ELSEVIER

Biophysical Chemistry 126 (2007) 65–79

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Thermodynamics of proteins in unusual environments

Giovanni Rialdi [a], Ezio Battistel [b,*]

[a] *via Caprera 8/14 – 16146 Genova, Italy*
[b] *Polimeri Europa, Istituto Guido Donegani, via Fauser 4, 28100 Novara, Italy*

Received 21 February 2006; received in revised form 26 May 2006; accepted 26 May 2006
Available online 30 June 2006

Abstract

Some aspects of protein thermodynamics in unconventional environments are addressed and discussed. Aqueous medium, especially dilute solution is the 'usual' ambient, which mediates all the interactions between protein and nearby molecules. When the water content is low, the surroundings may be considered 'unusual', exerting new stresses on the protein molecule and demanding different responses and property changes. The unusual systems considered in this article are low-water protein environments, including nearly dry state powders, organic solvent dispersions and reverse micelles' inclusions. The changes of hydration experienced by the protein after immobilization on solid supports are emphasized with respect to the free bulk solution state. Finally, the aqueous medium altered by water connectivity perturbing agents (polysaccharides) or in macromolecular crowding conditions (in the presence of polyols) is also considered as highly not ideal protein environments. The different responses elicited by the protein under the stress induced by drastic surrounding alterations may give insights for the controlled exploitation of the protein's biological and thermodynamic properties.

Keywords: Protein hydration; Thermostability; Protein unfolding; Domain decoupling

1. Introduction

Environment may be defined as the surrounding conditions and forces by which an entity (a living being, a cell or a macromolecule) is influenced and modified in its properties and development. The interaction is reciprocal: the entity acts upon the surrounding, which responds to the stress and, *vice versa*, alterations of the surrounding cause the entity to strain back. More precisely, the separation between entity and surrounding is formal since both actually constitute a single integrated system. However, the distinction may be useful for the foregoing discussion on chemical interactions.

At macroscopic level, emphasis is laid on the stress caused by human activity on the environment that gives origin to pollution, contaminants accumulation and possible climate alteration. Environmental issues are mostly related on how pollution and contamination can be detected, limited or reversed. Less emphasis is made on the studies on how the polluted environment will act back on human population, a problem that is often postponed as a long-term issue. At microscopic level, considering the system constituted by macromolecules and water solvent (the environment), we would like to invert this point of view: the emphasis will be placed on the consequences induced by the drastic changes of the chemical–aqueous environment on the protein molecule. Under controlled conditions, the studies will monitor how the macromolecules strain back and adopt novel properties as adaptation to new environmental stressing conditions.

Macromolecules such proteins, nucleic acids and polysaccharides are evolutionary fitted to respond to aqueous surroundings. The intracellular environment, in which proteins function *in vivo*, has little resemblance to the dilute solution usually employed in physical studies of proteins. The level of aqueous solvation of the proteins inside the cell may be considered of only 2–3 layers of covering water molecules, which is nevertheless more than enough to function [1]. Dehydration studies showed indeed that the onset of the enzyme activity requires as low water as 0.2–0.4 h (h is the ratio of grams of water per grams of protein, g/g) which is usually well below the amount needed for a monolayer water coverage of the enzyme molecule [2]. Bulk water starts to be observed at

* Corresponding author.
E-mail address: ezio.battistel@polimerieuropa.com (E. Battistel).

0301-4622/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2006.05.029

hydration level of 1.4 *h* or more that, for most tissues, corresponds to a water content of more than 60% by weight [1]. Thus, most tissues and cells may posses little, if any, bulk water. Moreover, within the cell it has been estimated that more than 50% of the proteins are associated with membrane that provide quite a hydrophobic environment [3]. Most of the other macromolecules are anyway associated in highly organized assembly of molecules, which, in most cases, experience little contact with the aqueous solvent.

Chemical environments that cause drastic alterations of the protein hydration may be defined as unusual, with respect to the situation where the macromolecule is dissolved in diluted aqueous solution [4]. Indeed water mediates all structural, functional and dynamical properties of the macromolecules. Dry proteins behave as rather rigid and glassy materials, which are plasticized and softened by water during hydration [5,6]. Internal water and first layer water coverage provide alternative mobile hydrogen-bond donors and acceptors for peptides and charged groups. This water is not bulk water, as assessed by many techniques [7–9]. Increasing hydration will guarantee the conditions for segmental motion, enhanced flexibility and free volume expansion and rearrangement, which bring forth the full set of protein structural and dynamic properties necessary for function.

A relevant concept in biotechnology is the possibility to control protein activities and functions at will. Enzymes are required to be active in certain conditions and for a certain amount of time but their activity often have to be shut off when the proper extent of the reaction is reached. An example, among many others in industrial biotechnology, is the use of cellulases as auxiliary agents in washing. These enzymes have to be stable in harsh conditions (concentrated detergents and additives, high temperature, etc.), but a much-prolonged enzymatic treatment can cause unwanted damage to the treated clothes. Therefore, their thermal stability, for instance, does not have to be very high, as may be achieved by genetically engineered mutants, but just high enough to carry on the desired activity as long as it is needed.

According to these concepts, the aim of this contribution is to address the topic of the property changes induced by drastic environmental alteration intentionally applied to stress protein behaviour. The more we understand these effects, the more we have the chance to shed light on protein response and to control enzyme activity and function. One should be reminded that in many industrial applications the reaction environment is often to be considered unusual, since it is far from the ideality observed in the diluted solution.

Unusual conditions may be schematically listed as follows: a) absence of bulk aqueous solvent as in hydrated protein powders or films; b) presence of organic solvents; c) insertion in reverse micelles; d) addition of either water soluble (polyethylene glycol and polysaccharides) or almost water insoluble (starch) polymers; e) immobilization onto supports characterized by different degree of surface hydrophobicity. Studies of protein behaviour are presented for each of these sections. As we will see, proteins respond in different ways to the stresses caused by the different environment. Perhaps, the only common underlying actor on the scene is water, which plays the universal role of 'co-reactant' or 'co-factor' in all the apparent changes of the protein thermodynamics.

The criterion to assess protein properties is the thermodynamic analysis of protein stability and function. If stability is defined as an equilibrium between the folded, native (N), and unfolded (U) forms of a protein, then the thermodynamic parameters of the transition $N \leftrightarrow U$ (free energy, enthalpy and entropy changes) allow a rigorous definition of the concept of stability. Therefore, in this contribution other criteria such the time dependence of the enzyme activity or inactivation rates will be not considered as a source of information on protein stability.

2. Materials and methods

2.1. Dry state

Ribonuclease A (RNAse type XII-A, Sigma) was adsorbed on Celite in order to obtain a homogeneously dispersed preparation and to avoid unwanted aggregation. A wetted paste of Celite and RNAse (previously dissolved in phosphate buffer 10 mM pH 7) was allowed to equilibrate for a week at 28 °C under isopiestic conditions in the presence of various H_2SO_4/water volume ratios. The final hydration level ranged from 0.24 to 1.4 *h*. Lower *h* values were obtained after treatment of the powder under high vacuum. A more detailed description of the sample preparation can be found elsewhere [10]. Differential scanning calorimeter (DSC) measurements were performed with a Perkin-Elmer DSC-7 instrument. High-pressure stainless steel pans were filled with up to 25 mg of sample. The scanning rate was 10 °C/min.

2.2. Organic solvents

After isopiestic equilibration at given water content, the Celite-adsorbed RNAse (20 mg, 0.09 mg protein/mg) was load into the DSC cell. The organic solvent (40 μl) was added directly into the cell. The sample was allowed to equilibrate at constant temperature (30 °C) for 1 h before starting the DSC measurement [10].

2.3. Reverse micelles

Protein micellar solutions were prepared by uptake of the protein powder by a 100 mM AOT (bis[2-ethyhexyl] sodium sulfosuccinate, Sigma) solution in isooctane (2,2,4-trimethylpentane, Fluka) at a given value of W_o [11]. W_o is the water to surfactant molar ratio, a parameter expressing the amount of water present in the system, normalized by the amount of surfactant. W_o ranged from 8 to 25. The micellar solution used for the protein uptake, at a given W_o, was prepared with diglycine buffer 10 mM pH 3.3. The final concentrations of RNAse (Sigma) and cytochrome *C* (Sigma) solubilized in the micellar phase was calculated by UV absorption spectra by using the extinction coefficients listed in the literature [11]. DSC measurements were performed with a MC-1 Microcalorimeter (Microcal Inc., MA). The scan rate was 0.5 °C/min.

2.4. PEG

Hen egg-white lysozyme (Fluka) dissolved in acetate buffer, 100 mM pH 4.2, in the presence of polyethylene glycol (PEG) 10 000 and 20 000 Da was used without purification [12]. DSC experiments were performed with a VP-DSC Microcal (Micro-Cal LLC, MA, USA) at constant protein concentration (0.4 mM). The PEG concentration was 26% w/v. The scan rate was 0.2 °C/min. From the calorimeter data, the excess molar heat capacity, ΔC_p, was also calculated. The density measurements were performed at 25 °C with an Anton Paar DMA 60/602 densimeter (Graz, Austria). From these data, the apparent molar volume, $V°$, was calculated.

2.5. Fluids for drilling in oil upstream operations

Water based drilling fluids were prepared by dissolving scleroglucan (Baroid, Hulliburton) 0.44% w/v in brine (1% KCl) under mild stirring with a Silverson Homogenizer (60 min). Maize starch (1.4%) was then added and the suspension stirred for 60 min. Finally, sized calcium carbonate (10.4%) was slowly introduced under higher stirring rate for 15 min. DSC scans were performed with a Setaram C80 (France) calorimeter at 0.2 °C/min. The sample cell was filled with 12 ml of solution or suspension to which 1.0 ml of a concentrated RNAse stock solution in acetate buffer 150 mM pH 5.1 was added. Typically, 45–50 mg of protein per scan were used. The final buffer concentration was ca. 10 mM. The reference cell was filled with the solution/suspension contained in the sample cell without protein (1 ml of buffer was added to obtain the same dilution of the sample). The RNAse concentration was calculated spectrophotometrically by using an extinction coefficient of $\varepsilon_{278}=9800$ l/mol/cm at 278 nm.

2.6. Covalent immobilization

RNAse was immobilized on Controlled Pore Carrier (CPC, aminopropyl silica beads, pore size of 500 Å; Fluka). The aminopropyl groups were activated with glutaraldehyde at pH 7. After washing, the protein was attached to the glutaraldehyde arm [13]. In order to obtain a highly hydrophobic surface, the silica beads were also silanized under standard conditions before protein immobilization. Eventually, the primary aminopropyl groups that did not react with glutaraldehyde were removed by means of the reaction with the monofunctional valerialdehyde. The concentration of the immobilized RNAse was determined by amino acid analysis or by the Lowry Folin method. It was estimated that the chemical links between protein and support was 1.2±0.3 [13]. The RNAse valerialdehyde derivative was obtained by following the same procedure used to link the protein to the glutaraldehyde-activated support. The amount of modified primary amino group was the same in both cases. Calorimetric and spectrophotometric isothermal binding experiments (25 °C) and DSC stability studies were carried on as described elsewhere [13–15].

