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Microcalorimetry and the molecular recognition of peptides and proteins

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Isothermal titration (ITC) and differential scanning calorimetry (DSC) techniques are now routinely applicable to the study of non-covalent interactions in biomolecular recognition. Examples from our own current work on peptide antibiotic interactions and protein folding illustrate what may be achieved. ITC binding studies of vancomycin antibiotics with model peptides give information about the thermodynamics of group interactions, and also demonstrate possible complexities due to ligand-induced aggregation processes. The thermal stability of proteins in mixed aqueous solvents, studied by DSC, shows how the balance of forces responsible for folding stability may switch, without markedly perturbing the native structure. Separate experiments on the molecular recognition of unfolded polypeptide chains by cyclodextrins are consistent with simple binding of these cyclic polysaccharides to exposed aromatic groups on the thermally denatured protein.

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1. Introduction

Biomolecular recognition processes rely on a subtle balance of non-covalent forces to control conformation and mediate binding. The folding of polypeptides and other biopolymers into unique conformations constitutes a molecular self-recognition process in which the system, comprising macromolecule plus solvent, recognizes that, for certain specific polymer sequences, limited regions of conformation space are more favourable. Once folded, these specific conformations are then likely to provide specific binding sites for the recognition of other molecules (ligands) that themselves depend on non-covalent interactions. This is fundamentally a thermodynamic problem. Although we might be able to name the sort of interactions involved (H-bonds, hydrophobic, electrostatic, van der Waals forces, etc.) we, as yet, have little success in predicting their relative contributions *ab initio*. This arises because, unlike covalent forces that are overwhelmingly enthalpic and can be computed with quite high precision, the free energy changes associated with non-covalent interactions involve a delicate balance between (often large) enthalpic and entropic contributions in which solvent molecules play a major role.

There are several consequences of this. From the molecular modelling point of view the lack of appropriate 2-body potential functions describing non-covalent forces leads to computational difficulties. Indeed, the inherently many-body nature of non-covalent interactions means that solvent must be explicitly included in any attempts to describe the overall energetics. And, indeed, even quite simple changes in solvent can alter the balance and nature of the interactions quite significantly. For example, as we shall see below, in recent work on the folding of a small protein we have shown

how transfer from aqueous to water/methanol mixtures might change the balance of the apparent stabilizing interactions: the hydrophobic effect is reduced, and H-bonding contributions increased – at least as a first approximation – without significantly affecting the native conformation of the protein (Woolfson *et al.* 1993).

In such a complex theoretical situation it is particularly important that we have reliable empirical approaches based on measurement of thermodynamic parameters for appropriate systems under relevant conditions. Although calorimetry gives the most direct thermodynamic information, until relatively recently the techniques were rather insensitive and tedious to use for most biological systems, and we had to rely on less satisfactory indirect techniques. However, instrumental speed and sensitivity has improved dramatically in recent years so that direct calorimetric studies of interesting biomolecular systems is now routine, and instruments are commercially available for both isothermal titration (ITC) and differential scanning (DSC) work (Privalov & Potekhin 1986; Sturtevant 1987; Wiseman *et al.* 1989; Wadsö 1991; Cooper & Johnson 1993, ch. 9–11). Here we will illustrate current applications and salient points of the technique with some recent examples from our own work on topics related to the folding and interaction of proteins and peptides, including studies on the effects of cyclic polysaccharides (cyclodextrins) and non-aqueous solvents on protein stability, and (in collaboration with Dr D. H. Williams and his group) measurements on the vancomycin class of antibiotics.

2. Isothermal titration microcalorimetry

ITC is used typically to study the thermodynamics of binding and other processes that can be initiated by mixing in solution. Earlier methods, based on the 'heat-leak' principle pioneered in the biomolecular field by Sturtevant (1974) and Wadsö (1983, 1991), have been superceded by more recent instruments using efficient mixing and active feedback methods (Wiseman *et al.* 1989), or kinetic deconvolution techniques (Bastos *et al.* 1991), that are much faster and rather more convenient in use. Typical biomolecular thermal titrations involving, say, 20 or more injections of ligand into a macromolecule solution may now be done in 1 h, or less, using reasonable amounts of material, and with an ultimate working sensitivity of the order of 5 μJ per injection. Current work in our laboratory includes measurement of enzyme–substrate complexes (McAuley-Hecht & Cooper 1993), protein–nucleic acid interactions, and, as described below, peptide antibiotic binding.

