Effect of Cyclodextrins on the Thermal Stability of Globular Proteins

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Since unfolding of globular proteins normally involves exposure of buried hydrophobic side chains,[1] the binding of cyclodextrins[2] to these exposed residues should destabilize native conformations by shifting the equilibrium in favor of the unfolded polypeptide chain. This indeed appears to be the case. Differential scanning calorimetry (DSC)[3] of a range of proteins shows that α-CD reduces the mean unfolding temperature (T_m) for all proteins examined (Figure 1), and increasing cyclodextrin concentration progressively reduces the thermal stability of the proteins in a manner consistent with weak non-covalent attachment of α-CD molecules onto the unfolded chain (Figure 2).

On a molar basis, α-CD is more effective in reducing protein stability than either urea or guanidine hydrochloride under similar conditions,[5] although it is much less soluble. Alongside this reduction in T_m there is a consistent decrease in the transition enthalpy (ΔH_m). Part of this arises from the normal temperature dependence of ΔH_m for globular proteins arising from heat capacity effects (ΔC_p).[1] However, even after correction for this effect, the observed enthalpies are consistently lower than expected by up to 10 kcal mol^{-1} at the highest α-CD concentrations available. The ΔC_p values themselves are also generally lower in the presence of α-CD, as indicated by the post-transition heat capacity base lines (Figure 1, except for PGK where this is obscured by exothermic aggregation). Both these effects are consistent with the binding of α-CD molecules to hydrophobic sites exposed on the unfolded polypeptide. Heats of complex formation between α-CD and aromatic groups are small but exothermic (−1 to −4 kcal mol^{-1}),[6] 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 ΔC_p.

A simple equilibrium model assuming n identical and independent α-CD binding sites on the unfolded protein shows that the shift in transition temperature ($\Delta T_m = T_m - T_{m0}$) should depend on ligand concentration [α-CD] thus:

$$(\Delta T_m)(\Delta H_{m0}) = -nRT_mT_{m0} \ln (1 + K_b[\alpha\text{-CD}]) \quad (1)$$

where K_b is the binding constant per site (at the transition temperature), R is the gas constant, and ΔH_{m0} is the enthalpy of unfolding at the transition temperature, T_{m0}, in the absence of ligand. For weak binding, at low ligand concentrations, this approximates to a linear form:

$$\Delta T_m \simeq -nK_bRT_{m0}^2[\alpha\text{-CD}]/\Delta H_{m0}$$

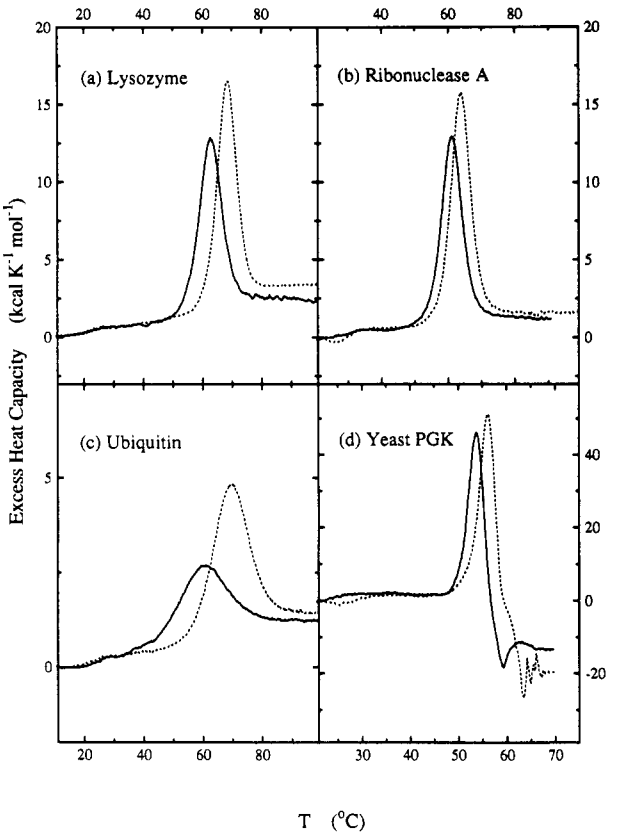

Figure 1. DSC of thermal unfolding of proteins in the presence (solid lines) and absence (dotted lines) of α-cyclodextrin, after base-line subtraction and concentration normalization. (a) Hen egg white lysozyme in 40 mM glycine hydrochloride, pH 3.0, [α-CD] = 138 mM. (b) Ribonuclease A (RNAse) in 0.1 M sodium citrate, pH 4.5, [α-CD] = 139 mM. (c) Ubiquitin in 40 mM glycine hydrochloride, pH 3.0, [α-CD] = 137 mM. (d) Yeast phosphoglycerate kinase (PGK) in 50 mM Pipes, 0.1 mM dithiothreitol, pH 7.0, [α-CD] = 69 mM. With the exception of PGK,[4] and provided samples are not kept at high temperature for too long, all thermograms are fully reversible.