3. Results and discussion

3.1. Dry state

The results of the differential scanning calorimetry (DSC) experiments with RNAse adsorbed on Celite are presented in Fig. 1. Celite has been used to obtain a homogenously dispersed sample and to minimize unwanted protein–protein association and interactions. Celite is an inert support with a minimal affinity with water. Consequently, it can be safely assumed that the water present in the sample is mostly adsorbed on the protein molecules [10], i.e., the presence of Celite does not interfere with the water redistribution within the sample under isopiestic equilibration.

The overall enthalpy change, ΔH, and the temperature, T_m (the highest value of the heat capacity peak), of the protein unfolding transition as a function of the hydration parameter, h (grams of water *per* grams of protein (g/g)) are shown in Fig. 1. At high level of hydration (1.4 h) the protein unfolding is characterized by an overall ΔH and T_m values similar to those observed in aqueous dilute solutions [10]. In phosphate buffer 10 mM pH 7, ΔH and T_m values are 480 kJ/mol and 65 °C, respectively. At 1.4 h, there is enough water to form 2–3 layers of water molecules around the protein, which guarantee that the necessary H-bond network for proper conformation, flexibility and function is completed. In these conditions, the protein has a thermodynamic behaviour similar to that observed in bulk water.

The enthalpy of unfolding slowly decreases (Fig. 1) as the water content diminishes from 1.4 to 0.5 h. The decrease becomes sharp below 0.30–0.35 h. T_m has a similar dependence on hydration. At high h T_m, increases slightly and then sharply turns upwards below 0.35 h, suggesting a strong stabilization effect. Indeed, hydration changes induce significant modifications of protein thermodynamics and structural integrity as the essential water is removed from the protein molecule.

Similar results have been observed by studying the unfolding of lysozyme [16], chymotrypsinogen A [17] and β-lactoglobulin [9] as a function of h. For comparison sake, the data of

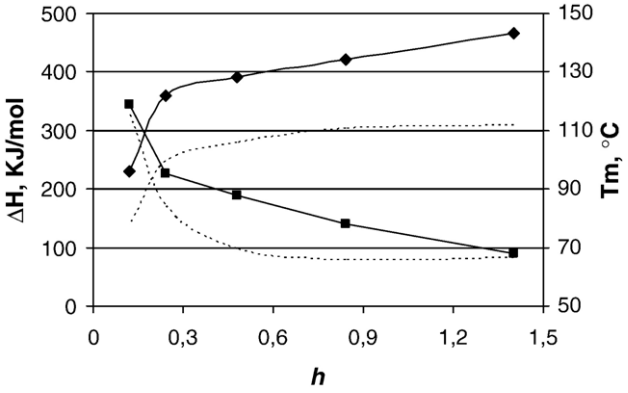

Fig. 1. Enthalpy change, ΔH, and transition temperature, T_m, of RNAse unfolding as a function of hydration, h (the ratio of grams of water per grams of protein, g/g) without solvent. RNAse adsorbed on Celite, from a phosphate buffer 10 mM pH 7 solution. T_m is the highest value of the heat capacity peak. The dotted lines schematically represent the data of Fujita and Noda [16] for lysozyme.

Fujita and Noda for lysozyme [16] are schematically reported as dashed lines in Fig. 1. From these studies, it was possible to define the presence of three main hydration regions. The first region, covering the range 0–0.33 *h*, is characterized by a drastic decrease of ΔH of unfolding and a concurrent increase of T_m. The second region is centered between 0.33 and 0.75 *h*, where ΔH and T_m changes smoothly, reaching the normal value observed in aqueous dilute solution between 0.75 and 1.4 *h*, which is the third hydration region. Fujita and Noda suggested that the first two regions are related to the building up of two different water phases, often referred to as A- and B-shell water phase by analogy with ion hydration [17]. The A-shell water corresponds to the non-freezing water [8,18], which is the amount of water required for the coverage of the charged and polar groups. At this hydration level there is 1–1.5 water molecule *per* H-bonding site on the protein. These water molecules are strongly influenced by the electric field of the charged ions. The B-shell water is not as strongly oriented as the A-shell molecules but still does not have the physical properties typical of bulk water. Probably it corresponds to the water needed to cover less polar or hydrophobic parts of the protein molecule. The differences of the extent of these two phases in the folded and unfolded state may bring about the observed drastic changes of the thermodynamic quantities especially at low *h*.

Rupley and Careri [2] have proposed an alternative and more appealing interpretation. They argued that the hydration dependence of ΔH and T_m is the sum of the contributions of both the folded and unfolded states. When the protein unfolds, the surface exposed to the solvent is expected to be more relevant with respect to the folded state. If the higher amount of water needed to hydrate the newly exposed sites is not available, then the unfolded state is less favoured with the respect the folded state where, at the same hydration level, the amount of water present is sufficient to saturate the solvation sites. If this is the case, the unfolded state is destabilized with respect the native state and this leads to an increase of T_m. It has been estimated that the full hydration of the unfolded state occurs around 0.75 *h* (this values has been calculated for lysozyme and can be slightly different for another protein). Below 0.75 *h*, an increase of T_m and a concurrent decrease of ΔH are expected, as indeed it has been observed. Moreover, the removal of structural water, i.e., internal bound water molecules, below 0.75 *h*, should be accompanied by a decreased of ΔH because of the decreasing number of intramolecular hydrogen bonds [9]. If dehydration below 0.75 *h* may induce a destabilization of the unfolded state of the protein due to the lack of the water required to satisfy newly exposed potential H-bond sites, then the conformation of the unfolded protein has to be rearranged in order to face the unfavourable situation. IR studies on dehydrated RNAse indeed showed that the most perturbed protein state upon dehydration is the unfolded state [10]. This effect is even more enhanced in the presence of perturbing agents, such as organic solvents.

It should be reminded that, in the case of lysozyme, the suggested level of 0.75 *h* for the fully hydration of the denatured state is equivalent to about 600 water molecules *per* protein molecule, which is less than the value estimated for an equivalent completely extended polypeptide chain [6]. This is why an unfolded protein rarely resembles a random coil or an extended chain, but rather a partially swollen polypeptide net with significant residual structure, although localized and partially stable. In fact, at 0.75 *h* lysozyme accommodates 200–300 more water molecule on unfolding, which corresponds to a quite small increase in expansion of the molecular volume.

Dehydration may lead to more or less significant conformational changes, which depend upon the protein studied. When observed, structural rearrangements occur essentially below 0.2–0.3 *h*. RNAse shows minor conformation changes on hydration as assessed by circular dichroism studies [19]. Raman and NMR studies of lysozyme unfolding lead to the conclusion that at very low hydration there is a loosening up of the molecule followed by more relevant conformation rearrangement at higher (0.1–0.3 *h*) hydration [7]. In general, dehydration-induced conformational changes appear to be driven by the need to compensate for the loss of hydrogen bonding with water [20,21]. This idea is supported by the hydration dependence spectroscopic studies on protein and model compounds. At low water content, the tendency to increase the extent of conformational states or secondary structures (such as β-sheets) with a lower degree of solvation and a higher degree of internal H-bonding (with respect to α-helix or random coil) has been observed. This is why additives containing hydroxyl groups, such sugars and polyols, which are able to act as water substitutes to form H-bonds with the protein groups, are effective protein stabilizers [20].

Below 0.2–0.25 *h* rigid-to-flexible state transitions are often observed, very much resembling glass-like transitions. Large-scale segmental motions are allowed only in the presence of water, which acts as plasticizer. Below a critical lubricant concentration (0.2 *h*) those motions do not occur anymore and only local (restricted to side-chain) and only small amplitude motions of few chain atoms are allowed. Dynamic glass-like rearrangements have been observed with various techniques such as hydrogen isotope exchange [22] and positron annihilation lifetime spectroscopy [23]. The decrease in flexibility has been correlated to the increase in protein thermostability [24].

In Fig. 2 a more detailed analysis of the consequences of dehydration on the protein organization is presented. Unlike at 1.4 *h*, the deconvolution analysis of the protein unfolding DSC peak at 0.48 *h* showed that the unfolding process could not be approximated by a single transition anymore but it has to be fitted with two partially overlapping subprocesses. These subprocesses can be attributed to the independent unfolding of the two domains of the RNAse molecule [13,25,26]. The corresponding values of the enthalpy change, ΔH_1 and ΔH_2, and of the transition temperature, T_{m1} and T_{m2}, are shown in Fig. 3 as a function of *h*. Domain decoupling is observed when one domain has a different intrinsic stability with respect to the other and/or the interdomain interactions are lost, with the concurrent decrease of the coupling free energy [26]. Conversely, complete coupling is observed at higher hydration level, where both domains unfold as a single cooperative unit. It

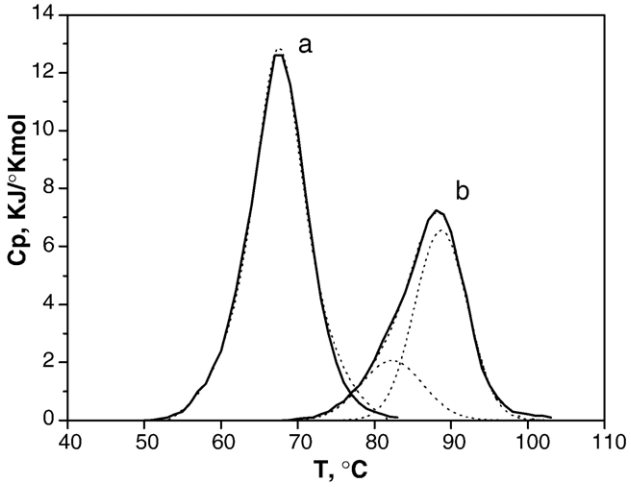

Fig. 2. Deconvolution analysis of the DSC curves. RNAse in the dry state at h 1.4 (a) and h 0.48 (b). The continuous lines are the experimental curves. The dotted lines are the curves calculated from the deconvolution fitting analysis. (Reprinted with permission from Battistel and Bianchi [10]. Copyright American Chemical Society).

is interesting to note that one of the two protein domains is more stabilized than the other, as it is shown in Fig. 3. ΔH_1 and T_{m1} associated with the unfolding of one domain are higher than the corresponding values of the other domain, suggesting an asymmetric stabilization effect. In conclusion, dehydration has a significant influence on the thermodynamic properties of the enzyme although at this hydration level (0.48 h) major conformational changes are not observed.