(a) Peptide antibiotic interactions

The binding of small peptide analogues of bacterial cell wall components to the vancomycin class of antibiotics is of both clinical and academic interest (Wright & Walsh 1992), providing a potentially simple experimental model for biomolecular recognition studies. Dudley Williams and his group have done much to clarify the structural details by high resolution NMR and are now showing how the basic interactions may be broken down into the component parts (Williams *et al.* 1990, 1991, 1993). To complement this work we are now doing systematic calorimetric measurements of these binding processes in solution.

The experiments are, in principle, relatively straightforward. Sequential addition of ligand (peptide) solution to the calorimeter cell containing antibiotic (or vice versa) gives a series of heat pulses associated with binding. In simple cases the size of each
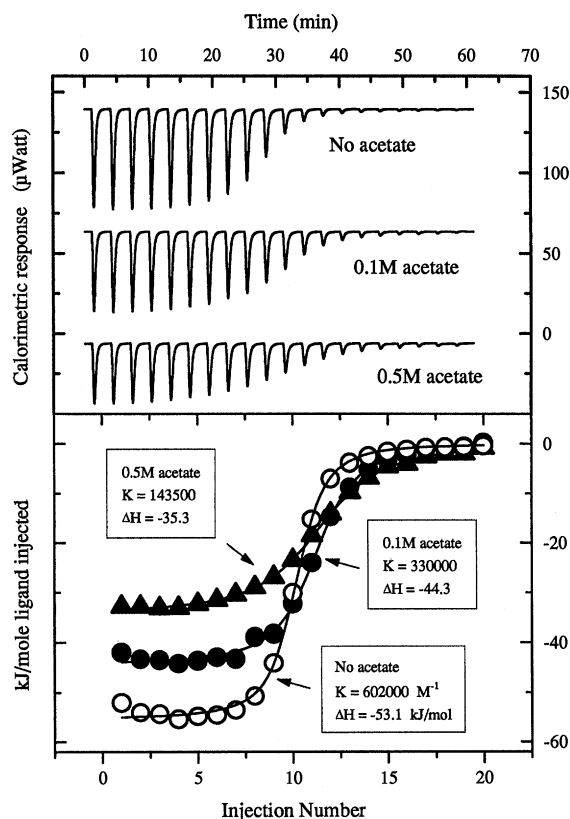
Figure 1. Isothermal calorimetry data for the titration of vancomycin (0.3 mM) with 20×5 µl injections of N,N′-diacetyl-L-lys-D-ala-D-ala (7 mM) in 0.1 M phosphate buffer, pH 7, at 25 °C. The upper part of the figure gives the raw data showing the sequence of exothermic heat pulses for three separate titrations at different acetate concentrations. The lower figure gives the integrated heat energy per injection, together with theoretical fits to each thermal titration curve, illustrating the competitive inhibition by acetate. The reaction cell volume of the calorimeter is about 1.4 ml.

heat pulse (after appropriate corrections for heats of mixing and dilution) is proportional to the extent of complex formation and the enthalpy (ΔH) of the process. Consequently one obtains a thermal titration curve which may be analysed to give both ΔH and the binding constant (K), which immediately leads directly to ΔG^0 and ΔS^0. Examples showing the isothermal titration microcalorimetry of binding of di-N-acetyl-L-Lys-D-Ala-D-Ala (DALAA) to vancomycin under various conditions are shown in figure 1. Measurements at different temperatures give the temperature dependence of ΔH (ΔC_p), which may be significant, and data for a range of ligands are given in table 1.

Though the range of ligands studied is still far from comprehensive, data such as these form the basis for attempts to determine the individual functional group components of the interaction, and the availability of enthalpy and entropy data, in addition to free energy, will be a stringent test of such predictions. Some such small group contributions can be obtained experimentally. For example, what is the contribution of the peptide $-COO^-$ group to the overall interaction? Binding of the acetate anion itself is rather too weak to measure directly by calorimetry – or, rather, the acetate concentrations required in the titration are so high that heats of dilution

Table 1. *Thermodynamic parameters for binding of assorted peptide ligands to vancomycin at 25 °C, determined by isothermal titration microcalorimetry at pH 7.0 in 0.1 M phosphate buffer*
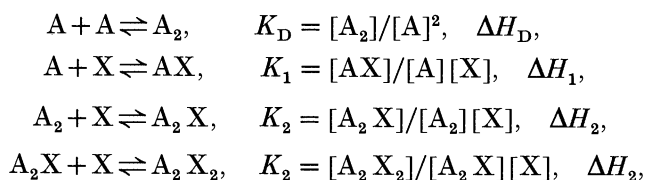
(Measured with an antibiotic concentration of 0.1 mM, or less, in cases where aggregation is significant.)

