

## The thermal unfolding of hevein, a small disulfide-rich protein

Andrés HERNÁNDEZ-ARANA<sup>1</sup>, Arturo ROJO-DOMÍNGUEZ<sup>1</sup>, Manuel SORIANO-GARCÍA<sup>2</sup> and Adela RODRÍGUEZ-ROMERO<sup>2</sup>

<sup>1</sup> Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, México

<sup>2</sup> Instituto de Química, Universidad Nacional Autónoma de México, México

(Received 2 September 1994/9 January 1995) – EJB 94 1314/3

Differential scanning calorimetry was used to study the thermal unfolding of hevein, a 43-residue disulfide-rich protein whose three-dimensional structure has been determined by X-ray diffraction. In the range pH 2.0–3.7 this process was approximately 75 % reversible as judged by repeated scans on the same sample. The ratios of van't Hoff to calorimetric enthalpies were considerably larger than one, suggesting that intermolecular cooperation is involved in the unfolding of this protein. Alternatively, it is possible that the partial irreversibility of this process may cause distortions of the endotherm that affect the calculation of the van't Hoff enthalpy. Experimental changes in heat capacity and enthalpy were compared with those calculated from polar and nonpolar surface areas buried in the native state. It was found that when the unfolded state is represented as an extended chain without disulfide cross-links, experimental and calculated parameters agree well. However, if the unfolded protein is modeled with the presence of disulfide bridges, the agreement between the two sets of parameters is lost. The entropy change/residue at 112 °C is considerably smaller than the average value for globular proteins, thus suggesting that, as expected, disulfide bonds strongly influence the entropy of the unfolded state of this protein.

**Keywords.** Hevein; disulfide-rich protein; thermal unfolding; differential scanning calorimetry.

During the last two decades, many efforts have been devoted to the study of the relationship between protein structure and stability. From these studies, important conclusions have recently emerged that seem to be generally applicable to the protein unfolding process [1, 2]. It is now accepted, for example, that thermodynamic parameters for unfolding can be parameterized in terms of the polar and apolar surface areas that are buried in the native state of the macromolecule and become exposed to the solvent in the unfolded state [1, 3]. According to this approach, hydrogen bonds, van der Waals' interactions, and the hydrophobic effect are considered as the main forces that contribute to protein stability [1, 3, 4]. It is recognized, however, that other factors like electrostatic interactions and disulfide bridges may have an important but less well understood role [5, 6].

In this study, we carried out a calorimetric investigation of hevein, a small disulfide-rich protein from *Hevea brasiliensis* [7]. The folding of the 43-residue polypeptide chain of this protein has been determined by X-ray diffraction [8]. It is remarkable that this molecule lacks a hydrophobic core, suggesting that its four disulfide bridges may play an important role in the stabilization of the native state. These molecular characteristics of hevein led us to study its thermal unfolding in order to test some of the current ideas about protein stability.

## MATERIALS AND METHODS

Hevein was purified from the latex of rubber (*H. brasiliensis*) trees by the method described previously by Rodríguez et al. [9]. The concentration of hevein was determined by means of its molar absorption coefficient at 278 nm ( $\epsilon = 12032 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) [10]. Care was taken to assure that this value does not change significantly within the pH range employed in this study. For the calculation of molar quantities, a molecular mass of 4700 Da [7] was used. Polyacrylamide gel electrophoresis experiments were carried out in a PhastSystem instrument (Pharmacia) using the running conditions suggested by the manufacturer.

Differential scanning calorimetry measurements were performed with a MicroCal MC-2 instrument (MicroCal, Northampton MA, USA). Experiments were conducted at several pH values in the range 1.8–4.5. Hevein solutions of 3.3–4.7 mg/ml were dialyzed against 0.05 M glycine at the appropriate pH. Solutions were degassed under vacuum for 15 min, after which the protein concentration and pH were confirmed, and samples were then loaded into the calorimeter. In all cases, the scanning rate was 60 °C/h, and a total pressure of  $2.2 \text{ kg} \cdot \text{cm}^{-2}$  was applied to the cell compartments. Buffer/buffer base lines were obtained under the same conditions and subtracted from sample tracings. Calorimetric,  $\Delta H_{\text{cal}}$ , and van't Hoff,  $\Delta H_{\text{vH}}$ , enthalpies were calculated by using the DA-2 software package (MicroCal).