3.2. Organic solvents

The thermodynamic parameters, ΔH and T_m, of RNAse unfolding in the presence of dodecane is shown in Fig. 4A and B, respectively, as a function of hydration. At 1.4 h, ΔH (466 kJ/mol) is as large as in dilute aqueous solution (480 kJ/mol), whereas T_m is slightly higher (68 °C instead of 65.9). As in the case of the dry protein without any solvent, ΔH decreases

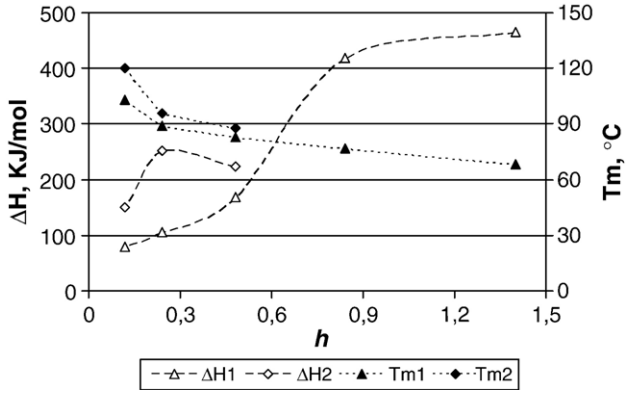

Fig. 3. Enthalpy changes, ΔH_1, ΔH_2, and transition temperatures, T_{m1}, T_{m2}, of RNAse domains unfolding as a function of hydration, h, without solvent. The transitions associated with the two domains unfolding were assessed by the deconvolution analysis of the DSC curves (see Fig. 2). (Reprinted with permission from Battistel and Bianchi [10]. Copyright American Chemical Society).

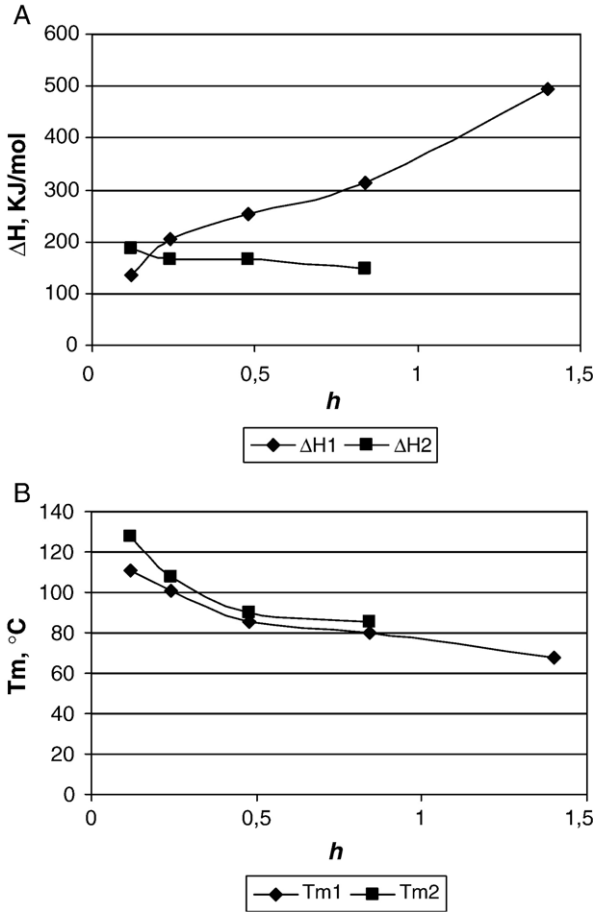

Fig. 4. Enthalpy changes, ΔH_1, ΔH_2 (A) and transition temperatures, T_{m1}, T_{m2} (B) of RNAse domains unfolding as a function of hydration, h, in the presence of dodecane. RNAse adsorbed on Celite. (Reprinted with permission from Battistel and Bianchi [10]. Copyright American Chemical Society).

smoothly from 1.4 h down to 0.3–0.35 h and then falls sharply below 0.3 h. Conversely, T_m increases slowly from 1.4 h to 0.35 h and then sharply increases, showing a significant stabilization effect. Seemingly, these dependencies on h suggest that the overall enhancement of the thermal stability in dodecane is due to the dehydrated state of the protein more than to a direct effect of the organic solvent. However, a more detailed analysis of the results reveals that dodecane has indeed some specific influence on the protein thermodynamics. For instance, domains decoupling occurs at higher hydration level than in the neat dry state. In fact, already at 0.84 h, the DSC unfolding transition has to be fitted with the sum of two independent subprocesses, whereas the same effect was observed at 0.48 h in the absence of any solvent. Moreover, as it is shown in Fig. 4A, the enthalpy change of unfolding of one domain (ΔH_2) is almost independent on hydration, whereas the other (ΔH_1) shows a remarkable dependence. Note that the same is not observed for T_{m2} (the middle point temperature of the domain transition) which changes as smoothly as T_{m1} by lowering h (Fig. 4B).

The effect of several organic solvents on RNAse thermal unfolding has been studied as a function of hydration. As it is shown in Table 1, the onset of domain decoupling occurs at

Table 1
RNAse unfolding in organic solvents

Solvent	Onset of domain decoupling, h	T_m (°C)	Solubility of water (%)
No solvent	0.48	87.0	–
Dodecane	0.84	80.6	0.006
Benzyl acetate	0.84	91.7	
Dipenthyether	0.84	89.0	
Toluene	0.24	97.0	0.03
Octanol	0.84	104	2.6 [a]
Cyclohexanone	0.84	123	8

RNAse adsorbed on Celite (reprinted in part with permission from Battistel and Bianchi [10]. Copyright American Chemical Society).
[a] 2-Ethyl-1-hexanol.

different hydration level depending on the solvent used [10]. Apparently, the break of the interdomain interactions is less sensitive to the presence of the organic solvent (the only exception is toluene) than to the hydration state. That is, as soon as the hydration drops below a critical value (about 0.8 h), the interdomain contact surfaces mismatch and the interactions are no longer favourable. However, also non-aqueous solvation may play a role on protein stability. In fact, apolar protein regions and hydrophobic patches can occupy a relevant portions of the protein surface, as inferred by the ratio of the polar to apolar residues (the value for RNAse is 1.73 [27], which is higher than for other typical globular proteins) and can interact directly with the organic solvent.

The overall T_m of the RNAse unfolding transition at 0.84 h in different organic solvents is listed in Table 1. As it is shown, T_m varies greatly with the solvent. Why T_m (and ΔH [10]) is so strongly solvent dependent at a given hydration level? Organic solvents, which have the lowest affinity towards water, have the least ability to solvate water molecules that remain adsorbed on the highly hydrophilic protein molecule. Conversely, more polar solvents do compete with the protein for water uptake, inducing a partitioning of water into the solvent and a partial protein dehydration. This effect is well represented by the data in Table 1. The increase of T_m correlates fairly well with the solvent capacity of dissolving water, which is stripped away from the protein. In this case, since the protein experiences a hydration level lower than the nominal one, T_m increases significantly, at level that in other solvents (less able to dissolve water) would correspond to a lower dehydration state.

Water miscible solvents have a completely different effect on the protein thermostability. Dimethylformamide destabilizes RNAse: at 1.4 h the unfolding transition occurs at 47.7 °C instead of 68 °C without solvent. The value of ΔH is as low as 250 kJ/mol, whereas in the absence of solvent is 470 kJ/mol. At lower h values the unfolding transition gradually disappears. The same destabilizing effect was observed with other polar water miscible solvents such as dioxane and pyridine. These effects suggest that these solvents are able to interact directly with the protein molecule, probably replacing water for H-bond formation or by direct binding to the protein. The replacement of the water molecules by the solvent results in the destabilization effect of the native protein structure [28,29].

Enhanced thermostability in organic water immiscible neat organic solvents has been observed mainly by studying enzyme activity and inactivation rates as a function of temperature [30–32] and only marginally by DSC [33]. All these studies have been performed with powdered enzyme suspensions by measuring the kinetics of the enzyme inactivation. It was concluded that enzymes are much more thermostable in hydrophobic solvents (which have weak interactions with the protein molecule) than in water miscible ones, which strip essential water away from the protein. The enhancement of the enzyme thermostability correlates with the increase of conformational rigidity in the dehydrated state. Moreover, deactivation rates are much slower in nearly dry organic solvents because water plays an essential role in many covalent reactions that cause enzyme irreversible thermoinactivation in aqueous solution. Besides thermostability, the use of enzymes in organic solvent brought forth novel enzyme properties as biocatalysts. Enzymes not only remain catalytically active but also show changes of the substrate specificity, regiospecificity, positional and stereo selectivity [34].

The role of organic solvent on enzyme thermostability is mediated by the water redistribution according to the affinity of the solvent towards water. Is there any other physical parameter of the solvent that correlates with protein stability? In order to answer this question, the following parameters have been considered: (a) log$P$: the partition coefficient of a given compound in the octanol/water two phases system. It is a parameter sensitive to the solvent hydrophobicity because all the molecular interactions between substance and solvent are reflected in the log$P$ value. (b) Hildebrand solubility parameter: this parameter is dependent on the heat of vaporization and therefore on the polar interactions and cohesive forces between the solvent molecules. (c) H-bonding parameter, Γ: it has been calculated from the shift of the IR frequency as a function of the strength of the H-bond. (d) Dipole moment: it is a measure of the electrostatic asymmetry of the molecule. Dipole–dipole and dipole–dipole induced interactions are part of the London dispersion forces that mediate molecular interactions in organic solvents. (e) Dielectric constant.

There is not a clear correlation between the ΔH and T_m of RNAse unfolding and the Hildebrand solubility parameter [35]. This suggests that intermolecular cohesive forces do not influence protein stability. A poor correlation was also obtained with the H-bonding parameter (Γ), which, seemingly, does not play a role on protein stability either. This is not surprising because the power of forming H-bond of the water immiscible organic solvent considered in this study is not very high. A general trend was not observed also as a function of the dipole moment. London dispersion forces, which mediate the interactions between solvent molecules and polar solvation sites of the protein, seem to have a minor impact on protein stability. The dependence of the ΔH and T_m of RNAse unfolding at 0.48 h on the last two parameters, log$P$ and dielectric constant, ε, are shown in Fig. 5A–D. In Fig. 5A and C a rather scattered dependence on log$P$ is observed. This is surprising because a direct correlation between thermostability (also operational stability and activity) and solvent hydrophobicity expressed by