Ligand	K M^{-1}	ΔG^0 kJ mol^{-1}	ΔH kJ mol^{-1}	ΔS^0 J K^{-1} mol^{-1}	ΔC_p J K^{-1} mol^{-1}
N-acetyl-gly-gly	*ca.* 200	*ca.* −13	*ca.* −35	*ca.* −74	—
N-acetyl-D-ala	300	−14.1	−35.8	−73	525
N-ac-D-ala-D-ala	6 600	−21.8	−35.6	−46	330
N-ac-gly-D-ala	5 600	−21.4	−35.6	−48	—
N,N′-diacetyl-L-lys-D-ala-D-ala	520 000	−32.6	−53.3	−69	—
N-fumaryl-D-ala	2 900	−19.8	−43.9	−81	—
N-succinyl-D-ala	1 250	−17.7	−42.9	−85	—
acetate[a]	6.5	− 4.6	−24	−65	—

[a] Determined by competitive binding with N,N′-diac-L-lys-D-ala-D-ala

obscure the desired binding heats – but it can be determined by competition experiments. Figure 1 shows that increasing concentrations of acetate in the reaction mixture progressively inhibit binding to vancomycin of the much stronger ligand, DALAA. The apparent enthalpy of DALAA binding is also reduced, and the effect is seen also with other antibiotics. The data are consistent with simple competitive binding of acetate and DALAA for the same antibiotic binding site, so that titration with DALAA in the presence of acetate requires displacement of the acetate, and may be analysed to give the acetate binding parameters shown in table 1.

Here is not the place to attempt any interpretation of these data, but it is important to point out some of the complications that can arise. For example, during the course of these experiments it became clear that the binding of many ligands was not a straightforward process. In particular, the apparent K and ΔH values showed some dependence on total antibiotic concentration and, in some cases at higher antibiotic concentration, the thermal titration curves showed anomalous behaviour (figure 2) inconsistent with simple 1:1 complex formation. Such observations suggest that aggregation of the antibiotics is significant even at the relatively low concentrations used in these experiments (0.04 to 1 mM), and it is for this reason that previous calorimetric measurements using less sensitive techniques show some differences with the results presented here (Rodríguez-Tebar *et al.* 1986; Arriaga *et al.* 1990). Ligand-induced changes in the aggregation state of the antibiotics have also been inferred from NMR experiments (Waltho & Williams 1989; Gerhard *et al.* 1993). The calorimetric observations may be described in terms of a simple ligand-induced dimerization or dissociation model which, in the simplest case, assumes two identical, non-interacting binding sites on the antibiotic dimer. This involves the following equilibria (A = antibiotic, X = ligand):
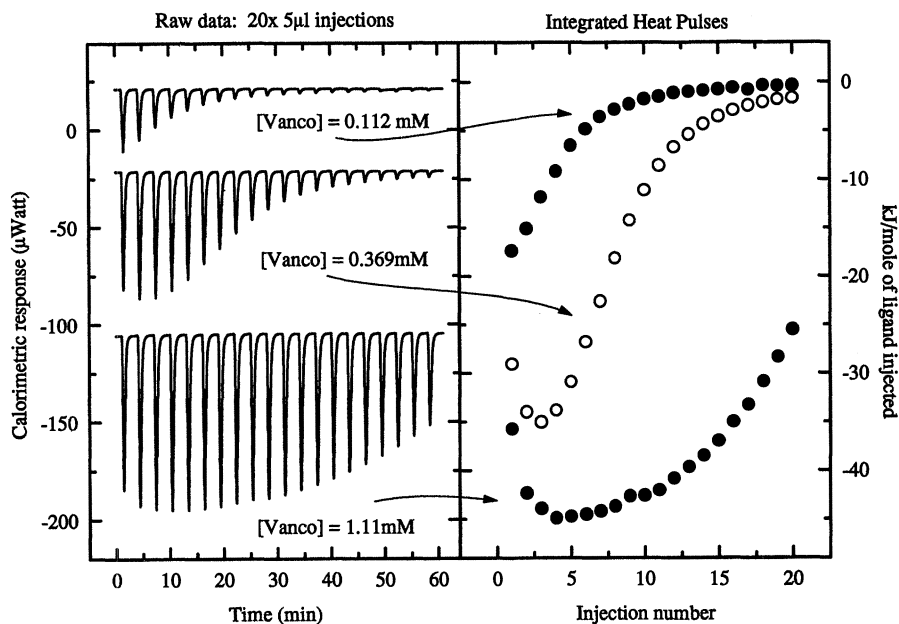
$$A + A \rightleftharpoons A_2, \qquad K_D = [A_2]/[A]^2, \quad \Delta H_D,$$

