The effect of gradual dehydration on the thermal stability of a protein entrapped in a polymeric network

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We report a significant enhancement in protein thermal stability associated with the gradual dehydration of a polymeric gel (in which a model protein is entrapped) and point out some general implications suggested by this result.

Thermostabilization is often required (or desirable) for technological applications of proteins, which explains the continuing interest in the characterization of proteins from thermophilic organisms and in the design of purposely modified proteins with enhanced thermal stability.[1] It is noteworthy that, in several studies,[2] dehydration has been shown to lead to significant protein thermostabilization; these studies have mostly employed fully dried or lyophilized enzymes, often suspended in anhydrous organic solvents. Here, on the other hand, we report on the stability enhancement associated with the gradual dehydration of a polymeric gel (polyacrylamide) in which a model protein (α-chymotrypsin) is entrapped.

Polyacrylamide gels employed in this work (25% monomer, 5% cross-linker) meet the following essential conditions.† (1) They are free from small amounts of the polymerization reactants, given that these very reactive substances (in particular, persulfate[3]) may irreversibly alter the protein; for the same reason, polymerization is carried out in the absence of protein, which is introduced into the gel by electrophoresis *only after* the gel has been prepared and exhaustively 'cleaned'.† (2) Once the protein has been introduced, gels with different degrees of dehydration (= percentage weight loss upon drying) are obtained by a controlled dehydration procedure;‡ dehydration is accompanied by gel shrinkage (20–25% reduction in each linear dimension for a dehydration degree of 50%), thus reducing the pore size of the polymeric network and contributing to the immobilization of the protein. (3) Absorption of the gels in the near-UV region is low and temperature-independent and, therefore, the denaturation of the entrapped protein may be characterized by following the temperature-dependence of the UV protein absorption§ (Fig. 1).

Thermal denaturation of α-chymotrypsin in solution, in glycine buffer pH 3, is reversible and conforms to the two-state equilibrium model.[4] Under the same solvent conditions, reversibility is also found for α-chymotrypsin entrapped in gels with dehydration degrees between 0% and about 50%; two-state analysis of the absorbance *versus* temperature profiles for these gels (Fig. 1) yields van't Hoff enthalpy values (505 ± 50 kJ mol^{-1}) in good agreement with the denaturation enthalpy determined for the protein in solution (540 kJ mol^{-1}) by differential scanning calorimetry;[4] nevertheless, the denaturation temperature values for the entrapped protein (54.5 ± 2.1 °C) are already a few degrees higher than that for the protein in solution: 49 °C.[4] (The denaturation temperature, T_m, is defined here as the temperature at which half of the protein has denatured.)

† Gel slabs (1 mm thick) were prepared by polymerization of a solution (25% monomer, 5% cross-linker) of acrylamide *plus* *N,N'*-methylenebisacrylamide (both ultrapure grade from Boehringer) in 50 mmol dm^{-3} glycine pH 3 buffer. Polymerization was initiated by addition of ammonium persulfate *plus* *N,N,N',N'*-tetramethylethylenediamine (TEMED) and was carried out at 30 °C, given that polymerization at lower temperatures may produce turbid gels with high absorption in the UV region (see: C. Gelfi and P. G. Righetti, *Electrophoresis*, 1981, **2**, 220). Gel slabs were exhaustively cleaned from residues of polymerization reactants by dialysis against eight 500 cm^3 changes of 50 mmol dm^{-3} glycine pH 3 (total dialysis time was four days). α-Chymotrypsin was purchased from Sigma and used without further purification. 1 mg amounts of the protein (initially dissolved in 100 μl buffer) were introduced in the gel by conventional electrophoresis and the protein band was located by UV-densitometry of the (unstained) gel.

‡ For gel dehydration, a small section of the slab (with or without protein) was cut and suspended inside a flask in such a way that it did not touch any surface; a water-pump vacuum was then applied and dehydration was allowed to proceed for a time ranging from a few minutes to about five hours.

§ For UV-spectroscopy, a section of the gel containing the protein (or free from protein for control experiments) was sandwiched between two quartz windows, sealed by using a silicone O-ring of the appropriate thickness and placed in the spectrophotometer cell holder. Protein-free polyacrylamide gels showed a low (and temperature-independent) absorption in the UV region and, after subtraction of the low absorbance due to the gel, UV spectra of entrapped α-chymotrypsin were essentially identical with those of the protein in solution. In general, the concentration of α-chymotrypsin in the gel area illuminated by the beam was of the order of 1 mg ml^{-1} (calculated from the absorbance at 280 nm, the thickness of the gel slab and the known extinction coefficient). In thermal denaturation experiments, the absorbance at $\lambda = 280$ nm or $\lambda = 291$ nm was measured as the temperature was increased at a heating rate of 0.5–1 °C min^{-1} (the temperature was controlled with a circulating-water bath and measured with a thermistor placed in contact with the sandwich). In order to check the reversibility of the denaturation process, the absorbance was also measured upon gel cooling and, often, a second heating experiment was also carried out. For a degree of dehydration below 50% the absorbance recovered its previous low-temperature value upon cooling and the second heating produced a transition essentially identical with that of the first heating; on the other hand, denaturation was irreversible for a degree of dehydration above 50% (no absorbance recovery upon cooling and no transition in the second heating were observed). Experimental absorbance/T profiles were analysed by using the following equation derived from the two-state, equilibrium model: $A = (A_N + A_D \cdot K)/(1 + K)$, where A_N and A_D are the absorbances of the native and denatured states (both assumed to change linearly with T) and K is the denaturation equilibrium constant given by the following integrated van't Hoff equation: $K = \exp[-(\Delta H^{vH}/R)\cdot(1/T - 1/T_m)]$, where ΔH^{vH} is the van't Hoff, denaturation enthalpy and T_m is the denaturation temperature. For irreversible denaturation (dehydration degree above 50%), an analysis of the data according to a two-state irreversible model (J. M. Sanchez-Ruiz, J. L. Lopez-Lacomba, M. Cortijo and P. L. Mateo, *Biochemistry*, 1988, **27**, 1648) was also carried out; this kinetic analysis led to T_m values in close agreement with those previously derived from the two-state, equilibrium analysis.