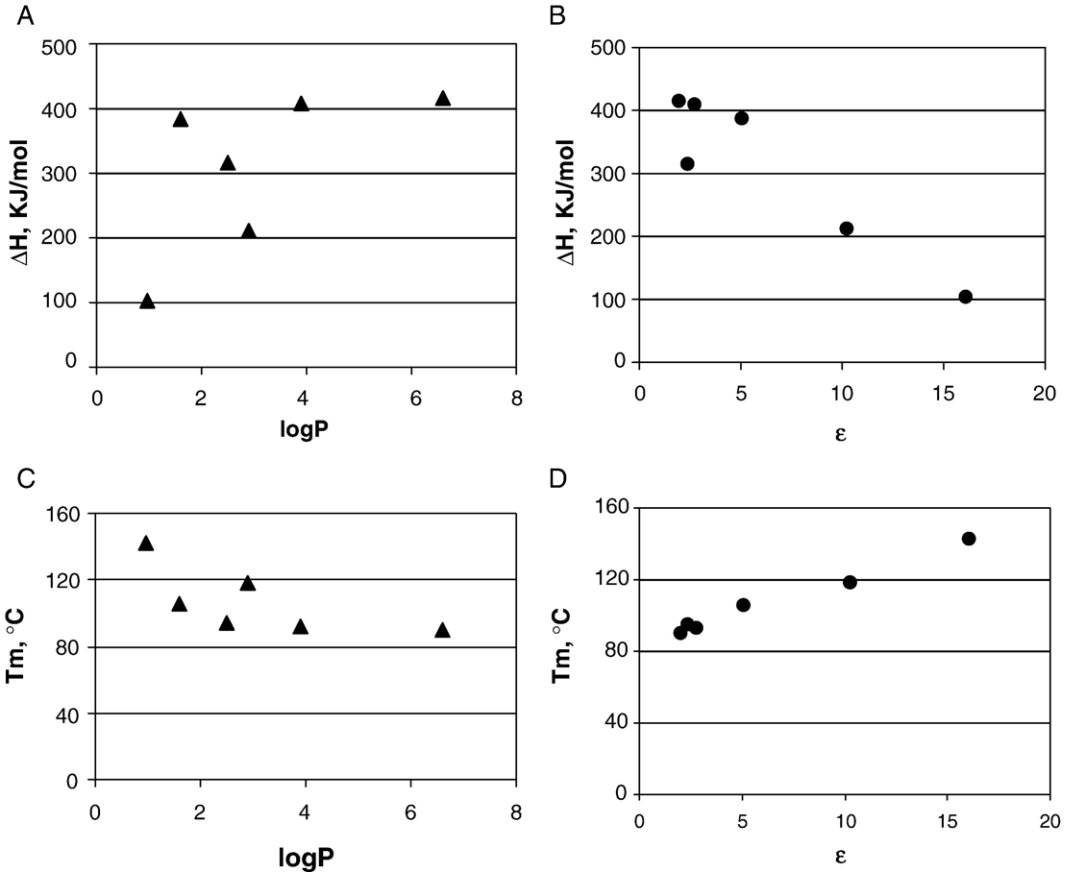

Fig. 5. ΔH and T_m of RNAse unfolding in organic solvents at 0.48 h as a function of log$P$ (A, C) and of the dielectric constant, ε, (B, D) of the solvent. (Reprinted with permission from Battistel and Bianchi [35]. Copyright Elsevier).

log$P$ has been reported [36,37]. If water solubility is accounted for, the correlation is even better [38]. All these results have been obtained from studies of the enzymes kinetics in organic solvents. In spite of the successful explanation of the kinetics data, log$P$ does not seem to give a good correlation with the thermodynamics of RNAse unfolding in organic solvents.

The parameter which gives the best correlation is the dielectric constant, ε, as shown in Fig. 5B and D, respectively. The data refer to the hydration level of 0.48 h but a similar trend is obtained at lower h values. Which kind of effects is reflected by the dependence of ΔH and T_m of RNAse unfolding on the dielectric constant? First, electrostatic forces (especially those independent of hydration such as dipole–dipole, dipole–charge, dipole–helix dipole, etc.) as well as the H-bonds are expected to be strengthened in low dielectric media. Consequently, if they play a significant role in protein energetics, an increase in the thermodynamic stability is expected with respect to aqueous solution. Second, as discussed in the previous section, if water redistribution upon unfolding is expected at low hydration level, the unfolded state would be significantly destabilized in organic media. In fact, the thermodynamic cost to leave an impaired H-bond is very high [39], much higher than in aqueous solution [40]. This is why the driving force to saturate potential H-bond sites upon unfolding (reflecting the need of the electrostatic balance) may become significant. As an evidence to support this assumption, EPR measurements showed that alcohol dehydrogenase dispersed in organic solvents at low hydration level becomes more rigid as the dielectric constant decreased. The degree of rigidity was also pH dependent, suggesting that the ionization state and polar interactions can have an important influence on protein dynamics in organic solvents [41].

In conclusion, the dependence of ΔH and T_m on the dielectric constant suggests that electrostatic forces may play a role on protein stability more relevant than that recognized before, in agreement with the new paradigm of proteins properties and functions [42]. Electrostatic forces may give a significant contribution to the stabilization of the hydrated protein, which has been probably underestimated in favour of the apparently more understood hydrophobic effect.

3.3. Reverse micelles

Reverse micelles are spheroidal aggregates formed by surfactants in organic solvents. They are called ‘reverse’ because the polar head of the detergent amphiphilic molecule is oriented towards the centre of the aggregate, whereas the apolar tail is immersed in the solvent. Inside the micellar restricted environment, limited by the polar heads (often formed by charged groups), water can be solubilized to form a micellar water pool. W_o, the water to surfactant molar ratio, is directly related to the dimension of the water pool, being a measure of the micelle water content. In the case of the micelles formed by

bis(2-ethylhexyl) sodium sulfosuccinate (AOT) in isooctane, W_o values ranging from 5 to 50 correspond to a micellar radius of 10 Å trough 80 Å [43]. The amount of AOT molecules varies from 11 to 1100 *per* micelle in the same W_o range. A small protein such as RNAse and lysozyme, which have a radius of 15–20 Å, may just be accommodated only above a certain value of W_o, unless water and AOT redistribution occur. Because of the small size of the water pool, the water dissolved inside the micelles has not the properties of bulk water even at W_o 30 [44,45]. Moreover, it has been estimated that at least 7 water molecules are needed to solvate the sulfonate group of AOT [46]. This implies that a fairly large amount of the water present (for instance, at least 30–40% at W_o 25) is strongly interacting with the charged surfactant heads.

In Fig. 6A and B the ΔH and T_m of RNAse and cytochrome *C* (Cyt *C*) unfolding in AOT reverse micelles are shown as a function of hydration, h', respectively. The hydration parameter h' (grams of water per grams of protein, g/g) was determined by using the amount of water inside the AOT micelles calculated

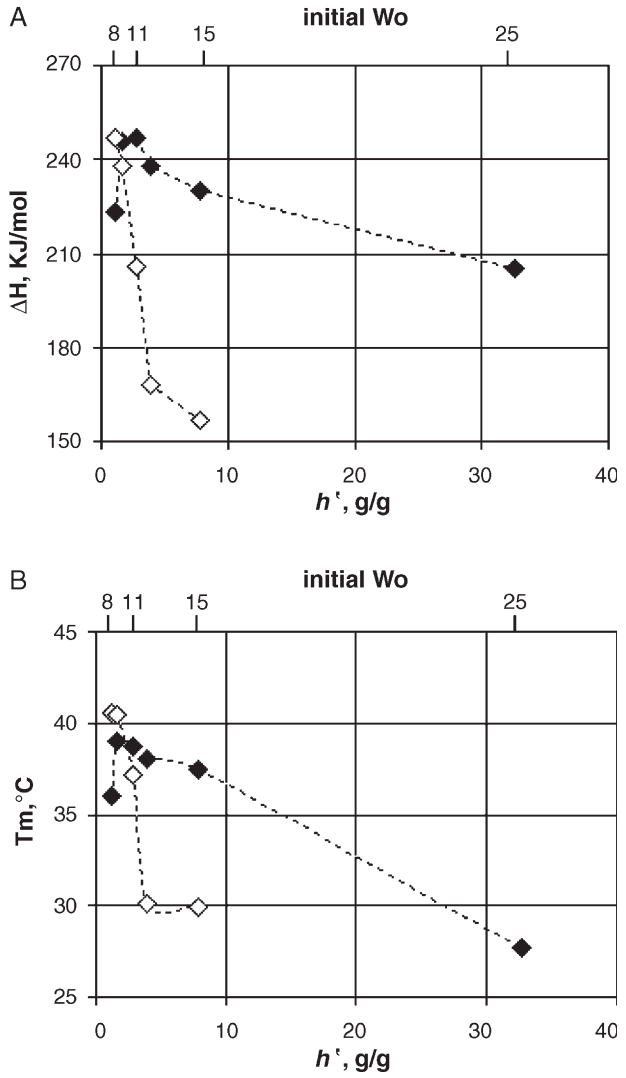

Fig. 6. ΔH (A) and T_m (B) of RNAse (◇) and cytochrome *C* (■) unfolding in reverse micelles formed by AOT 100 mM in isooctane as a function of the calculated hydration parameter, h', and of the initial W_o value.

from the micellar radii listed in the literature [43]. The amount of water was corrected by the change in water content due to the insertion of the protein inside the micelles, as described by Zampieri and Luisi [47]. As it is shown in Fig. 6, even at the highest hydration level, 32 h' (W_o 25) for RNAse and 7.8 h' for Cyt *C* (W_o 15.5), where micelles are significantly larger than the protein molecule, ΔH of unfolding is lower than in water solution. The measured values are 205 kJ/mol and 157 kJ/mol for RNAse, W_o 25, and cytochrome *C*, W_o 15.5, respectively, to be compared with corresponding ΔH in aqueous solution, 297 kJ/mol and 301 kJ/mol, respectively. The same effect is observed for T_m, which drops from 48.1 °C in water to 27.7 °C for RNAse and from 61 °C in water to 29.9 °C for Cyt *C*. These results suggest that, although the protein is largely hydrated, nevertheless the micellar aqueous environment is different from that in aqueous solution. The free energy change of unfolding, ΔG, calculated according to standard thermodynamic treatment, decreases from 17.8 kJ/mol in water to 2.0 kJ/mol at 32 h' for RNAse and from 17.6 kJ/mol in water to 3.0 kJ/mol at 7.8 h' for Cyt *C*. The values are still positive, but both proteins are clearly destabilized. The entropic term ($T\,\Delta S$) gives a large contribution to decrease the ΔG value but it is not compensated by the corresponding increase of the enthalpic term [11]. The destabilization effect seems to be related to the peculiar micellar protein surroundings that are dominated at pH 3.3 by electrostatic interactions between the positively charged proteins (the p*I* values of RNAse, cytochrome *C* and lysozyme are 9.4, 10.6 and 11, respectively) and the negatively charged AOT molecule. By changing the sign of the protein net charge, these electrostatic effects should be minimized. As a matter of fact, a fully reversible unfolding transition was observed with RNAse T_1 (p*I* 2.9) inserted in AOT micelles at pH 7. In this case the negatively charged protein has a thermodynamic behaviour very similar to that in water [48]. Similar conclusions were also suggested by the results of the elegant study of frequency domain fluorescence spectroscopy of proteins inserted in AOT reverse micelles. It was found that the perturbations observed were not due to hydration effects but to minor structural modification induced by the interactions with the micellar charged environment [49].