Accessible surface areas were estimated with the program ACCESS (Scott R. Presnell, University of California, San Francisco), an implementation of the Lee and Richard's algorithm [11], using a probe radius of 0.14 nm and a slice width of 0.025 nm. Accessible surface areas in the native state were calculated from the crystal structure of hevein [8], whereas two different denatured structures were modeled using BIOGRAF, version 3.1 (Molecular Simulations, Inc., Waltham MA) in order

Correspondence to A. Hernández-Arana, Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, Apartado Postal 55-534, 09340 México, DF, México

Fax: +52 5 7244666.

**Abbreviations.**  $\Delta H_{\text{cal}}$ , calorimetric enthalpy;  $\Delta H_{\text{vH}}$ , van't Hoff enthalpy;  $\Delta C_p$ , change in heat capacity upon protein unfolding;  $T_m$ , temperature at the maximum of the calorimetric curve;  $\Delta v$ , change in the number of protons bound upon protein unfolding;  $A_p$  and  $A_{\text{ap}}$ , change in the solvent-accessible polar and apolar areas due to denaturation, respectively;  $\Delta S_{T_m}$ , unfolding entropy at  $T_m$ .

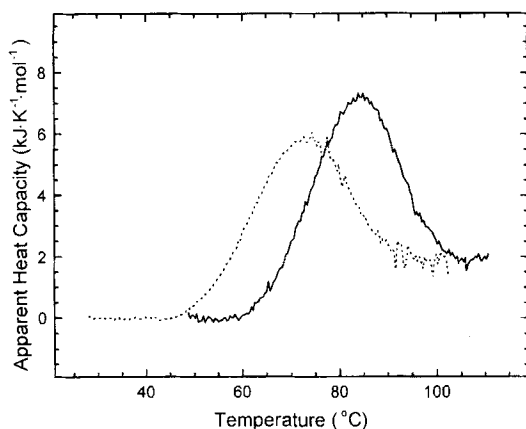


**Fig. 1.** Differential scanning calorimetry curves of hevein after subtraction of buffer/buffer baseline. Experiments were performed at pH 2.10 (.....) and 3.54 (—), with a scan rate of 60°C/h. The protein concentration was approximately 4 mg/ml.

to estimate their accessible surface areas. The first model of the denatured molecule did not consider the presence of disulfide bonds and consisted of a fully extended polypeptide chain with  $\phi$ ,  $\psi$  and  $\omega$  angles of 180° and  $\chi_i$  ( $i = 1-4$ ) equal to 60°. However, the second representation of the denatured state retained all four cystine residues joined by polypeptide single loops with proper amino acid sequence; the conformation of those loops was interactively oriented to achieve maximal solvent exposure in both main and side chains, and the geometry was relaxed by energy minimization. In all accessible surface areas calculations, nitrogen and oxygen atoms were considered polar whilst the solvent accessible surface of carbon and sulfur atoms determined the apolar areas.

## RESULTS AND DISCUSSION

Fig. 1 shows the differential scanning calorimetry curves for hevein at pH 2.10 and 3.54, after subtraction of the corresponding buffer/buffer tracings. In the pH range 1.82–3.67, denaturation endotherms were approximately 75 % reversible, as judged by repeated scans of samples that were previously subjected to a first heating and cooling cycle. Above pH 4.0, the reversibility drastically decreased and calorimetric curves showed a notable exothermic effect in the region of high temperature (data not shown), suggesting that aggregation of the unfolded molecules was taking place under these conditions [12, 13].

