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BEHAVIOUR OF PROTEINS IN ALTERNATE SOLVENT CONDITIONS

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Recent progress in thermodynamic aspects of protein function in unusual environments are described. Systems considered include enzymes incorporated in reverse micelles, immobilized onto solid support or suspended in low-water solvents.

Keywords: proteins, thermodynamic aspects of protein

Introduction

Since X-ray diffraction data of the structure of several globular proteins become available, it has been recognized that large portions of a single polypeptide chain of the protein may structurally form distinguishable parts, which are called domains [1, 2]. Increasing importance has been attributed to these structural features because numerous evidence shows that a single domain often constitutes a biological function unit [1, 3, 4]. Moreover, inter-domain interactions are the fine transmission mechanism for the coordination of different functional activities of either monomeric or multimeric proteins. Structural evidence suggesting the presence of two or more lobes of the protein molecule separated by deep cleft is often confirmed by the thermodynamic properties of the protein molecule. The thermal unfolding of some proteins, such as pepsin or fibrinogen, as studied by differential scanning calorimetry (DSC), can be separated in two or more transitions which correlates where there is a presence of different domains [5]. Conversely, other proteins such as RNAase, lysozyme and chymotrypsin, whose crystal structures show two domains separated by a deep cleft, have been reported to undergo to a single cooperative transition [6, 7]. Moreover, other proteins (i.e. hexokinase [8] and yeast phosphoglycerate kinase [9]), show a single or a

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double transition, depending on the experimental conditions. The intrinsic stability of the domains as well as the degree of thermodynamic coupling between them are the basis of this different behaviour. For example, by altering the interaction between two structural domains, i.e., by uncoupling two strongly interaction domains, the protein may unfold with two consecutive transitions instead of a single one. Interdomain interactions may be altered in several ways. For instance, by replacing water with a water miscible organic solvent, such as short aliphatic alcohols, the hydrophobic interactions, which give a large contribution to domain stabilities, are weakened. Asymmetric effects on domain stabilities may also be achieved by chemical modification or single point mutation of a key charged group, when the contribution of the electrostatic effects are the driving stabilization factors. Even the covalent attachment of the protein molecule to activated supports through charged amino acid side chains may give rise to a similar effect. It has been shown that the protein stability and conformational rigidity depend upon the subtle changes of structure modulated by protein/support interaction and number of covalent links [10, 11]. Finally, the role of hydration on changing protein conformational dynamics may be assessed by a drastic removal of bulk water to a level whereby the only water molecules present are those strongly interacting with the protein molecule. This may be achieved by replacing bulk water with organic solvents, such as long chain alkanes, or by solubilizing the protein in the core of reverse micelles formed by surfactants in organic solvents.

The thermal unfolding process of RNAase in aqueous dilute solution between *pH* 2 and 7.5 consists on a single cooperative transition in spite of the bilobal structure of the macromolecule inferred from X-ray crystallographic data [12]. The transition is almost completely reversible at *pH* around 6 and below. The enthalpy change, ΔH, associated with the unfolding process depends on T_m, which is the temperature of the middle point of the transition. Since the variation of T_m under different conditions (*pH*, ionic strength, etc.) spans a limited interval, a plot of ΔH *vs.* T_m can be well approximated by a straight line since the heat capacity change, C_p, can be considered constant in that temperature range. The ratio of the calorimetric enthalpy change, ΔH_c, and the van't Hoff enthalpy, ΔH_{vH} is almost equal to 1 in the whole pH range [6]. This ratio can be used as criterion of cooperativity of the unfolding transition, i.e. how much the average size of the cooperative unit differs from the size of the protein molecule. In other words, RNAase unfolds as a single cooperative unit and no significant accumulation of intermediates, partially folded species, is observed. The transition is close to a two-state process [13].