In all the experimental conditions the deconvolution of the DSC peak of both RNAse and cytochrome *C* shows the presence of one cooperative transition. Both proteins behave as a single thermodynamic cooperative units as in aqueous solution. The unfolding process is a two-state process in the whole h' range, as the ratio $\Delta H_{cal}/\Delta H_{vH}$ is close to 1 [50]. ΔH_{cal} is the measures calorimetric enthalpy change and ΔH_{vH} is the vant'Hoff enthalpy change associated with the transition. The domains decoupling process observed in neat organic solvents did not take place after protein solubilization in the reverse micelles, which, nevertheless, are surrounded by water immiscible organic solvents.

Apparently, the range of hydration, h', is considerably higher than that used for the organic solvents, h. It should be remembered, however, that a large amount of water is used up to hydrate the AOT molecules. For instance, at 2.8 h' (W_o 11.1) the calculated water molecules present are about

2100, but half of them are strongly interacting with the AOT molecules. Therefore, the actual water molecule available for protein hydration with respect to the total amount present is limited and corresponds to hydration level of about 0.7–0.9 h.

As the overall hydration decreases from 32 to 7.8 h' (W_o 25–15.5) to 1–2 h' (W_o 9–11), protein stability increases as indicated by the increase of both ΔH and T_m (Fig. 4), approaching the values in water. This is probably due to the progressive dehydration of the protein molecule, which, however, is still inserted in a highly charged environment that dominates its thermodynamics. ΔG significantly increases up to 10 kJ/mol at 2.8 h' for RNAse and up to 9.3 at 1.6 h' for Cyt C. The entropic term remains similar to the value calculated at 32 h' and 7.8 h', respectively, but the enthalpic term increases significantly, giving a positive contribution to the ΔG [11]. This confirms that the increase in stability might be not related to major water redistribution (which would have been mainly showed up in the entropic term) but rather to changes in the electrostatic balance and interactions inside the micelles as the water content is decreased. As W_o is further lowered (about 1 h'), the protein (RNAse) is destabilized by the progressive stripping of essential water.

It is interesting to note that the thermodynamic parameters of the RNAse unfolding at 2.8 h' (W_o 11.1) did not change over a period of 6 weeks. The enthalpy change remained constant within 0.5%. The protein showed a remarkable resistance to denaturation with respect to water solution.

3.4. Macromolecular crowding conditions

At significant high concentration of polyethylene glycol (PEG) in water, the solution cannot be considered a normal 'diluted' aqueous solution. Due to the presence of highly concentrated polymer, the chemical environment is different and crowding effects are expected. The macromolecules occupy most of the volume available and the contact with the solvent is minimal. This implies that the solvent volume is almost unavailable to dissolve a new macromolecule of the same size. For example, if a solution contains 30% of an identical polymeric species, less than 1% of the remaining volume is available to accommodate an additional molecule of a similar size. In other words, a new macromolecule will be inserted in the solution only by displacement of another macromolecule already present. It has been estimated that the concentration of macromolecules inside a living cell is within the range of 5–40%, far away from the dilute solution where usually biochemical processes are studied [51,52]. Besides the reduction of the diffusion rates, highly crowded conditions favour all the intermolecular reactions such as aggregation, binding, oligomerization, folding into compact conformations and so on.

What is the stability of proteins in artificial highly crowded solution conditions, which somewhat mimic internal cell environments? In order to inquire about this question, two crowded environment cases have been considered: one case will refer to the thermodynamics of hen egg-white lysozyme in concentrated (26%) PEG solution, and the other case is the study of the stability of RNAse in the thick mud-like fluids for drilling, artificial systems used in the oil upstream operations.

3.4.1. Concentrated PEG solutions

In Table 2 the enthalpy change, ΔH, and the middle point transition temperature, T_m, of the lysozyme unfolding transition in the presence of high molecular weight PEG (10 000 and 20 000 Da) at pH 4.25 are listed. The protein unfolds as a single cooperative unit and the transition can be well approximated by a two-state process in all the experimental conditions. Within the experimental error of the calorimeter experiments, ΔH can be considered constant. Actually, it was found to be independent on PEG molecular weight and concentration [12]. Conversely, T_m significantly decreases, suggesting a destabilizing effect induced by the presence of PEG. This was also observed earlier at lower PEG concentration by spectroscopic studies [53]. In Table 2 the apparent molar volume change, $\Delta V°$, and the apparent heat capacity change, ΔC_p, of unfolding are also listed. As it can be seen, a significant increase of both volume and heat capacity is observed. Based on these results, it seems that protein intermolecular aggregation, usually favoured in highly crowded states including PEG, can be ruled out. In fact, intermolecular association would be accompanied by a decrease, rather than by an increase, of the molar volume because the solvent molecules would be squeezed out from the intermolecular interface [54].

The data are more consistent with the hypothesis that there is an increase of the molecular volume due to the preferential hydration of the unfolded protein in the presence of high concentration of PEG. In other words, because of the high activity coefficient, the unfolded protein is able to compete favourably with the relatively hydrophobic PEG polymer for the few water molecules available. In this case an increase in molar volume is expected, as indeed has been observed. By assuming a hydrodynamic radius of 1.7 nm, it is possible to calculate that the addition of one monolayer of water molecules can induce a 30% increase of the molar volume, which is of the same order of the increment observed experimentally (Table 2). The preferential hydration hypothesis is consistent also with an increase of the molar heat capacity. In fact, bound water molecules have a specific heat capacity abnormally higher than free bulk water molecules [2,55].

It is not clear why T_m decreases in the presence of PEG. If the effects due to the change of the dielectric constant of the medium at high PEG concentration can be excluded, [12] the solute effect may be the net result of the destabilization of the native state and the stabilization of the unfolded state, two factors which are difficult to dissect. They may both depend upon some degree on the direct interaction between PEG and protein molecules.

Another possible explanation of the results is that PEG molecules are preferentially excluded from the surrounding of

Table 2
Thermodynamic parameters of lysozyme unfolding in 26% w/v PEG aqueous solution at pH 4.25

Conditions	ΔH (kJ/mol)	T_m (°C)	$V°$ (cm^3/mol)	ΔC_p (kJ/mol/K)
Water, pH 4.2	520	77	9833	21.0
PEG 10 000	506	72	12,300	34.0
PEG 20 000	540	74	12,084	42.0

$V°$ is the partial molar volume of lysozyme.

the protein native state but are preferentially bound to the unfolded state, as indeed it has been observed [56]. Since PEG is a partially hydrophobic polymer, it would interact favourably with the hydrophobic groups of the protein exposed upon unfolding, thus leading to a preferential stabilization of the unfolded form. This effect would be reflected by a decrease of the transition temperature, although ΔH would not be affected significantly. Moreover, the results of circular dichroism studies on protein–PEG systems showed that PEG does not induce any significant structural changes of the folded proteins, helping to maintain the native conformation [57].

A problem associated with this hypothesis is the observed increase of ΔC_p (Table 2). Hydrophobic interactions, as those occurring between the exposed apolar protein groups and the PEG molecule, should be characterized by a negative ΔC_p, as indeed has been observed experimentally for low molecular weight PEG 10 000 [56] and high molecular weight PEG 20 000 at relatively low concentration [12]. However, it is possible that in the presence of high concentration (26%) of PEG 20 000, the situation could be different. In these conditions, PEG molecule is larger than the protein, even in the unfolded state, and therefore, above some critical PEG concentration, steric and crowding effects may partially reverse the character of protein–PEG interactions after unfolding.

3.4.2. Fluids for drilling in oil upstream operations

Drilling of a well for oil/gas production requires the use of special fluids to counterbalance pressure gradients and, more generally, to control the pressure conditions. The water based drilling fluids are typically a mixture of starch (1–2% w/v), sized calcium carbonate (10–20% w/v) and scleroglucan or xanthan gum (0.1–0.5% w/v). The starch (usually chemically modified to improve thermostability) provides an elastic frame to control fluid loss to the rock (formation). Insoluble calcium carbonate is a bridging agent able to fill up the pores of the rock. The natural polymer scleroglucan (or xanthan gum) is used as viscosity enhancer (viscosifier). Even at low concentration this polysaccharide is able to increase the fluid viscosity, essential to improve transport capabilities. In fact, due to the presence of the viscosifier, the carbonate particles do not settle and form stable suspensions. This multicomponent fluid looks like a dense, thick and viscous mud. During the drilling, an impermeable thin layer, the so-called filter cake, is deposited on the borehole wall to avoid the invasion of the formation by the drilling fluids. The filter cake, only a few mm thick, is an elastic layer formed by a network of starch, scleroglucan and carbonate particles. The filter cake must be removed at the end of the drilling operations in order to start oil/gas production. The removal of the filter cake is accomplished by 'breakers', either chemical strongly oxidizers or hydrolytic enzymes, such as amylase, glucanase, etc. The first successful application of enzymes as breakers to clean up wells has been reported more than 10 years ago [58]. Since then, enzyme technology has been greatly improved [59].

Usually enzyme breakers are used at the end of the drilling phase, when the drilling fluids are removed from the well before starting the production. Ideally, it would be desirable to insert the enzyme directly into the filter cake as inactive catalyst during the drilling operations because in these conditions the catalyst would be evenly distributed along the whole well. The use of a suitable triggering event (such as a pH change) would then switch on the enzyme activity allowing the simultaneous activation of the filter cake breakage homogeneously along the well. This is a particularly important issue when operating in open-hole horizontal wells. In these wells, a difficult task is to optimize the production along the whole length of the well. In fact, the removal of the filter cake by chemical means (acid treatment, chemical oxidizers, etc.) is often obtained only in the initial part of the well, with the concurrent loss of production from the rest of it.

The control of the distribution of the enzyme catalyst along the well and the time-dependent regulation of its activity are key factors for their use in upstream operations. In these kinds of applications, the possibility to switch on and off the enzyme at will by some suitable triggering factor becomes an issue more important than the time or the temperature dependence of the activity (thermostability).

What is the stability of enzymes dispersed in the drilling fluids? What happens to the protein thermodynamic properties after solubilization in the highly crowded and heterogeneous environments of the drilling fluids? In order to answer this question, the unfolding of RNAse, used as a model protein, was studied in the presence of the typical drilling fluids components. These components were progressively added to the RNAse solution in water and after each addition the stability of the protein was monitored by DSC analysis. The normalized experimental DSC curves of RNAse unfolding, obtained in these conditions, are presented in Fig. 7. The corresponding ΔH and T_m are listed in Table 3. A single cooperative two-state process in all the conditions studied characterizes the unfolding transition. With respect to water, the addition of scleroglucan (0.4% w/w) shifts the transition to higher temperature (from 63.2 °C to 65.4 °C) accompanied by a slight increase of ΔH. It should be noted that, at this concentration, a viscous jelly-like

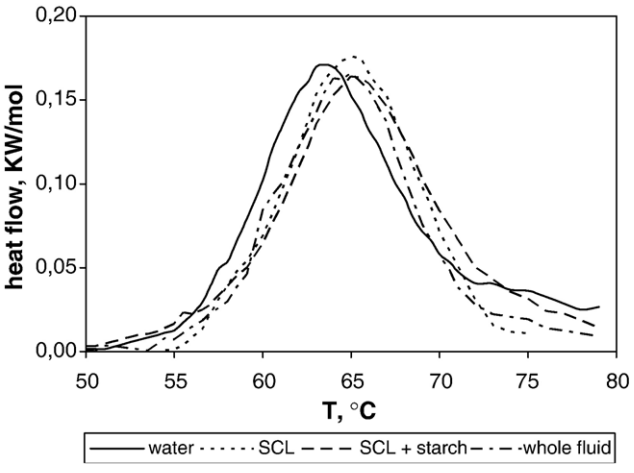

Fig. 7. Excess molar endothermic heat flow DSC curves of the RNAse unfolding transition in water (acetate buffer 150 mM, pH 5), in the presence of scleroglucan 0.4% w/w (SCL); scleroglucan 0.4% and maize starch 1%, (SCL + starch); scleroglucan 0.4%, starch 1% and calcium carbonate 10% (whole fluid). The RNAse concentration was approximately 4 mg/ml in each experiment.

