosen not to quote them here. More realistic error estimates for N are likely to be on the order of ±0.05 for experiments of this kind.

TABLE 4: Experimental Volumetric Parameters for Proteins, Ligands, And Their Complexes, Determined at 25 °C, 0.1 M Acetate, pH 5.5

	partial molar volume/cm^3 mol^{-1}	partial specific volume/cm^3 g^{-1}	expansion coefficient $\alpha_S^0/10^{-3}$ K^{-1}	molar expansivity E^0/cm^3 mol^{-1} K^{-1}	ΔE^0/cm^3 mol^{-1} K^{-1}
lysozyme	10 225 ± 29	0.72	0.582 ± 0.017	5.954 ± 0.175	
$(GlcNAc)_3$	453 ± 15	0.72	0.717 ± 0.067	0.325 ± 0.032	
Lys/$(GlcNAc)_3$	10 535 ± 45	0.71	0.553 ± 0.018	5.824 ± 0.190	−0.455 ± 0.06
RNase	9 645 ± 27	0.70	0.695 ± 0.024	6.704 ± 0.228	
2′-CMP	193 ± 12	0.60	1.594 ± 0.167	0.307 ± 0.037	
RNase/2′-CMP	9 859 ± 193	0.70	0.676 ± 0.024	6.662 ± 0.270	−0.349 ± 0.05

TABLE 5: Thermodynamic Parameters for Binding of Wild-Type and Mutant Pseudoazurin to Cytochrome *c* Peroxidase in 10 mM Cacodylate Buffer, pH 6.0, 10 mM NaCl, 2 mM $CaCl_2$

protein 1	protein 2	T/°C	N	$K_A/10^3$ M^{-1}	ΔH^0/kJ mol^{-1}	ΔS^0/J mol^{-1} K^{-1}	ΔG^0/kJ mol^{-1}
Cyt *c* peroxidase	WT-pseudoazurin	25	0.86	61 ± 2	16.1 ± 0.4	146	−27.3
Cyt *c* peroxidase	K109A-pseudoazurin	25	(1)[a]	1.8 ± 0.06	243 ± 6	878	−18.6

[a] ITC data fit assuming $N = 1$ in this weak binding case. Note that ΔH^0 and ΔS^0 estimates for the mutant protein should not be considered reliable in these circumstances since they are poorly defined by the MicroCal Origin fitting procedure (although the signs will be correct).

TABLE 6: Volumetric Parameters for Cytochrome *c* Peroxidase, Wild-Type and Mutant Pseudoazurin, and Their Complex Mixtures

	partial molar volume/cm^3 mol^{-1}	partial specific volume[b]/cm^3 g^{-1}	expansion coefficient $\alpha_S^0/10^{-3}$ K^{-1}	molar expansivity E^0/cm^3 mol^{-1} K^{-1}	ΔE^0/cm^3 mol^{-1} K^{-1}
Cyt *c* Peroxidase (200)[a]	27 452	0.7318	0.6060 ± 0.0005	16.637 ± 0.013	
WT-pseudoazurin (200)	9 896	0.7382	0.5943 ± 0.0012	5.881 ± 0.012	
CCP/PA mix (120)	37 348	0.7335	0.5950 ± 0.0004	22.238 ± 0.015	(−0.28)
corr to 100% complex[c]			0.5880 ± 0.0004	21.961 ± 0.015	−0.56 ± 0.04
Cyt c Peroxidase (40)			0.6191 ± 0.0009	16.995 ± 0.024	
K109A-pseudoazurin (40)			0.6832 ± 0.0034	6.761 ± 0.033	
CCP/K109A-PA mix (40)			0.6375 ± 0.0006	23.809 ± 0.03	0.053 ± 0.08

[a] Numbers of PPC pressure cycles in parentheses. [b] Partial specific volumes were obtained from amino acid compositions, as used in previous biophysical investigations of these interactions.[15] [c] After correction of the observed ΔQ for the fraction (28%) remaining uncomplexed in the mixture, taking into account the buffer displacement contributions.

with wild-type pseudoazurin is statistically significant at the $P < 0.0001$ level. Similar analysis of PPC data with the weakly binding K109A PA mutant (Figure 2B) shows no significant difference.