As can be seen in Fig. 1, the calorimetric curves for hevein indicate that there is a positive change in heat capacity upon unfolding of the macromolecule,  $\Delta C_p$ , in agreement with observations made on other proteins [14, 15]. This parameter was determined by linearly extrapolating the predenaturation and postdenaturation parts of each calorimetric recording and evaluating the difference between these parts at  $T_m$  (the temperature corresponding to the maximum of the curve) [15]. The average value found for  $\Delta C_p$  was  $1.91 \pm 0.33 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$  ( $0.41 \pm 0.07 \text{ J} \cdot \text{K}^{-1} \cdot \text{g}^{-1}$ ). In order to determine unfolding enthalpies, the calorimetric curves were corrected for heat capacity changes by subtraction of a straight line connecting initial and final temperatures of the transition. In this way, we constructed an excess heat capacity function from which  $\Delta H_{\text{cal}}$  and  $\Delta H_{\text{vH}}$  were evaluated by means of the DA-2 software. The results obtained are summarized in Table 1, where one can observe that at all pH values studied  $\Delta H_{\text{vH}} > \Delta H_{\text{cal}}$ . This finding suggests that intermolecular cooperation is involved in the unfolding pro-

**Table 1.** Unfolding parameters of hevein. Calorimetric and van't Hoff enthalpies are referred to  $t = t_m$ .

| pH   | $t_m$ | $\Delta C_p$                                          | $\Delta H_{\text{cal}}$           | $\Delta H_{\text{vH}}$ | $\Delta H_{\text{cal}} / \Delta H_{\text{vH}}$ |
|------|-------|-------------------------------------------------------|-----------------------------------|------------------------|------------------------------------------------|
|      | °C    | $\text{kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ | $\text{kJ} \cdot \text{mol}^{-1}$ |                        |                                                |
| 1.82 | 73.0  | 1.54                                                  | 121.2                             | 181.4                  | 0.67                                           |
| 2.10 | 74.2  | 1.86                                                  | 119.8                             | 174.2                  | 0.69                                           |
| 2.42 | 74.1  | 1.71                                                  | 126.6                             | 186.0                  | 0.68                                           |
| 2.92 | 77.8  | 2.09                                                  | 129.6                             | 173.5                  | 0.75                                           |
| 3.20 | 83.6  | 2.60                                                  | 142.3                             | 174.7                  | 0.81                                           |
| 3.33 | 82.6  | 1.76                                                  | 138.8                             | 181.8                  | 0.76                                           |
| 3.54 | 84.5  | 1.84                                                  | 152.6                             | 174.0                  | 0.88                                           |
| 3.67 | 90.1  | 1.88                                                  | 158.8                             | 177.7                  | 0.89                                           |

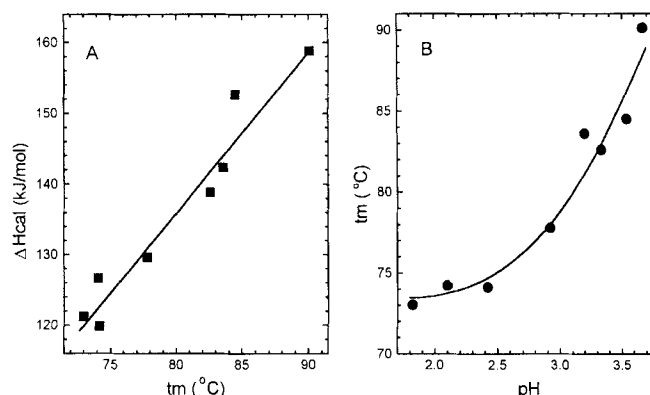


**Fig. 2.** Temperature dependence of  $\Delta H_{\text{cal}}$  (A) and the effect of pH on  $t_m$  (B) for hevein thermal unfolding. Data were taken from Table 1.