Table 3
Thermodynamic parameters of RNAse unfolding in the presence of the drilling fluids' components

Conditions	ΔH (kJ/mol)	T_m (°C)
Acetate buffer 15 mM pH 5.1	403	63.2
SCL 0.4% w/w	439	65.4
SCL 0.4%+Starch 1%	415	65.5
SCL 0.4%+Starch 1%+$CaCO_3$ 10%	405	65.0

SCL is scleroglucan. Starch was from maize.

aqueous 'phase' is formed. A further addition of starch 1% does not change T_m or ΔH. It should be remembered that maize starch in these conditions is sparingly soluble in water. The RNAse unfolding in the whole fluid, completed with insoluble calcium carbonate (10% w/w), is similar to that observed in the presence of scleroglucan. It can be concluded that the stabilization effect is mainly due to the presence of scleroglucan alone. The subsequent additions of starch and carbonate bring about drastic changes of the physical properties of the suspension but do not give any further contribution to the protein stability.

Scleroglucan is a water-soluble extracellular polysaccharide produced by the fungus *Sclerotium*. It is formed by a backbone chain of glucose molecule linked by a (1→3)-β bond. A (1→6)-β-D-glucopyranosyl residue is bound to every third main chain glucose unit, yielding a comb like branched primary structure. The polymer dissolves in water as a triple helix of about 1.5×10^6 Da [60,61]. Interstrand interactions are strong and induce the formation of aggregates. Consequently, scleroglucan forms viscoelastic aqueous solutions at concentrations as low as 0.02–0.2% and rigid gels at concentration above 0.3 w/v [62]. The efficiency as viscosity enhancer is associated with the persistence length of the triple helix, which is significantly larger than that of the single strand. The triple helix is remarkably stable at temperature as high as 130 °C.

Why scleroglucan stabilizes the protein? The stabilization effect may be explained in terms of the positive change of the chemical potential (free energy) of the protein induced by the addition of sugars or polyalcohol [27]. This stems from the preferential hydration of the protein molecule in the medium, that is, the solute molecules are preferentially excluded by the close surroundings of the protein, which experiences a higher concentration of water with respect to the bulk solution. This leads to the stabilization of the native protein state and this is why sugars are good protecting agents against denaturation. Since it has been shown that the positive free energy change is proportional to the surface area of the macromolecule, then the unfolded state of the protein should experience a even higher increase of the chemical potential because of the more largely exposed surface area upon unfolding [27]. This implies an even higher destabilization of the denatured state.

The mechanism of the protection against unfolding by sugars and polyhydric compounds has been well studied by using small molecules such as sucrose or lactose. However, it is possible that in the presence of a high molecular weight polymer such as scleroglucan additional effects are induced on the protein due to the crowding of macromolecules much larger than the proteins itself.

3.5. Covalent immobilization

Immobilization of proteins by covalent modification to solid supports is one way to modify proteins in order to enhance biotechnological useful properties. After the covalent attachment to a solid support, conformational changes, which drive changes of the protein stability and reactivity, are expected to take place because the protein surface is somewhat modified. This may be observed not only because of the modification of the protein chemical properties *per se*, but also because of the environment change induced by the forced confinement of the macromolecule near the support surface. In Fig. 8 a computer simulation of RNAse attached to a silica surface is shown. The protein is coupled to the silica by a single point glutaraldehyde-mediated attachment through the terminal amino group. As evident by visual inspection, the protein experiences a microenvironment, which is by far different from that perceived by a normal solvent-exposed molecule. Although free to rotate around the chemical link, nevertheless the macromolecule is forced to share a close contact with the support surface, which is not a chemically inert material. In Fig. 8 it is also possible to note the presence of the two structural domains, separated by a deep cleft which accommodates the substrate.

An important thermodynamic consequence of the covalent immobilization of proteins is the frequently observed enhancement of the thermal stability as in the case, for example, of penicillin G acylase [63], amylase [64], chymotrypsin [65,66]. These observations were mostly based on studies of the irreversible inactivation rates more than on thermal stability experiments at equilibrium. A correlation between the increase of the thermostability and the degree of the protein conformational

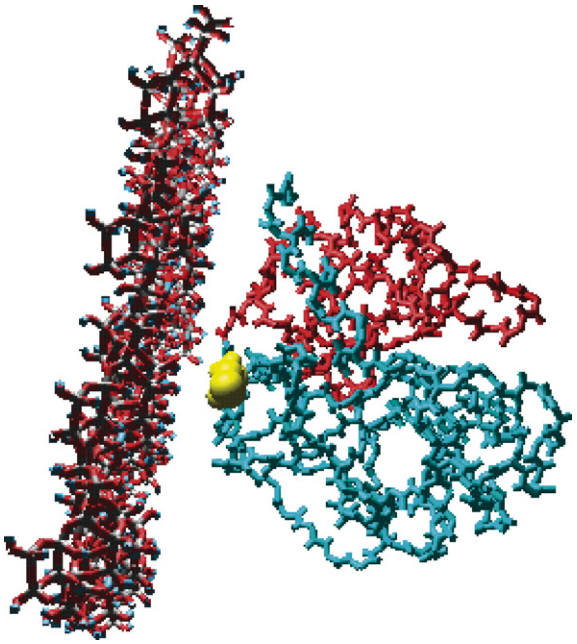

Fig. 8. Computer simulation of the ribonuclease A molecule linked to the silica controlled pore carrier (CPC, aminopropyl derivative) glass surface. The linking reagent is glutaraldehyde coupled to the terminal lysine amino group. The two differently coloured protein regions correspond to the two protein domains. The assembly has been simulated with Insight II (Biosym, San Diego, CA).

rigidity induced by the covalent attachment to a support was established. This underlines the importance of the entropic terms in the unfolding pathways of immobilized enzymes [24,67,68]. Conformational rigidity may have the thermodynamic consequence of stabilizing the functional equilibrium conformation, i.e., the native state, or decreasing the unfavourable conformational entropy gain of the random coil polypeptide chain during unfolding, i.e., kinetic stabilization of the unfolded state.

It has been observed that the properties of a protein significantly change after multipoint covalent attachment to a rigid support. Koch-Schmidt and Mosbach reported a progressive decrease of ΔH (the enthalpy change of unfolding) as a function of the number chemical links, from 2 to 8, between RNAse and the CNBr-activated Sepharose [69,70]. In the same interval, T_m increased by 5 °C and the activity (binding of inhibitor) decreased from 60% (1 link) to 15% (8 links) with respect to water. This suggests that the protein conformation was altered by progressively anchoring the protein to the support. The stability of the immobilized macromolecule was enhanced because T_m increased, but a concurrent 85% loss of the biological activity was also obtained. The decrease of ΔH is reminiscent of the similar decrease of the enthalpy of unfolding observed at low hydration level, where H-bond breaking is difficult and thermodynamically unfavourable.

In order to obtain information on the protein properties without inducing too much straining on the conformation, single point attachment techniques were developed [13,14]. The coupling between protein and controlled pore carrier (CPC) silica beads was obtained by the bifunctional glutaraldehyde which reacts with the protein primary amino groups on one side and with the aminopropyl groups of CPC on the other. Under controlled experimental conditions, the number of chemical linkages between RNAse and the aminopropyl silica beads can be reduced to 1.2±0.2. The thermodynamic analysis of the immobilized RNAse unfolding at pH 5 in various conditions is presented in Table 4. At this pH, CPC silica surface is slightly negatively charged (the pK_a of the siolic groups is 6.8) whereas RNAse is positively charged (pI 9.4). Unlike in the free state, the unfolding transition at pH 5 is well approximated by two independent processes, whereas the overall ΔH is almost unchanged. These processes are associated with the independent unfolding of the two protein domains. The overall cooperativity is lost and the uncoupling of the two domains occurs as a consequence of the immobilization [13]. It is interesting to note that each subprocesses is a single two-state transition because the ratio $\Delta H_{cal}/\Delta H_{vH}$ is very close to 1. Moreover, as shown in Table 4, one domain is significantly stabilized with respect the other because T_{m2} is 10 °C higher than T_{m1}, very close to the T_m value in water. The stabilized protein region is probably the domain containing the $-NH_2$ terminal group [13]. In fact, the RNAse terminal amino acid lysine is the most probable candidate for the linking reaction with the support because the pK of its primary amino group is 7.8, much lower than the other amino the primary amines (pK 10.2). The lower pK enhances the reactivity towards glutaraldehyde. The uncoupling of the protein domains was also observed in the presence of 3′-cytidine monophosphate (3′-CMP, Table 3), in spite of the slight stabilization effect (T_m increase) induced by the strength of the inhibitor binding. Indeed stabilization by ligand binding is often observed also for proteins in solution [71,72].

Table 4
Thermodynamic parameters of the unfolding of free, immobilized on silica carrier (CPC) and chemically modified RNAse

RNAse conditions	ΔH_1 (kJ/mol)	T_{m1} (°C)	ΔH_2 (kJ/mol)	T_{m2} (°C)	ΔH_{tot} (kJ/mol)
Free in acetate buffer 50 mM pH 5	375	58.9			375
Immobilized on CPC, pH 5	232	60.0	195	70.1	427
Immobilized on CPC, pH 5+3′-CMP	261	61.4	200	71.3	461
Immobilized on CPC treated with acetic anhydride	248	56.8			248
Immobilized on CPC treated with silane	0.0				0.0
Immobilized on CPC treated with silane+3′-CMP	284	59	213	75.4	428
Modified with valerialdehyde	394	59.3			394

3′-CMP is the inhibitor 3′-cytidine monophosphate.