Discussion

We have examined the thermodynamic and volumetric changes associated with noncovalent association of molecules in aqueous solution, utilizing three different systems covering a wide range of size and various kinds of interaction. Cyclodextrin host−guest complexation is usually described in terms of relatively nonspecific hydrophobic and van der Waals forces between the cyclodextrin cavity and the nonpolar guest (the adamantoyl group in this case), although, as others have pointed out,[13,30] the thermodynamic binding parameters (ΔH^0, ΔS^0, etc.) are not necessarily typical of conventional hydrophobic interactions. The lysozyme/$(GlcNAc)_3$ active site complex involves a more specific protein−ligand hydrogen bonding network, including bound waters, together with stacking interactions between aromatic residues and nonpolar faces of the trisaccharide inhibitor (PDB: 1hew[21] and 1lzb[22]), whereas RNase/CMP binding includes also significant electrostatic and hydrophobic components (PDB: 1rob[20]). All these interactions are observed here to be exothermic and enthalpy driven, with significant heat capacity changes. The cytochrome *c* peroxidase/pseudoazurin interaction involves a much larger intermolecular interface, comprising a broad spectrum of noncovalent forces, but thought to be guided predominantly by electrostatics. The binding in this case is endothermic and entropy-driven. We have suggested elsewhere,[15] in line with conventional wisdom, that the large positive ΔS^0 in this case is consistent with displacement of significant numbers of water molecules from the protein interfaces. However, changes in molecular surface hydration

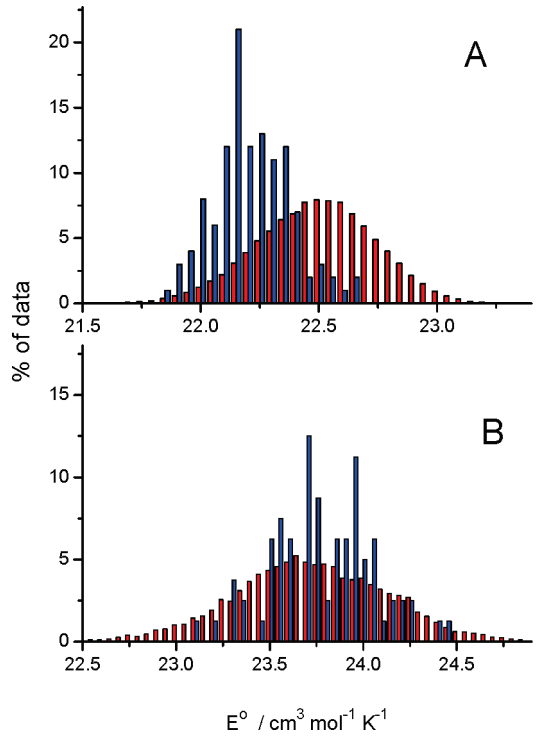

Figure 2. Bootstrap analysis of thermal expansivity data for cytochrome *c* peroxidase and pseudoazurins. (A) Distribution of 120 observed E^0 values for CCP/wild-type PA mixtures (blue) together with computed values for 10^7 random summed pairings of the thermal expansivities for the two separate proteins (red). The mean E^0 for the mixtures is 22.238 (±0.015) compared with 22.518 for the random pairings. (B) Similar distributions for CCP/mutant PA mixtures (40 samples). The mean E^0 for the mixtures in this case is 23.809 (±0.03) compared with 23.750 for the random pairings. Data were grouped at 0.05 E^0 intervals for histogram analysis.

must be involved in all the interactions looked at here, even though this is not always directly apparent in the thermodynamic parameters.