The gradients in Figure 2, coupled with the calorimetric T_{m0} and ΔH_{m0} data, give estimates of nK_b = 18.9, 10.6, and 19.4 M^{-1} for lysozyme, RNAse, and ubiquitin, respectively. (Nonequilibrium PGK data were not analyzed.) Nonlinear regression using eq 1 gives convergent fits with values ranging from n = 3.7 for ubiquitin and n = 5.4 for RNAse up to about n = 12 for lysozyme, with corresponding K_b values of order 2–7 M^{-1}. Primary targets for complexation are the aromatic amino acids, which are known to form weak α-CD complexes in solution.[6,7] Free phenylalanine, for example, has a relatively weak α-CD binding constant of about 9 M^{-1}, corrected to 65 °C using standard thermodynamic data,[6] which is likely to be reduced further by topological constraints in the polypeptide. The aromatic contents of the example proteins (lysozyme, 6 Trp, 3 Tyr, 3 Phe; RNAse; 0 Trp, 6 Tyr, 3 Phe; ubiquitin, 0 Trp, 1 Tyr, 2 Phe) are consistent with these estimates.

β-Cyclodextrin also reduces protein stability (by about 0.6 °C for lysozyme in 1% β-CD, for example), but the effect is limited by low β-CD solubility. No destabilization is observed with γ-CD nor, interestingly, with α-CD in acetate buffer, probably because of competitive CD binding by carboxylic acids.[2] We are currently exploring the use of more soluble cyclodextrin derivatives[2,8] and their potential role in reducing aggregation and irreversibility of protein unfolding. In conclusion, proposed uses of cyclodextrins in pharmaceutical protein preparations[8] may need to take into

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(2) (a) Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Springer: New York, 1978. (b) Saenger, W. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 344–362. (c) Szejtli, J. *Cyclodextrins and Their Inclusion Complexes*; Akademiai Kiado: Budapest, 1982. (α-, β-, and γ-CD refer to the six-, seven-, and eight-membered cyclic oligosaccharides, respectively.)

(3) Using a Microcal MC2-D instrument at protein concentrations in the range 0.4–4 mg mL^{-1}. Data were analyzed in terms of standard models (Sturtevant, J. M. *Annu. Rev. Phys. Chem.* **1987**, *38*, 463–488. Privalov, P. L.; Potekhin, S. A. *Methods Enzymol.* **1986**, *131*, 4–51). Protein concentrations determined using the following extinction coefficients ($\epsilon_{280nm}^{1mg/mL}$) and molecular weights: lysozyme, 2.65, 14 300; RNAse, 0.715, 13 700; ubiquitin, 0.150, 8565; PGK, 0.495, 45 000 (Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989**, *182*, 319–326).

(4) The noisy exothermic response of PGK (Figure 1d) arises from irreversible protein aggregation (Johnson, C. M.; Cooper, A.; Brown, A. J. P. *Eur. J. Biochem.* **1991**, *202*, 1157–1164).

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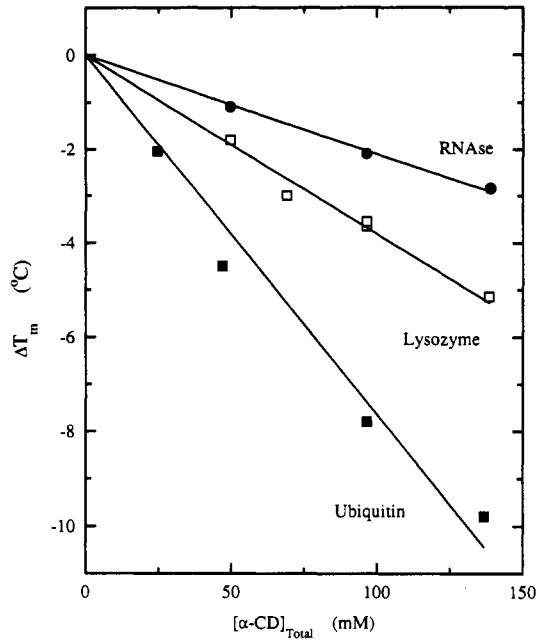

Figure 2. Decrease (ΔT_m) in transition temperatures with α-cyclodextrin concentration. Buffer conditions as in Figure 1. Transition temperatures (T_{m0}) and enthalpies (ΔH_{m0}) in the absence of α-CD were as follows: lysozyme, 67.7 °C, 114 kcal mol^{-1}; ribonuclease, 63.9 °C, 114 kcal mol^{-1}; ubiquitin, 69.2 °C, 59.3 kcal mol^{-1} (1 cal = 4.184 J).

account the effects reported here.