Does the surface of the support have an influence on protein thermodynamics? In order to answer this question, the aminopropyl-CPC silica beads were modified in two ways: (A) removal of the aminopropyl primary amino groups and reduction of the surface charge density by treatment with acetic anhydride *after* glutaraldehyde activation (and before enzyme coupling); (B) silanization with dichlorodimethylsilane *before* glutaraldehyde activation and protein attachment in order to make the surface highly hydrophobic. In case A, RNAse glutaraldehyde-linked to CPC treated with acetic anhydride shows a single unfolding transition, as it is shown in Table 4. The magnitude of ΔH suggests that, after immobilization, only one domain is in the native folded state and is able to unfold as the temperature is raised, whereas the other domain is probably collapsed into an unstructured state. In the second case, B, RNAse, attached to a highly hydrophobic silanized CPC surface, does not give raise to any unfolding transition. The protein conformation is completely destabilized. The addition of the inhibitor 3′-CMP brings back the structural integrity of the protein although not completely because the splitting into two subprocesses is still observed (Table 4). This has two main implications. First, the destabilizing effect of the modified surface is not strong enough to induce irreversible changes of the protein conformation. Second, the binding of the inhibitor to the active site, located in the cleft between the two domains, has a gluing effect on the two protein regions, restoring the folded conformation but not the interdomain interactions.

When the protein was chemically modified with the monofunctional valeraldehyde, which reacts with the terminal amino groups mimicking the reaction with the bifunctional glutaraldehyde, the RNAse unfolding was identical to that in aqueous solution (Table 4, last entry). The loss of a single

surface charge (the amino group of the terminal lysine) does not seem to alter the protein thermodynamic stability and conformation in any significant way. All these observations lead to the conclusion that the chemical modification of the protein through the glutaraldehyde linkage *per se* does not alter significantly the protein stability. Conversely, it seems that the forced vicinity to the support surface induces more significant changes in protein thermodynamic state, ranging from domains decoupling (immobilization on unmodified CPC), through unfolding of a single domain (partially folded intermediate, observed with CPC treated with acetic anhydride) to complete unfolding (with silanized CPC).

In Table 5 the equilibrium constant, K_b, and the related free energy change, ΔG_b, of the binding reaction between the inhibitor 3′-CMP and CPC immobilized RNAse at pH 5, 25 °C, are listed as a function of the length of the spacer arm used to link the protein to CPC. The binding has been followed by calorimetry and spectrophotometry [14,15]. As it is shown, in the presence of a very short arm (the two carbon atoms of glyoxal, C_2) the protein is still able to bind the ligand but the affinity is low as indicated by the decrease of K_b with respect to water. The C_5 glutaraldehyde-coupling arm is sufficient to restore the accessibility of the ligand, as assessed by the value of K_b closer to the value in water. A longer spacer, the C_{22} glutaraldehyde–diaminododecane–glutaraldehyde bridge, does not improve the efficiency of the binding reaction. Scatchard plot analysis of the data shows that for the C_5 and C_{22} derivatives all the immobilized protein molecules were competent for ligand binding, whereas for the C_2 derivative only 40% of the molecules were active [13]. These results may suggest that, even in the case of very long coupling bridges, the protein does not experiences a complete accessibility to the substrate as in the free state in solution. Somehow the presence of the carrier surface is still perceived even if the apparent distance allowed by the length of the spacer arm between protein molecule and surface seems enough to guarantee a complete rotational and orientation freedom.

The effects of the covalent immobilization have been also studied with other proteins. Chymotrypsinogen is a typical protein, which is folded into two structural domains as inferred by inspection of the X-ray diffraction data. The protein unfolds as a single cooperative unit in aqueous solution. After immobilization on CPC, domains decoupling is observed and the two domains unfold separately [73]. Also in this case, the stabilization effect is asymmetrical because one domain is more stabilized than the other, showing a T_m value higher than that of the overall transition in water. The ΔH has remarkable pH dependence. By lowering the pH below 5, the unfolding transition of one domain is lost, whereas the other remains present down to pH 2.5. It seems that the collapse of part of the molecule at low pH is favoured by the covalent linkage to the support, which amplifies the conformational rearrangement on protonation also observed in the case of the free enzyme. In fact, by lowering the pH below pH 5, free chymotrypsinogen (but not chymotrypsin) has an abnormal excess heat of protonation associated with a conformational change induced by the protonation of 10 carboxylic acid groups [74]. A similar pH dependence is observed with immobilized papain [75]. Domain decoupling occurs after immobilization and the transition associated with one domain is lost below pH 5.5. Conversely, chymotrypsin does not show the same behaviour [76]. After immobilization, domain decoupling is accompanied by the stabilization of one domain with respect the other. However, unlike chymotrypsinogen, both domains are stable down to pH 3.

Table 5
RNAse immobilized on CPC. Effect of the length of the spacer arm on the binding reaction between of RNAse and the inhibitor 3′-CMP in acetate buffer 50 mM pH 5, 25 °C

Spacer length, C atoms	K_b (l/mol, $\times 10^3$)	ΔG_b (kJ/mol)
None (acetate buffer 50 mM pH 5)	50.0	26.9
C2 (glyoxal)	13.2	23.6
C5 (glutaraldehyde)	37.5	26.2
C22 (glutaraldehyde–diaminododecane–glutaraldehyde)	33.2	25.8

K_b, binding equilibrium constant. ΔG_b, free energy change.

4. Conclusions

The insertion of a protein in unusual chemical environments induces a variety of conformational responses as an adaptation to the new perturbing influence. The macromolecule strains back to cope with the new stress. The protein structural architecture shows a great deal of flexibility on responding to the different environmental changes, shifting the thermodynamic behaviour along the chemical coordinates of the protein–solvent integrated system. This versatility is observed in spite of the glass-like structural and physicochemical properties of the protein macromolecule. Water plays an essential role to modulate protein structural responses. Thermodynamic studies on proteins in the dry state clearly showed that water is the molecular lubricant of the protein structure. Low hydration levels change protein properties with respect to the highly hydrated state in solution, regulating stability, rigidity and function. On dehydration it was shown that subtle conformational changes cause the onset of the domains decoupling well before the loss of enzyme activity or the induction of major conformational changes. Dehydration effects are also fundamental to understand protein thermodynamics in neat water immiscible organic solvents. However, the non-aqueous environment has a well-defined influence on the protein stability, as it was shown by the dependence on the dielectric constant of the stability parameters. This dependence emphasizes the role of electrostatic interactions rather than hydrophobic interactions as source of protein stability also in aqueous solutions. The addition of detergent to form reverse micelles does not cause domain decoupling, which is the first sign of alteration of protein cooperativity, leaving unchanged the conformational integrity of the protein. However, the micellar high-charge-density environment alters water pool properties influencing protein stability, which is different from that in aqueous solution. On the other hand,

non-ionic molecular crowded environments have different effects on protein stability, ranging from destabilization caused by preferential binding, as in the PEG system, to stabilization induced by preferential hydration as in the case of the heterogeneous complex mixtures used as drilling fluids. Covalent immobilization brings about domain decoupling and a variety of conformational changes. The protein experiences a forced proximity of the support surface, which in turn induces protein adaptation responses. These responses range from domains uncoupling with asymmetric stabilization of one domain with respect to the other (attachment to unmodified CPC carrier), to complete loss of protein conformation (silanized CPC) through the loss of a single domain structure (acetic anhydride treated CPC or pH dependence). This is a peculiar situation where it is possible to trap unfolding intermediates, which is usually a quite difficult task in biochemical kinetic studies. All these effects are well characterized by thermodynamic point of view and may constitute the basis for the rational and controlled exploitation of proteins and enzymes in biotechnological applications.

References

[1] G.D. Fullerton, V.A. Ord, I.L. Cameron, An evaluation of the hydration of lysozyme by an NMR titration method, Biochim. Biophys. Acta 869 (1986) 230–236.

[2] J.A. Rupley, G. Careri, Protein hydration and function, Adv. Protein Chem. 41 (1991) 37–172.

[3] L.G. Butler, Enzymes in non-aqueous solvents, Enzyme Microb. Technol. 1 (1979) 253–259.

[4] G. Rialdi, E. Battistel, B.G. Barisas, Thermodynamics of enzymes in unusual environments, Thermochim. Acta 193 (1991) 349–361.

[5] S. Bone, R. Pethig, Dielectric studies of the binding of water to lysozyme, J. Mol. Biol. 157 (1982) 571–575.

[6] R.B. Gregory, Protein hydration and glass transition behaviour, in: R.B. Gregory (Ed.), Protein–Solvent Interactions, Marcel Dekker, New York, 1995, pp. 191–264.

[7] P.L. Poole, J.L. Finney, Hydration induced conformational and flexibility changes in lysozyme at low water content, Int. J. Biol. Macromol. 5 (1983) 110–308.

[8] I.D. Kunz, Hydration of macromolecules: III. Hydration of polypeptides, J. Am. Chem. Soc. 93 (1971) 514–516.

[9] M. Ruegg, U. Moor, B. Blanc, Hydration and thermal denaturation of β-lactoglobulin: a calorimetric study, Biochim. Biophys. Acta 400 (1975) 334–342.

[10] E. Battistel, D. Bianchi, Thermostability of ribonuclease A in organic solvents: a calorimetric and spectroscopic study, J. Phys. Chem. 98 (1994) 5368–5375.

[11] E. Battistel, P.L. Luisi, G. Rialdi, Thermodynamic study of globular protein stability in microemulsions, J. Phys. Chem. 92 (1988) 6680–6685.

[12] W. Zielenkiewicz, R. Swierzewski, F. Attanasio, G. Rialdi, Thermochemical, volumetric and spectroscopic properties of lysozyme-polyethylene glycol system, J. Therm. Anal. Calorim. 83 (2006) 587–595.

[13] G. Rialdi, E. Battistel, Decoupling of melting domains in immobilized ribonuclease A, Proteins, Structure, Function, and Genetics 19 (1994) 120–131.

[14] E. Battistel, D. Bianchi, G. Rialdi, Thermodynamics of immobilized A, Pure Appl. Chem. 63 (1991) 1483–1490.

[15] E. Battistel, P. Sabbioneta, G. Rialdi, Binding studies on 3′ cytidin monophosphate and ribonuclease A derivatives by flow and batch calorimetry, Thermochim. Acta 172 (1990) 21–30.

[16] Y. Fujita, Y. Noda, Effect of hydration on the thermal denaturation of lysozyme as measured by differential scanning calorimetry, Bull. Chem. Soc. Jpn. 51 (1978) 1567–1568.

[17] Y. Fujita, Y. Noda, Effect of hydration on the thermal stability of protein as measured by differential scanning calorimetry. Chymotrypsinogen A, Int. J. Pept. Protein Res. 18 (1981) 12–17.

[18] P.H. Yang, J.A. Rupley, Protein–water interactions. Heat capacity of the lysozyme–water system, Biochemistry 18 (1979) 2654–2661.

[19] Y.N. Chirgadze, A.M. Ovsepyan, Hydration mobility in peptide structures, Biopolymers 11 (1972) 2179–2186.

[20] S.J. Prestrelski, N. Tedeschi, T. Arakawa, J. Carpenter, Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers, Biophys. J. 65 (1993) 661–671.

[21] J.F. Carpenter, J.H. Crowe, An infrared spectroscopic study of the interactions of carbohydrates with dried proteins, Biochemistry 28 (1989) 3916–3922.