$$A + X \rightleftharpoons AX, \qquad K_1 = [AX]/[A][X], \quad \Delta H_1,$$

$$A_2 + X \rightleftharpoons A_2X, \qquad K_2 = [A_2X]/[A_2][X], \quad \Delta H_2,$$

$$A_2X + X \rightleftharpoons A_2X_2, \qquad K_2 = [A_2X_2]/[A_2X][X], \quad \Delta H_2,$$

Figure 2. ITC data for binding of N-acetyl-D-ala-D-ala to vancomycin at 25 °C, pH 7. Each experiment consists of an identical sequence of $20 \times 5\mu l$ injections of peptide ligand (12.3 mM) into vancomycin solutions at three different concentrations (0.112, 0.369 and 1.11 mM).

and

$$AX + AX \rightleftharpoons A_2 X_2, \quad K'_D = [A_2 X_2]/[AX]^2, \quad \Delta H'_D.$$

Not all these parameters are independent, of course. For example:

$$K'_D = K_D(K_2^2/K_1^2) \quad \text{and} \quad \Delta H'_D = \Delta H_D + 2(\Delta H_2 - \Delta H_1),$$

which emphasises an important principle of thermodynamic linkage (Wyman 1964) that, in this case for instance, if ligand binding is more favourable to the dimer $(K_2 > K_1)$ then ligand binding will also induce dimerization $(K'_D > K_D)$, and vice versa.

With so many free variables it has not yet been possible for us to obtain unique sets of parameters by fitting the data. We can, however, model the observations with physically reasonable numbers that may be tested by other kinds of experiment. For example, the vancomycin/N-ac-D-ala-D-ala data of figure 2 may be modelled approximately by the following parameters:

$$K_1 = 5000 \, \text{M}^{-1}, \qquad \Delta H_1 = -37 \, \text{kJ mol}^{-1},$$
$$K_2 = 100000 \, \text{M}^{-1}, \quad \Delta H_2 = -58 \, \text{kJ mol}^{-1},$$
$$K_D = 10 \, \text{M}^{-1}, \qquad \Delta H_D = 0,$$

giving

$$K'_D = 4000 \, \text{M}^{-1} \qquad \Delta H'_D = -42 \, \text{kJ mol}^{-1}.$$

This implies that this ligand binds preferentially to the vancomycin dimer, and that the relatively weak tendency toward dimerization of the free antibiotic is significantly enhanced by the presence of peptide ligand. We should emphasize that these are model parameters only, but similar order of magnitude estimates are found for other ligands studied.

Although we have concentrated here on the observations with vancomycin, we have also done most of these experiments with other members of the vancomycin family, including ristocetin, eremomycin, and derivatives. However, despite their familial relation, their behaviour towards ligand binding can be quite different. For example, with ristocetin we also observe anomalous concentration-dependent ligand binding isotherms but, in this case, the data are more consistent with ligand-induced dissociation, rather than the association we see with vancomycin. This illustrates the potential complexity of even apparently simple molecular recognition systems, and emphasises the difficulties in generalizing from one system to another.

3. Differential scanning calorimetry

Differential scanning calorimetry is a relatively simple and familiar technique involving measurement of the differential heat energy uptake in the sample during a change in temperature. For systems showing a sharp thermal transition, such as lipid bilayer phase changes, DNA/RNA melting, or protein unfolding transitions, the resultant peak in excess heat capacity may be analyzed to give overall enthalpy, heat capacity, and other thermodynamic components of the process. The main technical difficulty with biological materials is that one ideally needs to work in dilute (less than 1 mg cm^{-3}) aqueous solutions where the heat capacity of the sample may be overwhelmed by that of the water, but specialist instruments based on the designs of Privalov and Brandts are available (Privalov & Potekhin 1986; Cooper & Johnson 1993).