cess of hevein [16]. It must be noted that X-ray studies of hevein, performed on crystals grown at pH 7.5, show no evidence of extensive intermolecular contacts [8]. Furthermore, gel-filtration experiments indicated that hevein behaves as a monomer in solution either at pH 7.0 or at pH 3.0 (unpublished results from our laboratories). Of course, it is possible to explain the calorimetric results by assuming that the unfolded state is oligomeric. In this respect, it must be noted that the electrophoretic behavior of heat-denatured hevein in the presence of sodium dodecyl sulfate (but without reducing agents) showed that a considerable part of the protein is present as an oligomer of molecular mass much higher than that of monomeric hevein. In control experiments, where a reducing agent was added, the amount of oligomer decreased somewhat. This evidence indicates that some intermolecular disulfide bonds can be formed in the unfolded state of hevein; however, other mechanisms of covalent oligomerization could also be taking place.

A further explanation for the difference between  $\Delta H_{\text{cal}}$  and  $\Delta H_{\text{vH}}$  can be found in the lack of full reversibility. Indeed, it has been shown that when the unfolding of a protein is followed by a relatively slow irreversible step, which occurs with a negligible enthalpy change, the calorimetric curves can be distorted to some extent [17]. This effect may conduce to a small error in the determination of  $T_m$ , but otherwise it can produce considerable variations in the height of the heat capacity curve at  $T_m$ , which is the value employed to calculate  $\Delta H_{\text{vH}}$ .

Fig. 2A shows the variation of the unfolding enthalpy with temperature. There is a good linear relationship between  $\Delta H_{\text{cal}}$  and  $T_m$ , as has been observed for several other proteins [14, 15].

Furthermore, it is generally accepted that the slope of a plot of  $\Delta H_{\text{cal}}$  versus  $T_m$  is equal to  $\Delta C_p$ , provided that the ionization enthalpy of protein groups is adequately compensated by the ionization enthalpy of the buffer [15]. From Fig. 2A, a  $\Delta C_p$  of  $2.34 \pm 0.23 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$  was calculated for hevein, in good agreement with the value determined directly from the calorimetric curves (see above). However, pH had an important effect on the stability of hevein, as indicated by the variation of  $T_m$  in the range pH 2.4–3.7 (Fig. 2B). This effect is a reflection of the change in the number of protons bound,  $\Delta v$ , that occurs upon unfolding of the protein. For a two-state transition  $\Delta v$  can be estimated by means of the equation [15, 18]:

$$\Delta v = \frac{\Delta H}{2.303 RT_m^2} \cdot \frac{\partial T_m}{\partial \text{pH}} \quad (1)$$

Using the assumption that hevein denaturation is a two-state process, it would be found (from data in Fig. 2B and data in Table 1) that the unfolding of this protein results in the uptake of approximately one proton/protein molecule. This figure should be taken cautiously since deviations from a reversible two-state transition might lead to a different number of protons bound. Nevertheless, the value of  $\Delta v$  obtained for hevein agrees with that reported in the case of bovine pancreatic trypsin inhibitor, another small disulfide-rich protein [19].

**Comparison of hevein unfolding parameters with those for other proteins.** *Heat capacity and enthalpy changes.* In order to compare the unfolding thermodynamics of hevein with results obtained for larger proteins, it is necessary to relate thermodynamic parameters to molecular properties such as the number of residues or changes in accessible surface areas. It is now recognized that the unfolding heat capacity change has both polar and apolar contributions [1, 3]. It has been proposed that  $\Delta C_p$  (in  $\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ) can be calculated by the relationship [1]:

$$\Delta C_{p,\text{calc}} = 188 (A_{\text{ap}}) - 109 (A_{\text{p}}), \quad (2)$$

where  $A_{\text{ap}}$  and  $A_{\text{p}}$  are the apolar and polar surface areas buried in the folded state, respectively. Furthermore,  $A_{\text{p}}$  is the main determinant of the enthalpy change when extrapolated to 100°C, the so-called convergence temperature [1]. At this temperature, the enthalpy change arises only from polar interactions and has an average value of  $14.6 \pm 1.3 \text{ kJ} \cdot (\text{mol} \cdot \text{nm}^2)^{-1}$  of buried polar surface for the vast majority of globular proteins studied so far [1].