Despite this diversity in molecular size and nature of the interactions, all share similar volumetric signatures. In particular, formation of the noncovalent complex is in all cases accompanied by a measurable decrease in molar expansibility, and it was anticipated that this might reflect, at least in part, solvation/hydration changes. Following Chalikian,[10] we can imagine the apparent volume of a molecule in solution to be made up of two components:

$$V^0 = V_M + \Delta V_h = V_M + n_h(V_h - V_o)$$

where V_M is the intrinsic (i.e., physical) volume of the solute molecule, and ΔV_h is the total hydration-induced change in the volume of solvent. The latter might be visualized as arising from a solvation layer of n_h solvent molecules with average molar volume (V_h) compared to the bulk solvent (V_o). It then follows that the apparent thermal expansivity also comprises two components, one arising from thermal expansion of the intrinsic volume of the molecule itself, plus a second term reflecting changes in the solvation layer:

$$E^0 = \partial V^0/\partial T = \partial V_M/\partial T + \partial \Delta V_h/\partial T = E_M + n_h(E_h - E_0)$$

where E_h is the average molar expansivity of the n_h water molecules in the hydration shell, and E_o is the expansivity of water molecules in the bulk phase. Thermal expansion generally arises from temperature-induced changes in mean interatomic or intermolecular distances, specifically, at least in the case of solids, from the anharmonicity of interatomic potentials. Consequently, because covalent bond vibrations are not excited at normal temperatures, changes in intrinsic volume ($E_M = \partial V_M/\partial T$) should be very small for rigid, covalent molecules (for example, the cyclodextrin:adamantane system). It therefore follows, at least for rigid molecules in solution, that observed expansibility (E^0) effects should reflect predominantly the volumetric properties of the solvation layer (i.e., $\partial V_M/\partial T = 0$, $E^0 \approx \partial \Delta V_h/\partial T$). Moreover, of particular significance in the present context, changes in expansibility during complexation (ΔE^0) would then be directly related to solvation changes.

$$\Delta E^0 \approx \Delta n_h(E_h - E_0)$$

where, to the extent that this underlying assumption is valid, Δn_h would be the number of water molecules displaced from the solvation layers of the solute molecules during binding.

Estimates for $(E_h - E_o)$ are difficult to obtain unambiguously, but typically, on the basis of the volumetric properties of small molecules in water,[28,31] lie in the range 0.006−0.01 cm^3 mol^{-1} K^{-1}. For the β-CD complexes, using the PPC-derived ΔE^0 data from Table 2, this leads to estimates of Δn_h in the range of 15−25 water molecules released upon adamantane binding. These numbers are consistent with expectations from structural considerations and are in agreement with estimates obtained for the β-CD/Ad-COOH complex using volumetric and osmotic stress-related techniques, even though different techniques might sample different populations of hydrated water.[28,32] We see no significant differences in Δn_h estimates for the different adamantanes interacting with β-CD, indicating that the number of water molecules displaced by insertion of the adamantyl group into the cyclodextrin cavity is not particularly sensitive to the nature of the polar substituents which, on structural grounds, are expected to lie outside the β-CD cavity.[13]

In contrast, the Δn_h estimate (5−10) for the modified cyclodextrin complex, β-CD-Me$_2$/Ad-COOH, appears lower than observed for unmodified β-CD. This may reflect the interesting observations from crystal structures that hydrated dimethyl-β-cyclodextrin shows just one water molecule in the CD cavity[33] compared with about seven waters in the cavity of unmodified β-CD.[34] Consequently, assuming that the hydrated crystal structures are reasonable representations of the situation in solution, the total number of water molecules displaced upon complexation should be less for β-CD-Me$_2$, as observed.

Similar calculations for protein−ligand and protein−protein expansibilty changes lead to estimates of Δn_h in the range 46−76 (HEWL/(GlcNAc)$_3$), 35−58 (RNase/CMP), and 56−93 (CCP/PA). Interestingly, these estimates are somewhat smaller than those obtained for similar protein−ligand binding systems using other volumetric techniques,[10,35−38] again reflecting the possibility that different techniques sample different levels of solvation.[10,28,32] Rough estimates for the numbers of water molecules potentially displaced can be obtained from calculations of the decrease in solvent accessible surface areas (ΔASA) in the complexes. Assuming an area of 10 Å^2 for each water molecule in a solvation monolayer,[32] together with ΔASA estimates from crystal structures, we calculate Δn_h = 97 (HEWL/(GlcNAc)$_3$), 52 (RNase/CMP), and 205 (CCP/PA), somewhat higher than estimates from our experimental expansibility changes. It should be borne in mind here that interfacial solvation effects are likely to extend beyond the first hydration

layer, so naive monolayer estimates such as these can only be a rough guide.