(a) Protein folding forces

Protein folding and unfolding has been extensively characterized by DSC studies, in parallel with other methods, though the precise balance of thermodynamic forces responsible for the stabilization of protein conformations is still unclear: see Dill (1990) for a review.

Building upon earlier work by Velicelebi & Sturtevant (1979) we have recently looked at the thermal unfolding of a small protein, ubiquitin, in mixed water/methanol mixtures (Woolfson *et al.* 1993). High resolution NMR of this protein has shown that it retains its native conformation even up to 40% MeOH. In the absence of methanol, ubiquitin is quite stable, but undergoes a cooperative thermal unfolding transition at elevated temperatures (figure 3). The details of this endothermic transition are typical of small globular proteins in aqueous solution, including the distinctive increase in excess heat capacity of the unfolded chain with respect to the folded protein. This positive ΔC_p mimics the thermodynamics of transfer of organic molecules from non-polar to aqueous phases, and is usually taken as the signature of hydrophobic stabilization of the folded protein (Kauzmann 1959). In the presence of methanol, although its stability is reduced somewhat, the protein still undergoes a cooperative endothermic transition but with $\Delta C_p = 0$, in contrast to the behaviour in pure water. The transition is also significantly more endothermic (ΔH more positive) than would be expected in water at the same temperature. NMR experiments indicate that, although thermal unfolding is not apparently as complete as it is in water, aromatic side chains do become exposed in the high temperature state of ubiquitin in water/methanol mixtures. Consequently, although the protein may fold entirely adequately in mixed solvents, such folding lacks the thermodynamic characteristics of the hydrophobic interaction.
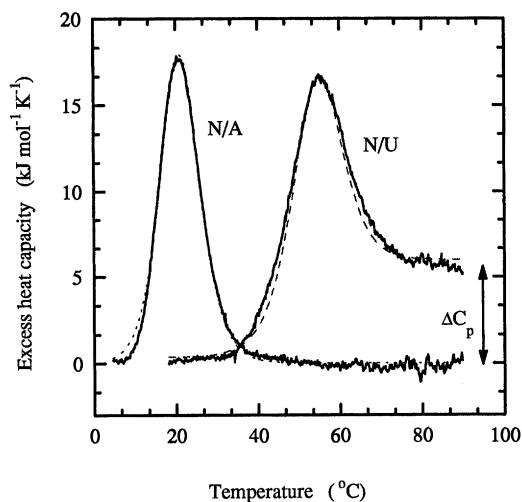
Figure 3. Baseline-corrected DSC data for the thermal transition of ubiquitin in aqueous buffer (N/U) and 40% methanol/water mixtures (N/A).

This illustrates, perhaps, some of the important principles and major difficulties in describing the thermodynamic basis of protein folding. Although much structural emphasis is placed on hydrogen bonding within protein structures, the thermodynamic contribution of such interactions to the stability of the folded protein in water is relatively small. This is because polar, H-bonding groups usually have a strong affinity for water that, in small molecules at least, makes them more soluble in water than in non-polar environments. Consequently, although it is energetically important that all H-bonds be made, it is relatively unimportant whether these are intra-molecular bonds with other peptide or side chain groups, or inter-molecular with solvent water. The tightly packed, extensive H-bonded network we see within proteins is a necessary but not sufficient thermodynamic condition for folding, and the driving force is (probably) provided by the hydrophobic contribution. However, replace some of the solvent water with less polar molecules, and this balance may change. Hydrophobic residues may now be less unfavourable in the unfolded state, exposed to the non-polar solvent, but transfer of hydrogen-bonding groups would be less favourable because they would now be unable to make as satisfactory H-bonds with solvent. As far as the protein is concerned, at low temperature, the compact extensively H-bonded structure is favourable in either solvent, though the thermodynamic characteristics might be quite different. Experimentally it is quite difficult to separate different contributions such as these since it is impossible to find solvent mixtures that change hydrophobicity, for example, without at the same time altering hydrogen bond affinities: indeed this is inevitable since the 'hydrophobic effect' is really only another consequence of the hydrogen bond in water.