Table 1 Transition temperatures and enthalpy changes for ribonuclease a and it s valeraldehyde derivative

	pH	T_m / °C	ΔH_c / kJ/mole	ΔH_{vH} / kJ/mole
RNAase	3.0	47.95	300.7	303.8
	3.3	48.1	297.	294.
	4.3	56.9	361.	354.
	5.0	59.1	369.	368.5
	5.1	60.2	392.	392.
	6.9	64.6	438.	418.
	7.0	65.6	463.	459.
(riscan)	5.0	59.1	360.	368.5
VA-RNAase	3.0	53.3	336.	332.
	4.0	55.8	404.	405.
	5.0	59.3	378.	385.
	7.0	63.6	441.	437.

errors: $pH = \pm 0.2$; $T_m = \pm 0.1$; $\Delta H = \pm 4\%$

The chemical modification of selected amino acid side chains may not significantly change the thermodynamic properties of the protein. RNAase has been acylated with valeraldehyde (VA-RNAase) at *pH* 7 [14], where the amino terminal group (*pK* 7.8) reacts more readily than the other primary amino groups (*pK* 10.2). Under these conditions, 1.4 ±0.3 groups per protein molecule were modified. The biological activity of the modified RNAase changes slightly: the enthalpy and entropy changes (ΔH, ΔS) of binding 3'CMP are different but largely compensate each other, leaving the binding free energy change, ΔG, almost uneffected. This suggests that the active site was only partially perturbed. On the other hand, the thermal unfolding of the modified protein shows only minor changes with respect to the native proteins. A single cooperative transition is observed. As shown in Table 1, the ΔH_c for the two forms of RNAase are similar, but the VA-RNAase shows a smaller *pH* dependence, due to the removal of 1–2 positive charges from the protein surface, which have been substituted with a short hydrophobic chain. The cooperative unit is still close to unity, as shown by the ratio of $\Delta H_c/\Delta H_{vH}$ as in the case of the native protein. ΔH_c is linearly related to T_m for both protein forms. The slopes of the two curves, ΔC_p, are similar, therefore sug-

gesting that protein-solvent interaction were not greatly perturbed by the addition of a short aliphatic chain in the protein surface. In conclusion, RNAase covalently modified with VA does not show any significant change in either the biological activity (as far as the binding of a ligand is concerned) or the thermodynamic stability. Moreover, the modification of the terminal amino group which is located at the entrance of the binding cleft between the two domains of the proteins, is not sufficient to alter the interdomain interactions or domain stability.

The perturbation of the properties of the solvent water may lead to drastic changes in protein intersubunit and interdomain interactions. Short aliphatic alcohols, such as methanol or ethanol, at high concentrations, lower the dielectric constant of the aqueous medium strengthening electrostatic effects and weakening hydrophobic interactions. Brandts *et al.* [9] found (using DSC) that the RNAase unfolding process can be split into two overlapping transitions by lowering the apparent *pH* of the solution from 3.8 to 2.0, in the presence of 50/70% methanol. The overall unfolding process is shifted to a lower temperature as the *pH* is dropped, while the two transitions broaden and separate from one another. The fitting of the DSC curve is consistent with two overlapping reversible two-state processes. Since spectroscopic studies suggest that the structure of RNAase does not change much in 50% methanol [15], it is possible that the two transitions correspond to the unfolding of the two structural domains of the protein. This may be due to either a weakening effect on interdomain interactions or a difference of the intrinsic stability of the two domains brought about by the presence of the co-solvent. It should be noted that the density of charged amino acids on the two domains is quite asymmetric. The net result is the uncoupling of the two structurally different parts of the molecule, which in dilute aqueous solution strongly interacts and behaves as a single cooperative unit. Makarov *et al.* [16] also found that the size of the cooperative unit of pepsin changes in presence of 20% ethanol. Only two structural domains are distinguishable according to the X-ray diffraction data [17], but the DSC unfolding process can be fitted with 4 transitions. In 20% ethanol, the size of the cooperative unit, i. e. the ratio $\Delta H_c/\Delta H_{vH}$, decreases from 4 to 2 by decreasing the *pH* from 7 to 2, while the overall transition shifts to a higher temperature. The domain uncoupling at neutral *pH* may be attributed to electrostatic repulsive effects due to the numerous carboxyl side chains present in the protein surface in an unprotonated form.