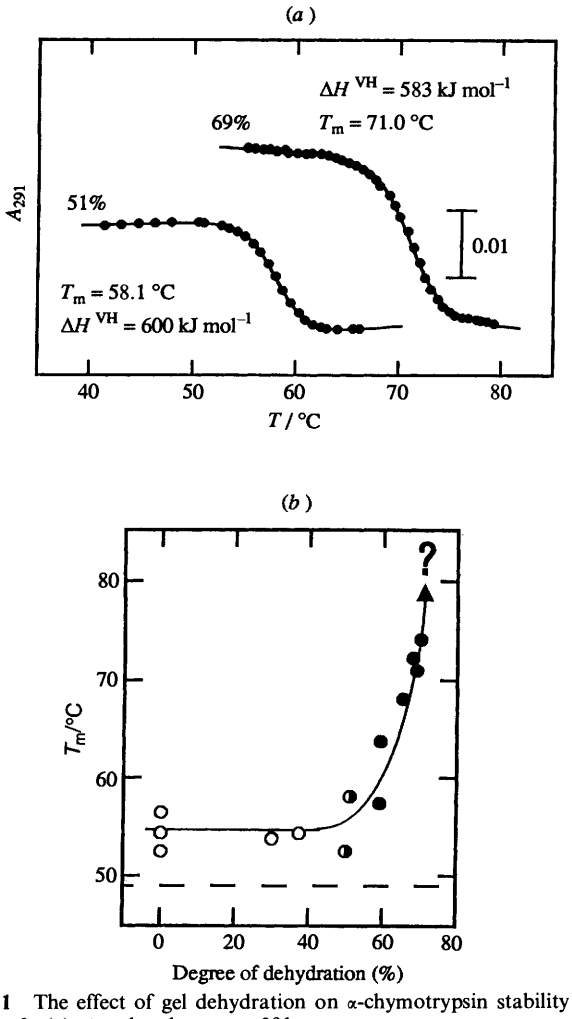

Fig. 1 The effect of gel dehydration on α-chymotrypsin stability at pH = 3: (*a*) ○, absorbance at 291 nm *versus* temperature, experimental data for protein entrapped in gels with dehydration degree of 51% and 69%; ——, best fits of the two-state equilibrium equation;§ (*b*) effect of gel dehydration on the denaturation temperature of α-chymotrypsin [T_m values were calculated from non-linear, least-squares fittings, such as those shown in (*a*)]. The broken line corresponds to the T_m value for α-chymotrypsin in solution, determined by differential scanning calorimetry.[4] The different types of symbols refer to the reversibility of the denaturation process: ○, highly reversible; ◑, partially reversible; ●, irreversible.

For dehydration degrees above 50%, the following two features are apparent (Fig. 1). (1) T_m increases sharply and a value of 75 °C is obtained for a dehydration degree of 71%,¶ and (2) denaturation becomes irreversible, suggesting that the native structure is kinetically trapped and that stabilization might correlate with a decrease in the rate of denaturation.[5] In fact, a restricted conformational mobility in the absence of water (leading to a high kinetic barrier for unfolding) has been adduced[2] to explain the enhanced thermal stability of dry proteins suspended in organic solvents; in our case, the steric constraints imposed by the polymeric network might also contribute to this restricted conformational mobility.

Some technological applications of proteins (biosensors, molecular electronics, optical processing of information, *etc.*) may require a solid-like support[6] (such as a thin film). The present work suggests that, in these cases, immobilization of the protein in an appropriate support followed by a controlled dehydration process is an interesting possibility if thermostabilization is required, as we have previously pointed out.[7]

Recent work[8] indicates that some proteins are likely to be partially denatured upon lyophilization, a result which has obvious implications for lyophilized protein pharmaceuticals and may explain why enzymes are usually much less active in organic solvents than in water.[8] We suggest, therefore, that alternative (and potentially gentler) dehydration procedures, such as that employed in the present work, deserve attention.

¶ Attempts to reach a higher degree of dehydration by using our simple (and, admittedly, crude) dehydration procedure resulted in 'hard gels' (dehydration degree around 80%) that gave complex and difficult to interpret absorbance *versus* temperature profiles. Note, however, that the shape of the T_m *versus* dehydration degree plot [Fig. 1(*b*)] suggests that very high T_m values may be reached by carefully tuning the amount of residual water (or, better, the water activity) in the gels. Possibly, this could be achieved by equilibrating the gels with solutions of known water activity (see V. A. Parsegian, R. P. Rand, N. L. Fuller and D. C. Rau, *Methods Enzymol.*, 1986, **127**, 400).

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