One possible explanation for the apparent discrepancy in estimated Δn_h values is that the value of $(E_h - E_0)$ used here, obtained from published studies on small molecules, is not appropriate for hydration layers around larger molecules, such as proteins. Unfortunately, it is difficult to probe this directly, especially because such considerations are contingent upon the assumption that changes in intrinsic volume/expansibilty are small enough to be ignored in comparison with solvation effects. This is probably reasonable in the case of cyclodextrin/adamantane complexes, as has also been assumed by others.[28] However, it is not clear that this is necessarily true for other systems. Indeed, recent work has shown that even with cyclodextrins, binding of Ad-COOH to the larger and more flexible γ-cyclodextrin results in a more highly compressible complex compared with the equivalent β-CD/adamantane complex.[39] This will be generally true in most situations, and especially in the case of interactions involving inherently more flexible macromolecules, such as proteins, in which changes in intramolecular packing, conformation, and dynamics could all contribute to volumetric changes. For example, in their study of compressibility and volume changes of lysozyme on inhibitor binding, Gekko and Yamagami interpreted their findings in terms not only of solvent displacement but also of diminished structural fluctuation in the protein.[40] The situation with proteins is further complicated by uncertainties relating to possible contributions from void volumes within the protein structure and surrounding solvent: the so-called thermal volume contribution.[11,35]

One way to address this issue is to examine how expansibility data vary with the extent of molecular surface area involved in the interaction. To a first approximation, if all contributions other than solvation changes were negligible, one might anticipate that the numbers of solvent molecules displaced during binding, and consequently their contribution to ΔE^0, should be correlated, possibly linearly, with the change in solvent accessible surface area (ΔASA) upon binding. A plot of experimental ΔE^0 values against computed ΔASA (Figure 3) reveals some interesting features. First, although there appears to be a general trend toward a larger ΔE^0 effect with an increase in buried accessible surface area, the variation is not what one might naively expect to arise from solvation changes alone. In particular, for the protein-based systems, the variation in expansibility differences (ΔE^0) is relatively small and does not extrapolate to zero at zero ΔASA, as one might otherwise expect for purely hydration layer contributions. Moreover, for the intrinsically more rigid cyclodextrin/adamantane complexes, the observed ΔE^0 values deviate from the trend, being smaller in magnitude than one would interpolate for protein systems of similar ΔASA.

It is, of course, naive to assume that all hydration waters behave the same. As noted above with regard to Δn_h estimates, E_h may be different for hydration layers around polar groups, as compared with nonpolar groups, for example, and this may be a sufficiently plausible, if unsatisfactory (because hard to prove or disprove), rationalization of the trends illustrated in Figure 3. There may also be particular instances when water molecules may be retained or incorporated into the hydrogen-bonded or solvated lattice during binding (rather than displaced) for specific structural or functional requirements. Nonetheless, the trends are interesting and demand closer consideration of possible additional contributions from intrinsic expansibility (E_M) effects.