One may ask the question as to what would happen if water is completely replaced with a neat organic solvent. In the case of long chain alkane, for example, no hydrogen bonds between protein molecule and the surrounding medium would be either possible in native or unfolded states. Therefore, the

hydration of the protein powder suspended in dry organic solvent is expected to play an important role in protein stability. Indeed, RNAase shows a cooperative unfolding DSC transition in dry undecane. The T_m is dependent on the degree of hydration of the protein molecule. As shown in Table 2, RNAase exhibits greatly enhanced unfolding denaturation temperature compared to aqueous solution. However, the increase of hydration has a detrimental effect on RNAase thermal stability. By studying RNAase in organic solvents, a similar effect has been reported by Volkin *et al.* [18]. They showed that the effect of water content on enzyme thermostability is similar in the dry state and in neat nonane or other hydrophobic organic solvents due to the conformational rigidity of the enzyme in both states. By increasing the hydration of the enzyme powder, the mobility and flexibility of the protein molecule increases because water acts as a molecular lubricant due to its ability to form H-bonds. Thermal stability decreases in accordance to an increase of molecular dynamics and fluctuations. It is interesting to note that *pH* of the buffer from which the enzyme has been liophylized, influences the activity [19] and the thermal stability of enzymes in organic hydrophobic solvents. The immobilization of RNAase through a covalent link to an activated silica support does not further enhance the thermal stability in undecane, which remains close to that of the free enzyme.

Table 2 The effect of water content of ribonuclease powder on protein denaturation temperature in organic solvent

Solvent	T_m /°C		
% Water (*w/w* protein)	16	20	23
dodecane (from *pH* = 7)	94	90	87.5
dodecane (from *pH* = 4)	90	85	82
nonane (a)	98	92.5	87.5
dodecane covalently bound to Silica (CPC)	95	92.5	89

a) extrapolated from (18).
Error T_m (°C) = ±1

The effect of hydration on protein stability can also be studied by solubilizing enzymes in organic solvents inside reverse micelles formed by surfactants. In this case, instead of a suspension, a homogeneous solution can be readily obtained. The proteins are located inside the water pool of the micelle surrounded by one or few layers of water molecules that protect them

