

Fundamentals of protein stability

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Abstract - Protein stability is quantitatively described by the standard Gibbs energy change, ΔG_D^0 , involved in unfolding the unique, three dimensional structure to randomly coiled polypeptide chains. However, for construction of new proteins or improving stability of existing proteins knowledge of the overall ΔG_D^0 is not sufficient. Rather it is desirable to have parameters at hand that permit estimates of the various contributions to stability of individual interactions resulting from amino acid replacements. This presentation refers to some basic experiments and summarizes the present picture of principal forces governing protein stability.

NATURE COPES WITH EXTREMES

The frontiers of viability of living organisms are marked by extreme conditions: 1 - 12 for pH, -5 - 110°C for temperature, 0.1 - 120 MPa for hydrostatic pressure, and 0.6 - 1.0 for water activity. While organisms existing at extreme pH usually maintain neutral pH in their cytoplasm by active proton pumps, other extremophiles, such as thermophilic or barophilic organisms, cannot evade the external stress but have to adapt for survival (ref. 1). Prominent candidates for adaptational changes are proteins that carry out a great many of the important tasks in living cells. Consequently there is great interest in understanding the forces involved in stabilization of the native structure of proteins. In view of today's genetic engineering capabilities a solution of that problem is also of cardinal importance to rational protein design in medical and technical applications.

THE STANDARD GIBBS ENERGY OF DENATURATION, ΔG_D^0 , IS THE APPROPRIATE MEASURE OF STABILITY. IT CAN BE OBTAINED FROM DSC-CURVES

A comparison of stability between proteins having different transition temperatures must be based on ΔG_D^0 values that refer to the same temperature. In general 25°C is the reference temperature. Proper extrapolation from the transition temperature T_m to 25°C requires knowledge of the heat capacity change, ΔC_p , between the native and unfolded protein. For a protein undergoing a two state transition $N \rightleftharpoons D$, the temperature dependence of ΔG_D^0 is given by eq. 1.

$$\Delta G_D^0 = \Delta H_D^0(T_m) \cdot \{1 - T / T_m\} + \Delta C_p \cdot \{T - T_m - T \cdot \ln(T / T_m)\} \quad (1)$$

where $\Delta H_D^0(T_m)$ is the standard enthalpy change at the transition temperature, T_m , and ΔC_p the molar temperature independent heat capacity difference between the native and unfolded form of the protein. The above formula is only valid for the simple $N \rightleftharpoons D$ equilibrium. Other stoichiometries require addition of a mass law ratio term, Q_m , for the standard state to be properly represented.

$$\Delta G_D^0 = \Delta H_D^0(T_m) \cdot \{1 - T / T_m\} + \Delta C_p \cdot \{T - T_m - T \cdot \ln(T / T_m)\} - R \cdot T_m \cdot \ln Q_m \quad (2)$$

For a two-state transition $N_2 \rightleftharpoons 2D$ this term is $-R \cdot T_m \cdot \ln(c)$ where c refers to the total monomer concentration of the protein. All parameters necessary for calculation of ΔG_D^0 can be obtained from differential scanning calorimetry (DSC) curves such as that given in Fig. 1a. Figure 1b gives a magnified

view of the linear predenaturational heat capacity increase to provide an idea of the quality of the C_p measurements necessary for a meaningful application of fitting procedures. As a matter of fact Fig. 1a exhibits both the experimental and calculated transition curve which at this scale are indistinguishable. Simulation of the curve is based on the assumption of linear heat capacity functions before ($C_{p,N} = a + b \cdot T$) and after ($C_{p,D} = c + d \cdot (T - T_m)$) the transition and the thermodynamic parameters given in the figures.