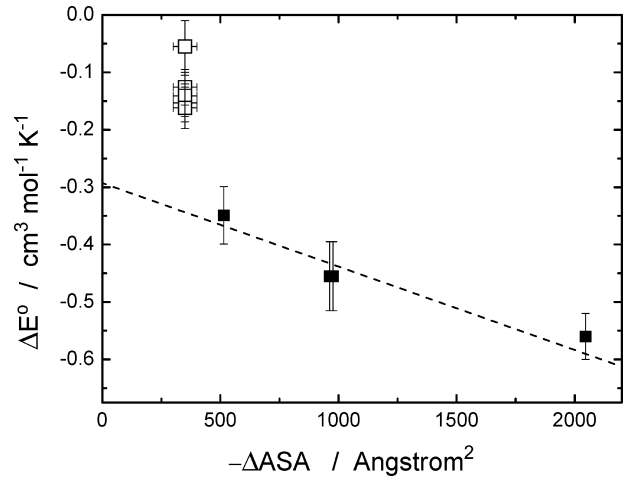

Figure 3. Correlation of changes in molar expansivity (ΔE^0) with loss of solvent accessible surface area (ΔASA) upon binding. Accessible surface area data for protein−ligand and protein−protein complexes (solid symbols) were estimated from available crystallographic structures using EBI-PISA software (protein interfaces, surfaces and assemblies service at the European Bioinformatics Institute[19]) and protein structure database codes: 1rob (RNase[20]); 1hew and 1zb (HEWL[21,22]); 2c1u (CCP[23]) and 3erx (PA[24]) together with published modeling/docking predictions for the Cyt *c* P-pseudoazurin case.[15] Appropriate structural data are not available for cyclodextrin/adamantane complexes (open symbols), but calculated ΔASA values for a range of similar guests all cluster around 300−400 Å^2.[49,50] The dashed line is simply a guide to the eye, indicating approximate linear extrapolation of protein data. Error bars are estimated from standard deviations of multiple ΔQ measurements, omitting absolute systematic errors due to concentration uncertainties, etc., that cancel in ΔE^0 differences.

Thermal expansion happens because of heat-induced increases in interatomic or intermolecular distances that might arise from thermal excitation of anharmonic molecular motions (dynamic effects), thermal redistribution of molecules or groups into different structural environments (conformation/packing effects), or both. Expansibility changes upon binding will involve all these contributions, although so far we have considered only possible contributions from redistribution of solvent molecules. Although this is a reasonable approximation for rigid small covalent molecules, it is unlikely to be valid for proteins and other macromolecules that are stabilized by weak noncovalent interactions and prone to significant thermodynamic fluctuations and a multiplicity of thermally induced soft anharmonic motions.[41−44] Moreover, it is now well established that binding to proteins almost invariably leads to a reduction in conformational dynamics and fluctuations.;[41,42,45,46] exactly the same kind of thermally excited fluctuations that determine expansibility.

But here we encounter an apparent paradox. Judging from surface area (ΔASA) correlations (Figure 3), the ΔE^0 values that we observe for protein binding systems are larger (more negative) than would be anticipated in comparison with the more rigid complexes. It is tempting to speculate that this represents the additional contributions from intrinsic expansibility changes in the protein systems, and it would be consistent with the more rigid structures indicated (in part) by the reduction in heat capacities upon binding. On the other hand, our estimates of the numbers of displaced waters obtained from experimental ΔE^0 values are less than would be expected from surface hydration considerations and volumetric measurements on comparable protein binding systems. As already mentioned, this is possibly due to the use of inappropriate values of $(E_h - E_o)$ (0.006−0.01 cm^3 mol^{-1} K^{-1}), with the indication being that smaller values might be more appropriate for water layers

around proteins to rationalize our observations. Interestingly, this is in line with the lower estimate of $(E_h - E_o) \sim 0.002$ cm^3 mol^{-1} K^{-1} obtained by Chalikian[10] from consideration of lysozyme hydration, including estimates of intrinsic volume effects.

However, this still leaves unresolved the potential contributions of intrinsic expansibility changes to our ΔE^0 observations. Assuming Δn_h is proportional to ΔASA, the protein data in Figure 3 indicate either that no single value for $(E_h - E_o)$ fits all cases or we are still missing contributions from changes to intrinsic macromolecular expansibility that cannot be determined from these experiments alone. This adds to the growing consensus that estimates of other parameters based on ΔASA correlations may also be in error if contributions from conformational dynamics are not properly accounted for. In particular, this may apply to estimates of thermodynamic binding and stability parameters in which initially promising correlations with ΔASA have not proved universally applicable.[47−49] This may be especially relevant for proper understanding of heat capacity (ΔC_p) effects, since, like expansibility, these depend on thermally accessible molecular motions as well as solvation changes.

Conclusions

1. Pressure perturbation calorimetry can be used to measure thermal expansibilities of noncovalent complexes in water in comparison with their uncomplexed species.

2. Results show that a small reduction in molar expansibility is seen in all cases, regardless of the size or nature of the interacting species.

3. Although expansibility changes can be related to changes in solvation, giving physically reasonable estimates for the numbers of displaced water molecules in cyclodextrin/adamantane complexes, the interpretation is less convincing with more flexible protein−ligand and protein−protein interactions.

4. Comparison of expansibility changes with changes in solvent accessible surface areas indicates that additional global effects must be considered in these more flexible molecules.

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