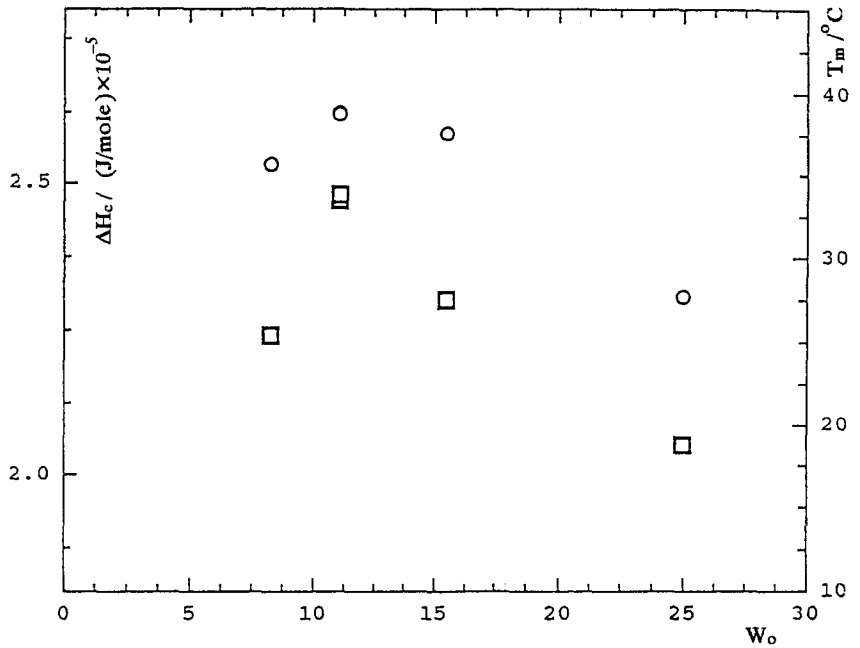

Fig. 1 Dependence on W_o of the apparent calorimetric enthalpy (□) and the transition temperature (o) for ribonuclease A inserted in reverse micelles

from the denaturating effects of the surfactant and the organic solvent [20]. The reverse micelles formed by AOT in isooctane is a typical system which can solubilize up to 6–7% *v/v* of water. The DSC analysis of RNAase micellar solutions in AOT/isooctane shows a cooperative unfolding of the enzyme. Both T_m and ΔH_c depend on the amount of water present in the system (expressed as W_o, i.e. the ratio of the molar concentration of water and AOT) [21]. As shown in Fig. 1, the ΔH_c of the unfolding process goes through a maximum, where the peak at $W_o = 10$. The transition is partially reversible and shows a two-state behaviour, since the ratio $\Delta H_c/\Delta H_{vH}$ is close to unity. It should be noted that the cooperative unit and interdomain interactions, do not change, even at very low water content ($W_o = 8.3$) where the enzyme molecule is barely surrounded by a single layer of water molecules. The thermal stability (and ΔH_c) decreases as the hydration of the enzyme/micelle system increases: as in the case of neat organic solvents, water may be considered as a molecular plasticizer of the protein structure, increasing mobility and flexibility of the protein/AOT system. In fact, at low water content (for RNAase around $W_o = 10$), globular proteins have a higher degree of

'conformational rigidity' in respect to water, which is progressively lost at higher W_o [20]. It is interesting to note the case of lysozyme. No unfolding transition is observed at any W_o or *pH*. However, in the presence of a ligand, *N'*, *N''*, *N'''*-triacetylglucosamine, lysozyme gives rise to a sharp DSC transition [21]. This suggests that the ligand strongly stabilized the native conformation of the enzyme, which is denaturated after solubilization inside the micelle.

It is clear from the above discussion that the stability and thermodynamic state of a protein are strongly affected and finely tuned by environmental conditions, including protein-solvent and protein-ligand interactions. The attachment onto a solid support can also be expected to bring about conformational changes, which may alter protein stability [22]. The immobilization can be accomplished in several ways, including

i) covalent atteachment to water soluble and insoluble support through a single or multipoint links of various lengths,

ii) adsorption by ionic or hydrophobic interactions on organic or inorganic supports,

iii) entrapment in polymeric gels or microcapsules, including copolymerization between protein and matrix.

RNAase can be covalently attached to aminopropyl controlled pore silica beads through the bifunctional glutaraldehyde, which links the amino group of the glass with an amino group of the protein. At *pH* 7 the most reacting group of RNAase is the amino terminal due to the fact that its *pK* [7, 8] is lower than the other primary amino groups (*pK* 10.2) [23]. Since the arm joining the enzyme with the surface of the silica particle is short and flexible, the enzyme may experience a local environment which may be different from that of the free enzyme. The presence of slightly acidic sialic –OH groups may favour hydrophilic and electrostatic interactions between the positively charged protein and the particle surface. RNAase covalently immobilized undergoes a single unfolding DSC transition in the *pH* range from 3 to 7 [14]. A general stabilization effect is observed, especially at low *pH*, where T_m is higher than that of the native enzyme. Deconvolution analysis reveals the presence of two overlapping processes (Fig. 2). Each transition can be approximated by the all-or-none two state model, which does not hold for the overall processes. The $\Delta H_c/\Delta H_{vH}$ ratio is close to unity for each of the two transitions. This is consistent with the consecutive unfolding of the two domains of the protein molecule. After immobilization, the presence of one or two covalent bonds may change the relative intrinsic stability of one of the two domains or may alter their coupling free energy. In addition, the interactions between protein and support may enhance the decoupling effect. The biological activity of RNAase as studied by the binding of 3'-CMP is almost