(b) Molecular recognition of unfolded proteins

The standard picture of the unfolding of a globular protein involving exposure of non-polar residues leads directly to a simple method to recognize the unfolded state using cyclodextrins. These cyclic polysaccharides can bind to a wide range of non-polar molecules in water, usually by complexation within the relatively hydrophobic
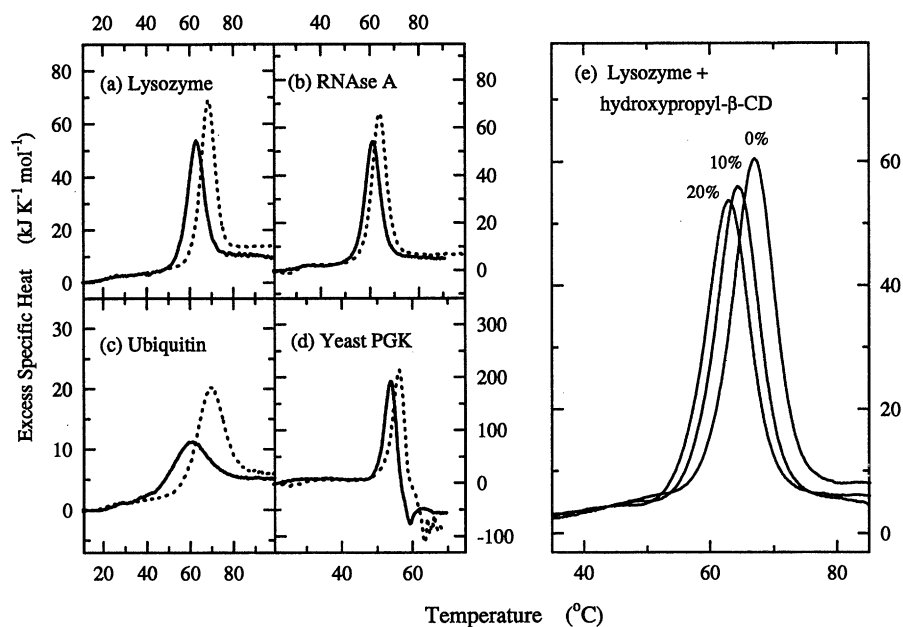
Figure 4. (*a*)–(*d*) DSC data for the thermal unfolding of proteins in the absence (dotted lines) and presence (solid lines) of α-cyclodextrin. (*e*) shows similar data for lysozyme with various concentrations of hydroxypropyl-β-cyclodextrin.

cavity of the toroidal molecule (Bender & Komiyama 1978; Saenger 1980; Szejtli 1982). The broad specificity of this process is well established and has stimulated a wide range of applications, including recently such delightful examples as the formation of 'molecular necklaces' of α-CD molecules threaded onto single polymer chains (Harada & Kamachi 1992), and the inclusion of C_{60} into γ-CD to give water-soluble fullerenes (Andersson *et al.* 1992). This remarkable versatility prompted the thought that cyclodextrins might also affect the thermal stability of proteins and other macromolecules (Cooper 1992). Since the unfolding of a globular protein normally involves the exposure of hitherto buried hydrophobic amino acid side-chains (Kauzmann 1959; Privalov & Gill 1988), the binding of cyclodextrins to these exposed residues might destabilize its native conformation by shifting the equilibrium in favour of the unfolded polypeptide chain. This indeed appears to be the case. Differential scanning calorimetry (DSC) measurements of a series of proteins shows that α-CD promotes unfolding, and that increasing cyclodextrin concentrations progressively reduce the thermal stability of the proteins in a manner consistent with weak non-covalent attachment of α-CD molecules onto the unfolded chain. This novel means of perturbing conformation in solution is proving useful in probing the thermodynamics and kinetics of protein folding.