[22] J.E. Shinkel, N.W. Downer, J.A. Rupley, Hydrogen exchange of lysozyme powders. Hydration dependence of internal motions, Biochemistry 24 (1985) 352–366.

[23] R.B. Gregory, Free-volume and pore size distributions determined by numerical Laplace inversion of positron annihilation lifetime data, J. Appl. Phys. 70 (1991) 4665–4670.

[24] M. Vihinen, Relationship of protein flexibility to thermostability, Protein Eng. 1 (1987) 477–480.

[25] K.P. Murphy, V. Bhakuni, D. Xie, E. Freire, The molecular basis of co-operativity in protein folding: III. Identification of cooperative folding units and folding intermediates, J. Mol. Biol. 227 (1992) 293–306.

[26] J.F. Brandts, C.Q. Hu, L.-M. Lin, M.T. Mas, A simple model for proteins with interacting domains. Applications to scanning calorimetry data, Biochemistry 28 (1989) 8588–8596.

[27] T. Arakawa, S.N. Timasheff, Stabilization of protein structure by sugars, Biochemistry 21 (1982) 6536–6544.

[28] J. Hermans, D. Puett, G. Acampora, On the conformation of denatured proteins, Biochemistry 8 (1969) 22–30.

[29] G. Velicelebi, J.M. Sturtevant, Thermodynamics of the denaturation of lysozyme in alcohol–water mixtures, Biochemistry 18 (1979) 1180–1186.

[30] A. Zacks, A.M. Klibanov, Enzymatic catalysis in organic media at 100 °C, Science 224 (1984) 1249–1251.

[31] A.M. Klibanov, Enzyme that work in organic solvents, Chemtech (1986 (June)) 354–359.

[32] F.H. Arnold, Engineering enzymes for non aqueous solvents, Tibtech 8 (1990) 149–244.

[33] D.B. Volkin, A. Staubli, R. Langer, A.M. Klibanov, Enzyme thermo-inactivation in anhydrous organic, Solvents Biotechnol. Bioeng. 37 (1991) 843–853.

[34] J.S. Dordick, Principles and applications of nonaqueous enzymology, Appl. Biocatal. 1 (1991) 1–51.

[35] E. Battistel, D. Bianchi, Influence of the solvent properties on protein stability in organic media, in: W.J.J. van den Tweel, A. Harder, R.M. Buitelaar (Eds.), Stability and Stabilization of Enzymes, Elsevier, Amsterdam, 1993, pp. 13–20.

[36] L.E.S. Brink, J. Tramper, Optimization of organic solvent in multiphase biocatalysis, Biotechnol. Bioeng. 27 (1985) 1258–1269.

[37] M. Reslow, P. Adlercreutz, B. Mattiason, Organic solvents for bioorganic synthesis: 1. Optimization of parameters for a chymotrypsin catalyzed process, Appl. Microbiol. Biotechnol. 26 (1987) 1–8.

[38] C. Laane, S. Boeren, R. Hilhorst, C. Veeger, Optimization of biocatalysis in organic media, in: C. Laane, J. Tranper, M.B. Lilly (Eds.), Biocatalysis in Organic Media, Elsevier, Amsterdam, 1987, pp. 65–84.

[39] A.R. Fersht, J.-P. Shi, J. Knill-Jones, D.M. Lowe, A.J. Wilkinson, D.M. Blow, P. Brick, D.M. Carter, M.M.Y. Waye, G. Winter, Hydrogen bonding and biological specificity analysed by protein engineering, Nature 314 (1985) 235–238.

[40] K.A. Dill, Dominant forces in protein folding, Biochemistry 29 (1990) 7133–7155.

[41] R.M. Guinn, P.S. Skerker, P. Kavanaugh, D.S. Clark, Activity and flexibility of alcohol dehydrogenase in organic solvents, Biotechnol. Bioeng. 37 (1991) 303–308.
[42] R. Lumry, The new paradigm for protein research, in: R.B. Gregory (Ed.), Protein–Solvent Interactions, Marcel Dekker, New York, 1995, pp. 1–141.
[43] M.P. Pileni, T. Zemb, C. Petit, Solubilization by reverse micelles: solute localization and structure perturbation, Chem. Phys. Lett. 118 (1985) 414–418.
[44] J.P. Morel, N.M. Desrosiers, C. Lhermet, Heat capacities and volumes in the reverse micellar phase of the AOT–water–decane systems, J. Chim. Phys. Phys-Chim. Biol. 81 (1984) 109–112.
[45] P.L. Luisi, B.E. Straub (Eds.), Reverse Micelles, Plenum, New York, 1984.
[46] M. Zulauf, H.-F. Eicke, Inverted micelles and microemulsions in the ternary-system H_2O–aerosol–OT–isooctane as studied by photon correlation spectroscopy, J. Phys. Chem. 83 (1979) 480–486.
[47] G.G. Zampieri, H. Jäckle, P.L. Luisi, Determination of the structural parameters of reverse micelles after uptake of proteins, J. Phys. Chem. 90 (1986) 1849–1854.
[48] M.C.R. Shastry, M.R. Eftink, Reversible thermal unfolding of ribonuclease T_1 in reverse micelles, Biochemistry 35 (1996) 4094–4101.
[49] P. Marzola, E. Gratton, Hydration and protein dynamics: frequency domain fluorescence spectroscopy on proteins in reverse micelles, J. Phys. Chem. 95 (1991) 9488–9495.
[50] P.L. Privalov, Stability of proteins. Small globular proteins, Adv. Protein Chem. 33 (1979) 167–241.
[51] S.B. Zimmerman, A.P. Minton, Macromolecular crowding: biochemical, biophysical, and physiological consequences, Annu. Rev. Biomol. Struct. 22 (1993) 27–65.
[52] R.J. Ellis, A.P. Minton, Join the crowd, Nature 425 (2003) 27–28.
[53] D.A. Knoll, J. Hermans, Effect of poly(ethylene glycol) on protein denaturation and model compound p*K*a, Biopolymers 20 (1982) 1747–1750.
[54] T.V. Chalikian, Volumetric properties of proteins, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 207–235.
[55] J.J. Suurkuusk, Specific heat measurements on lysozyme, chymotrypsinogen, and ovalbumin in aqueous solution and in solid state, Acta Chem. Scand., A 28 (4) (1974) 409–417.
[56] J.C. Lee, L.L.Y. Lee, Thermal stability of proteins in the presence of poly (ethyleneglycols), Biochemistry 26 (1987) 7813–7819.
[57] J.C. Lee, L.L.Y. Lee, Preferential solvent interactions between proteins and polyethylene glycols, J. Biol. Chem. 256 (1981) 625–631.
[58] A.R. Richards, R.M. Tjon Joe Pin, J.L. Boles, Enzymatic breaker system for nondamaging removal of cellulose-based blocking gels, Paper SPE 25488 Presented at the 1993 Production Operations Symposium Held in Oklahoma City, OK, March 21–23, 1993.
[59] H.D. Brannon, R.M. Tjon Joe Pin, P.S. Carman, W.D. Wood, Enzyme breaker technologies: a decade of improved well stimulation, Paper SPE 84213 Presented at the 2003 SPE Annual Technical Conference and Exhibition held in Denver, CO, October 5–8, 2003.
[60] T. Yanaki, T. Norisuye, H. Fujita, Triple helix of *Schizophyllum commune* polysaccharide in dilute-solution: 3. Hydrodynamic properties in water, Macromolecules 13 (1980) 1462–1466.
[61] B.H. Falch, A. Elgsaeter, B.T. Stokke, Exploring the (1→3)-beta-D-glucan conformational phase diagram to optimise the linear to macrocycle conversion of the triple-helical polysaccharide scleroglucan, Biopolymers 50 (1999) 496–512.
[62] A.J. Stipanovic, P.J. Giammatteo, Curdlan and scleroglucan. NMR characterization and gel properties, in: J.E. Glass (Ed.), Polymers in Aqueous Media: Performance Through Association, Advances in Chemistry Series, vol. 223 American Chemical Society, 1989, pp. 75–87.
[63] J.M. Guisan, G. Alvaro, R. Fernandez-Lafuente, C.M. Rosell, J.L. Garcia, A. Tagliani, Stabilization of heterodimeric enzyme by multipoint covalent immobilization: penicillin G acylase from *Kluyvera citrophila*, Biotechnol. Bioeng. 42 (1993) 455–464.
[64] R. Ulbrich, A. Schellenberger, W. Damerau, Studies on the thermal inactivation of immobilized enzymes, Biotechnol. Bioeng. 28 (1986) 511–522.
[65] V.V. Mozhaev, N.S. MelikNubarov, V.Y. Levitski, V.A. Siksnis, K. Martinek, High stability to irreversible inactivation at elevated temperatures of enzymes covalently modified by hydrophilic reagents. alpha-Chymotrypsin, Biotechnol. Bioeng. 40 (1992) 650–662.
[66] R.M. Blanco, P.J. Halling, A. Bastida, C. Cuesta, J.M. Guisan, Effect of water immiscible organic solvents on activity/stability of soluble chymotrypsin and immobilized derivatives, Biotechnol. Bioeng. 39 (1992) 75–84.
[67] P. Zhuang, D.A. Butterfield, Optimization of covalently coupling. enzyme to poly membranes: EPR studied of papain, J. Appl. Polym. Sci. 47 (1993) 1329–1342.
[68] J.M. Sturtevant, Heat capacity and entropy changes in processes involving proteins, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 2236–2240.
[69] A.C. Koch-Schmidt, K. Mosbach, Studies on conformation of soluble and immobilized enzymes using differential scanning calorimetry: 1. Thermal stability of nicotinamide adenine dinucleotide dependent dehydrogenases, Biochemistry 16 (1977) 2101–2105.
[70] A.C. Koch-Schmidt, K. Mosbach, Studies on conformation of soluble and immobilized enzymes using differential scanning calorimetry: 2. Specific activity and thermal stability of enzymes bound weakly and strongly to Sepharose CL4B, Biochemistry 16 (1977) 2105–2109.
[71] H. Fukada, J.M. Sturtevant, F.A. Quiocho, Thermodynamics of the binding of L-arabinose and of D-galactose to the L-arabinose-binding protein of *Escherichia coli*, J. Biol. Chem. 258 (1983) 13193–13198.
[72] J.M. Sturtevant, Biochemical applications of differential scanning calorimetry, Annu. Rev. Phys. Chem. 38 (1987) 463–488.
[73] E. Battistel, F. Attanasio, G. Rialdi, Thermal stability of immobilized α-chymotrypsinogen, J. Therm. Anal. 61 (2000) 513–525.
[74] D.D.F. Shiao, J.M. Sturtevant, Heat of binding protons to globular proteins, Biopolymers 15 (1976) 1201–1211.
[75] G. Rialdi, E. Battistel, Unfolding mechanism and stability of immobilized papain, J. Therm. Anal. 47 (1996) 17–25.
[76] G. Rialdi, E. Battistel, Melting domains in proteins. Free and immobilized α-chymotrypsin, J. Therm. Anal. 45 (1995) 631–637.