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Thermochemical Confirmation of the Mechanism of Action of Vitamin K

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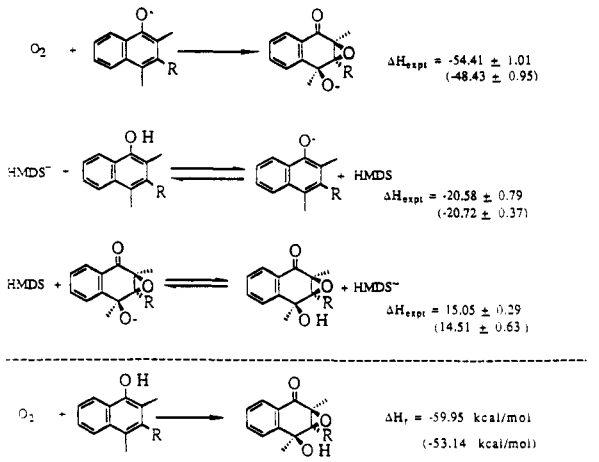
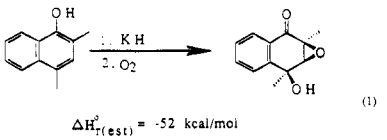
Vitamin K plays a key role in the complex cascade of steps on which the crucially important phenomenon of blood clotting depends.[1] Recently, two of us (P.D. and S.W.H.) proposed[2] a unique principle of base strength amplification to explain how the relatively weak naphthoxide anion ($pK_a \approx 10$) immediately available from vitamin K could be converted into a base strong enough ($pK_a \approx 27$) to effect carboxylation of glutamic acid to γ-carboxyglutamic acid in the presence of oxygen and carbon dioxide. The essence of the mechanism is the reaction of molecular oxygen with the biologically active hydroquinone form of vitamin K, vitamin KH_2. The oxidation leads to a powerfully basic α-epoxy tertiary alkoxide anion that is able to produce a carbanion at the γ-position of glutamate within the water-poor environment of the cell membrane or the hydrophobic interior of the carboxylase.

Dowd and Ham[2] used the oxygenation of an α-naphthol to a keto epoxy alcohol to construct a model system. The efficacy of the model was demonstrated by inducing a base-promoted Dieckmann ring closure of diethyl adipate simply by pumping oxygen into a solution of potassium 2,4-dimethylnaphthoxide in THF in the presence of 18-crown-6 polyether to sequester the potassium ion (eq 1).[2]

Scheme I[a]



[a] Bracketed numbers apply to the trimethyl compounds; R = H for the dimethyl series.



Stereochemical, structural, and labeling experiments are consistent with a strictly intramolecular oxygenation mechanism.[2,3] Thermochemical estimates for the reaction in eq 1 were made on the basis of reasonable interpolations from published heats of formation of intermediates and products required by the mechanism.[2] Specifically, the mechanism requires that the transformation in eq 1 be exothermic by about 52 kcal/mol and that the heat of deprotonation of the model epoxy alcohol should be approximately 10 kcal/mol less exothermic than its parent naphthol.

To test these proposals, the reaction in eq 1 was carried out in a Tronac 450 solution calorimeter at 25 °C using the dimethyl- and trimethylnaphthoxides shown in Scheme I. A 0.17 M solution of the 18-crown-6/potassium naphthoxide was prepared in THF, and successive increments of 99.8% oxygen (Matheson) were injected through a Teflon cannula from a 10-mL gas-tight syringe.[3] Reaction with oxygen was instantaneous and clean by NMR examination of products. In conformity with prediction,[2] the heats of reaction (ΔH_{ox}) for two complete runs of 11 measurement replicas were −54.41 ± 1.01 kcal/mol for oxygenation of the dimethylnaphthoxide and −48.43 ± 0.95 kcal/mol for the trimethylnaphthoxide. Since ion-pairing effects within the enzyme are unknown, it is interesting to note that $\Delta H_{ox} = -47.75 \pm 0.60$ kcal/mol for the former reaction in the absence of the crown ether.

Following the procedure of Arnett and Moe,[4] the heats of deprotonation (ΔH_{dep}) with lithium hexamethyldisilazide (LiHMDS) in THF at 25 °C for the epoxy alcohols and their naphthol precursors are as shown in Scheme I; each value is based on 12 replica measurements. Using 18-crown-6/KHMDS, $\Delta H_{dep} = -12.72 \pm 0.41$ kcal/mol for the trimethyl epoxy alcohol and $\Delta H_{dep} = -18.68 \pm 0.40$ kcal/mol for the 2,3,4-trimethylnaphthol were obtained. For comparison with a model tertiary alcohol, $\Delta H_{dep} = -13.41 \pm 0.21$ kcal/mol for dimethylbenzylcarbinol. An attempt was also made to compare the ΔH_{dep}'s of the alcohols with K^+DMSYL^- in DMSO.[5] However, the $DMSYL^-$ anion im-

† Duke University.

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