According to the procedures described in the Materials and Methods section, we found that when the unfolded state is modeled as an extended chain with broken disulfides, native hevein buries  $12.25 \text{ nm}^2$  and  $17.76 \text{ nm}^2$  of polar and apolar surface areas, respectively. Using these values in Eqn (2),  $\Delta C_{p,\text{calc}}$  amounts to  $2.01 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ , which is close to the experimental  $\Delta C_p$ . However,  $\Delta H_{\text{cal}}$  extrapolated to 100°C is  $183.9 \text{ kJ} \cdot \text{mol}^{-1}$  (compare Fig. 2A) and when normalized by the polar buried area the value of  $15.0 \text{ kJ} \cdot (\text{mol} \cdot \text{nm}^2)^{-1}$  is obtained, in excellent agreement with the average value for other globular proteins. The coincidence between experimental and calculated parameters suggests that in the fraction of unfolded hevein molecules that occur as oligomers, the contact surface between monomers is rather minimal. In addition, the above results also suggest that disulfide bridges have only a minor influence on the unfolding enthalpy and heat capacity changes. It should be stressed that Eqn (2) was derived relating experimental  $\Delta C_p$  values obtained from a set of proteins, with their  $A_{\text{p}}$  and  $A_{\text{ap}}$  values estimated under the consideration of a fully extended chain with no disulfide bonds for the denatured state. This fact requires that any  $\Delta C_p$  prediction using Eqn (2) must be performed with the same conditions as the data-set proteins. However, it is reason-

able to expect that the presence of cross-links might lead to a reduction of accessible surface area in the unfolded state and, therefore, to a diminution of the area buried in the native state. Since hevein is smaller and possesses more disulfide bonds than the proteins used to propose Eqn (2), it was considered interesting to compare the results obtained above with those estimated with a cross-linked denatured state model. Calculations performed in this way showed that  $A_{\text{p}}$  and  $A_{\text{ap}}$  are reduced to  $10.08 \text{ nm}^2$  and  $8.26 \text{ nm}^2$ , respectively. If these latter values were employed in Eqn (2),  $\Delta C_{p,\text{calc}}$  would be  $0.46 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ , in disagreement by a large amount with the experimental  $\Delta C_p$ . Similarly, the normalized enthalpy change at 100°C would be modified to  $18.2 \text{ kJ} \cdot (\text{mol} \cdot \text{nm}^2)^{-1}$ , which is outside the range observed for larger globular proteins. The use of a cross-linked model for unfolded hevein thus gives results in contradiction with experimental parameters. A plausible explanation for these results is that the model of unfolded hevein with disulfide bridges is conformationally too rigid to adequately represent a dynamic, real unfolded state, thus causing an underestimation of the surface area exposed in the unfolded state and, consequently, of  $A_{\text{p}}$  and  $A_{\text{ap}}$ .

**Entropy change.** For globular proteins, unfolding entropies/residue have a common value of  $18.0 \pm 0.4 \text{ J} \cdot \text{K}^{-1} \cdot (\text{mol residue})^{-1}$  when extrapolated to 112°C [1, 15]. Since it is assumed that at this temperature entropy changes are primarily of configurational origin [1], deviations from the average value that occur in particular cases may reflect the effect of disulfide cross-links on the unfolded state entropy. In this study, the unfolding entropy of hevein at 112°C (385.15 K) was calculated by using the known relationship:

$$\Delta S = \Delta S_{T_m} + \Delta C_p \ln \left( \frac{385.15}{T_m} \right), \quad (3)$$

where  $T_m$  is a reference temperature at which  $\Delta S_{T_m} = \Delta H_{T_m}/T_m$ . Experimental values of  $T_m$  and  $\Delta H_{T_m}$  were chosen as those at pH values less than 2.42, because in this region ionization effects are negligible (compare Fig. 2B). The unfolding entropy so obtained was  $13.9 \pm 0.3 \text{ J} \cdot \text{K}^{-1} \cdot (\text{mol residue})^{-1}$ , which is well below the average value mentioned above. This result indicates that disulfide bonds have a strong influence on the unfolding entropy of hevein.