Figure 4*a*–*d* gives examples of DSC traces showing the endothermic unfolding transitions of a representative series of proteins in aqueous buffers, with and without α-CD. With the exception of PGK, and provided samples are not kept at high temperature for too long, all these thermograms are fully reversible and are typical of simple cooperative thermal unfolding transitions of proteins. α-Cyclodextrin reduces the mean unfolding transition temperature (T_m) for all proteins examined, and the effect is approximately linear with [α-CD] up to saturating concentrations
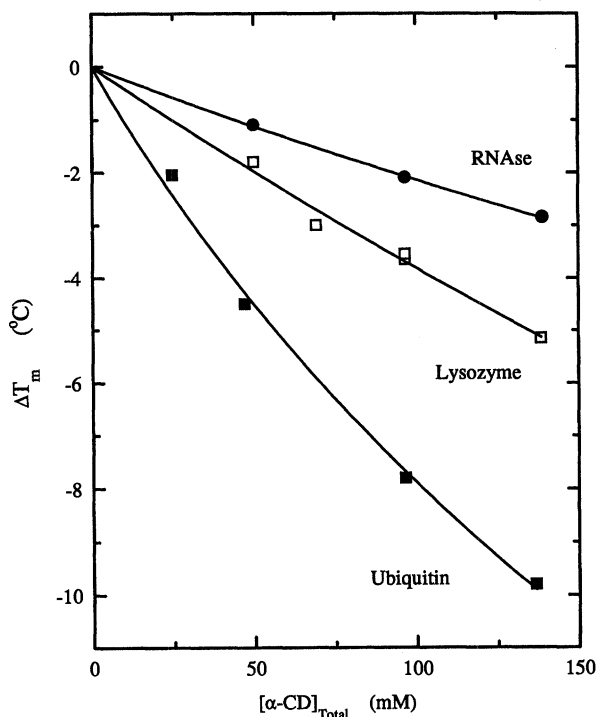
Figure 5. The decrease in thermal transition temperatures (ΔT_m) as a function of α-cyclodextrin concentration for three different proteins. The lines are nonlinear regression fits to (2) with the parameters given in table 2.

(about 14% mass (by volume) at room temperature), as illustrated in figure 5. Similar effects are seen with β-cyclodextrin and with its more soluble hydroxypropyl derivative (figure 4*e*).

Two other effects are apparent in figure 4. First, alongside the reduction in T_m there is a consistent decrease in the transition enthalpy (ΔH_m) with increasing cyclodextrin concentration. Part of this arises from the normal temperature dependence of ΔH_m for globular proteins, which is a manifestation of the heat capacity increment (ΔC_p) on unfolding (Kauzmann 1959; Privalov & Gill 1988). However, even when corrected for this effect, the observed transition enthalpies are consistently lower than expected at these temperatures in the absence of CD, by up to about 40 kJ mol^{-1} at the highest available concentrations. Secondly, the ΔC_p values themselves are also consistently lower in the presence of CD, as indicated by the post-transition heat capacity baselines (except for PGK where this is obscured by the exothermic aggregation of the protein). Both these effects are consistent with the binding of cyclodextrin molecules to hydrophobic sites exposed on the unfolded polypeptide. Heats of complex formation between α-CD and aromatic groups are small but exothermic (typically -4 to -20 kJ mol^{-1}; Lewis & Hansen 1973; Cooper & MacNicol 1978) and complex formation with such groups on the unfolded polypeptide would both reduce the overall transition enthalpy and, by burying these groups within the CD cavity, also reduce the ΔC_p effect.
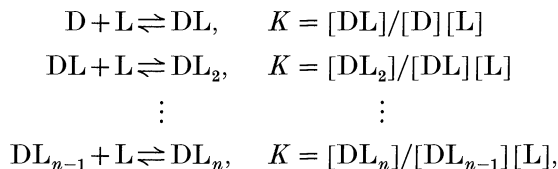
Mole-for-mole, the destabilizing effects of cyclodextrins are comparable with or even greater than conventional chemical denaturants such as urea or guanidine

hydrochloride under similar conditions. Unlike urea or GuHCl, however, where the denaturing mechanism is far from clear, the cyclodextrin destabilizing effect can be described in terms of a quite simple binding model.