fully preserved after immobilization [23]. The ligand interacts with the active site with an affinity similar to that of the free enzyme. Furthermore, the binding enthalpy and entropy changes differ only slightly. At *pH* 5, the unfolding of the lower temperature domain is fully reversible, whereas the other one is only partially reversible. At *pH* 7, only the reversibility of the first transition is complete, whereas the other one is practically irreversible.

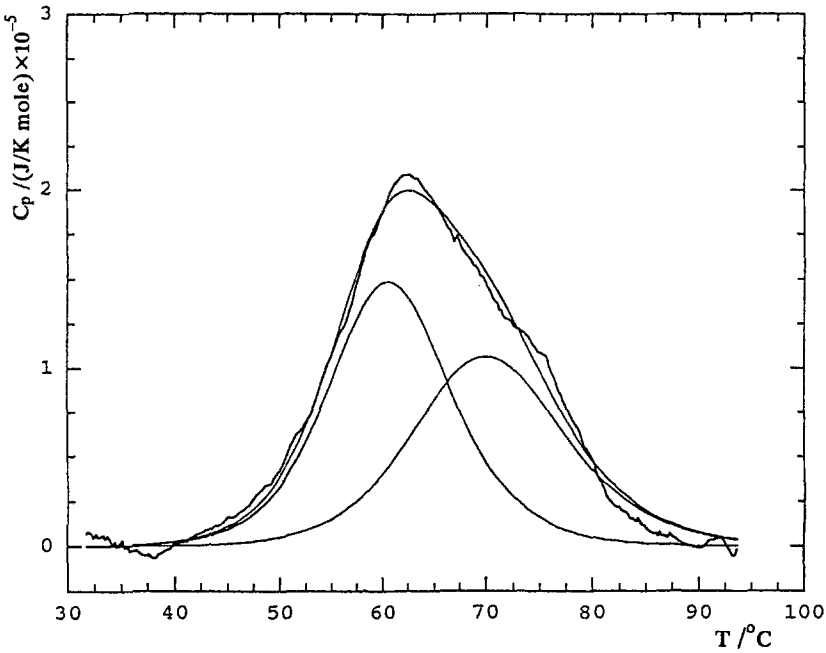

Fig. 2 Temperature dependence of the specific heat capacity, C_p, of immobilized RNAase on CPC-Silica in 0.05 *M* acetate buffer *pH* = 5. Two overlapping transitions have to be considered in order to obtain the best fit of the overall experimental transition. Calorimeter: Microcal Inc., MA, USA Scan rate = 40 deg/hr

The perturbation of the interdomain energy by immobilization is not a general rule. For example, lysozyme has been immobilized under the same conditions used for RNAase with silica beads. Unlike RNAase, the immobilized lysozyme unfolds with a single cooperative DSC transition, without showing any splitting of the two structural domains which can be recognized from the X-ray data. Conversely, immobilized α-chymotripsin (another two-domain protein) behaves like RNAase, showing the presence of a high temperature peak which is not present in the unfolding process of the free enzyme.

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Zusammenfassung — Es werden die bisherigen Ergebnisse bei der Untersuchung thermodynamischer Aspekte von Proteinfunktionen in ungewöhnlicher Umgebung beschrieben. Untersuchte Systeme beinhalten Enzyme, die in reversen, auf fester Unterlage immobilisierten oder in Lösungsmitteln suspendierten Mizellen eingeschlossen wurden.