This study was supported in part by the *Consejo Nacional de Ciencia y Tecnología, México* (Convenio no. 0609-N9110).

## REFERENCES

- Murphy, K. P. & Freire, E. (1992) Thermodynamics of structural stability and cooperative folding behavior in proteins, *Adv. Protein Chem.* **43**, 313–361.
- Murphy, K. P., Privalov, P. L. & Gill, S. J. (1990) Common features of protein unfolding and dissolution of hydrophobic compounds, *Science* **247**, 559–561.
- Spolar, R. S., Livingstone, J. R. & Record, M. T. Jr (1992) Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water, *Biochemistry* **31**, 3947–3955.
- Privalov, P. L. & Gill, S. J. (1988) Stability of protein structure and hydrophobic interaction, *Adv. Protein Chem.* **39**, 191–234.
- Dill, K. A. (1990) Dominant forces in protein folding, *Biochemistry* **29**, 7133–7154.
- Betz, S. F. (1993) Disulfide bonds and the stability of globular proteins, *Protein Sci.* **2**, 1551–1558.
- Walujono, K., Sholma, R. A., Beintema, J. J., Mariono, A. & Hahn, A. M. (1975) in *Atlas of protein sequences and structure* (Dayhoff, M. O., ed.) vol. 5, supplement 3, p. 308, National Biomedical Press, Washington DC.

8. Rodríguez-Romero, A., Ravichandran, K. G. & Soriano-García, M. (1991) Crystal structure of hevein at 2.8 Å resolution, *FEBS Lett.* **291**, 307–309.
9. Rodríguez, A., Tablero, M., Arreguín, B., Hernández, A., Arreguín, R., Soriano-García, M., Tulinsky, A., Park, C. H. & Seshadri, T. P. (1988) Preliminary X-ray investigation of an orthorhombic crystal of hevein, *J. Biol. Chem.* **263**, 4047–4048.
10. Archer, B. L. (1960) The proteins of *Hevea brasiliensis* latex. 4. Isolation and characterization of crystalline hevein, *Biochem. J.* **75**, 236–240.
11. Lee, B. & Richards, F. M. (1971) The interpretation of protein structures: estimation of static accessibility, *J. Mol. Biol.* **55**, 379–400.
12. Goins, B. & Freire, E. (1988) Thermal stability and inter-subunit interactions of cholera toxin in solution and in association with its cell-surface receptor ganglioside Gm1, *Biochemistry* **27**, 2046–2052.
13. Edge, V., Allewell, N. M. & Sturtevant, J. M. (1988) Differential scanning calorimetric study of the thermal denaturation of aspartate transcarbamoylase of *Escherichia coli*, *Biochemistry* **27**, 8081–8087.
14. Privalov, P. L. & Khechinashvili, N. N. (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study, *J. Mol. Biol.* **86**, 665–684.
15. Privalov, P. L. (1979) Stability of proteins. Small globular proteins, *Adv. Protein Chem.* **33**, 167–241.
16. Sturtevant, J. M. (1987) Biochemical applications of differential scanning calorimetry, *Annu. Rev. Phys. Chem.* **38**, 463–488.
17. Sánchez-Ruiz, J. M. (1992) Theoretical analysis of Lumry-Eyring models in differential scanning calorimetry, *Biophys. J.* **61**, 921–935.
18. Ptitsyn, O. B. & Birshtein, T. M. (1969) Method of determining the relative stability of different conformational states of biological macromolecules, *Biopolymers* **7**, 435–445.
19. Schwarz, H., Hinz, H. J., Mehlich, A., Tschesche, H. & Wenzel, H. R. (1987) Stability studies on derivatives of the bovine pancreatic trypsin inhibitor, *Biochemistry* **26**, 3544–3551.