Assume, for simplicity, that there are n identical and independent ligand (L) binding sites exposed when the native protein (N) undergoes the 2-state transition to the unfolded state (D). The unfolding transition may be described by the simple equilibrium:

$$N \rightleftharpoons D, \qquad K_0 = [D]/[N],$$
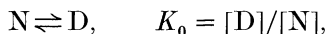
with subsequent ligand binding given by

$$D + L \rightleftharpoons DL, \qquad K = [DL]/[D][L]$$
$$DL + L \rightleftharpoons DL_2, \qquad K = [DL_2]/[DL][L]$$
$$\vdots \qquad\qquad\qquad \vdots$$
$$DL_{n-1} + L \rightleftharpoons DL_n, \qquad K = [DL_n]/[DL_{n-1}][L],$$

where K is the intrinsic binding constant for ligand at any one site. Following standard methods, and bearing in mind the combinatorial factors, one may derive an overall apparent equilibrium constant for the unfolding process:

$$K_{App} = [D]_{tot}/[N]_{tot} = K_0(1 + K[L])^n,$$

which shows, as expected, that unfolding of the protein is promoted by increasing ligand concentrations. For the interpretation of DSC experiments, this is best expressed in terms of the effect on T_m, the mid-point temperature of the transition, obtained as follows.

The Gibbs free energy of unfolding at any temperature (T) is given by

$$\Delta G = -RT \ln K_{App}$$
$$= \Delta G_0 - nRT \ln(1 + K[L]), \tag{1}$$

where R is the gas constant, and $\Delta G_0 = -RT \ln K_0$ is the free energy of unfolding in the absence of ligand. This may be further expressed in terms of the enthalpy of the transition (ΔH_0) at the mid-point temperature (T_{m0}) in the absence of ligand:

$$\Delta G_0 = \Delta H_0(1 - T/T_{m0}).$$

(Ignoring, for simplicity, any possible temperature dependence of the enthalpy. This is a reasonable approximation over the narrow temperature ranges considered here, but ΔC_p effects may be incorporated if required.)

In the presence of ligand, the mid point of the unfolding transition will occur at temperature T_m when, by definition, $\Delta G = 0$. Re-arrangement of (1) under these conditions gives

$$\Delta T_m/T_m = -(nRT_{m0}/\Delta H_0) \ln(1 + K[L]), \tag{2}$$

where $\Delta T_m = T_m - T_{m0}$ is the shift in transition temperature of the protein brought about by the presence of ligand.

At low concentrations, with a weakly-binding ligand ($K[L] \ll 1$), this takes on the approximate linear form

$$\Delta T_m = -nKRT_{m0}^2[L]/\Delta H_0. \tag{3}$$

These simple expressions allow one to estimate the number of ligand binding sites

Table 2. *Protein-cyclodextrin binding parameters determined from ΔT_m against $[\alpha\text{-}CD]$ using (2)*

| | aromatic side chains | | | | | |
	Trp	Tyr	Phe	total	n_{expt}	K_b $\text{M}^{-1\text{a}}$
lysozyme	6	3	3	12	*ca.* 12	*ca.* 2
RNAse	0	6	3	9	5.4	2.2
ubiquitin	0	1	2	3	3.7	7.4

[a] For comparison, the typical α-CD binding constant for an aromatic amino acid (phenylalanine) in solution is approximately 9 M^{-1} at 65 °C (estimated from Cooper & MacNicol 1978).

and their average intrinsic binding constants. (Equivalent expressions for a model in which ligand binds to the native, rather than unfolded form of the protein are identical to (2), and (3), except for a change of sign.)

Figure 5 shows data for three different proteins plotted according to (2), from which one may estimate the apparent numbers of binding sites and binding constants listed in table 2. Despite the simplifying assumptions, it is clear that this basic model is reasonably successful in explaining the observations, with estimates of n comparable to the number of available aromatic side-chains in these proteins and with weak K values expected for such cyclodextrin complexations at higher temperatures (Cooper & MacNicol 1978).

Further work in this area is concentrating on the effects of these and chemically-modified cyclodextrins on the kinetics and reversibility of folding, and the use of polymerized cyclodextrins both to improve the recognition selectivity and to separate folded and unfolded proteins by chromatographic techniques.

4. Experimental methods

Calorimetric experiments reported here were done using Microcal MC2-D (DSC) or Omega (ITC) instruments as described elsewhere (Cooper 1992; Cooper & Johnson 1993, ch. 9–11; McAuley-Hecht & Cooper 1993), and analysed using the ORIGIN suite of programs from Microcal Inc. Proteins, peptides, and cyclodextrins were from commercial suppliers (Sigma, Aldrich), with the exception of some modified peptides and antibiotics courtesy of Dr D. H. Williams and his group. Protein concentrations were determined from 280 nm absorbance measurements (Gill & von Hippel 1989).

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