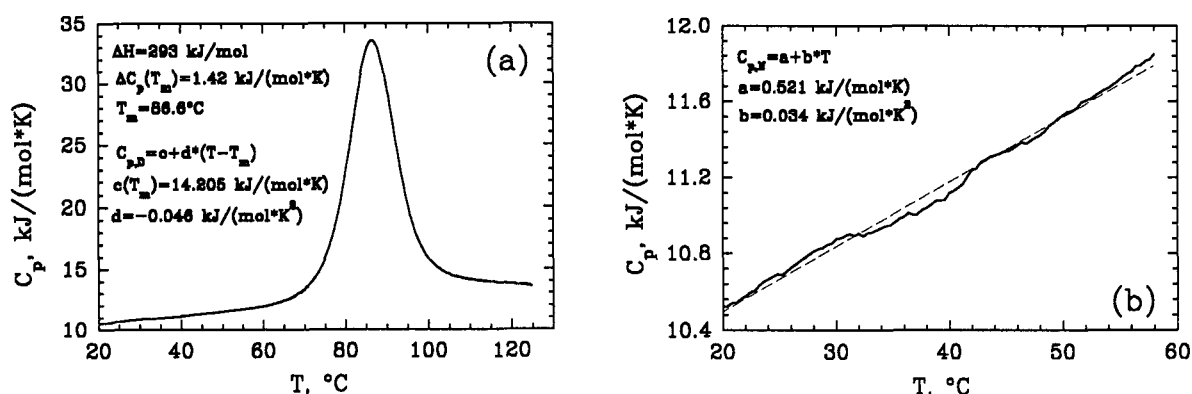


Fig. 1. (a) DSC transition curve of basic pancreatic trypsin inhibitor (BPTI) (10 mM sodium phosphate, 10 mM sodium sulphate, pH 1; [BPTI] = 6.35 mg/ml; heating rate = 1 K/min). (b) enlarged view of predenaturational baseline.

The very close identity of the experimental and calculated transition curve is a valid proof of the correctness of the model. These high quality transition curves enable us to improve our results of the heat capacity change involved in unfolding of native BPTI (ref. 2). Due to instrumental limitations of the DASM 1 calorimeters used previously it was not possible to measure beyond 110°C. Therefore ΔC_p values could not be obtained from single transition curves as can be done now. The presently most accurate value is 1.42 ± 0.5 kJ/(mol·K) for denaturation at pH 1 of native BPTI having three intact disulfide bridges. The ΔC_p value given in ref. 2 had been obtained from the variation with temperature of the transition enthalpy. The value was lower probably due to a lack of accuracy in the determination of the postdenaturational baseline resulting from the instrumental limitations mentioned above. Since the present ΔH_D^0 values at low pH are identical within error limits to those reported previously the ΔG_D^0 value at 25°C is 42.2 kJ/mol. Such ΔG_D^0 values are important properties for a quantitative comparison of stabilities of different proteins.

PHYSICAL NATURE OF A PROTEIN MOLECULE IN SOLUTION: MOLECULAR CRYSTAL OR OIL DROP?

Over the years various models have been proposed that liken the protein interior to an oil drop or a molecular crystal. This is not a question of semantics only, since the answer could be decisive in choosing thermodynamic model reactions for transfer of amino acid side chains. Depending on the physical nature of the protein interior transfer parameters from hydrophobic liquids to water or hydrophobic crystals to water could be more appropriate for a simulation of the various individual contributions to stability. The question cannot be answered unambiguously yet. However, there are some good reasons in favour of a crystalline interior of proteins. Among them are the existence of high resolution crystal structures, the low compressibility, and the high packing density of the amino acids in native proteins. Another interesting aspect that might be instrumental in defining the physical nature of protein is the variation with temperature of the partial specific volume. Figure 2 shows a DSC curve of lysozyme together with the dependence on temperature of the partial specific volume. The \bar{v} vs. T curve has been obtained from high precision differential scanning density (DSD) studies, employing a scan rate of 0.5 K/min in the temperature range 10 - 90°C. The intriguing result which is characteristic for a functionally and structurally unrelated group of proteins is the abrupt change in the expansion coefficient of the protein. Although it is not the case for lysozyme, for practically all other proteins studied so far (about 10) the change in expansion coefficient appears to occur in the early stages of unfolding seen by calorimetry.

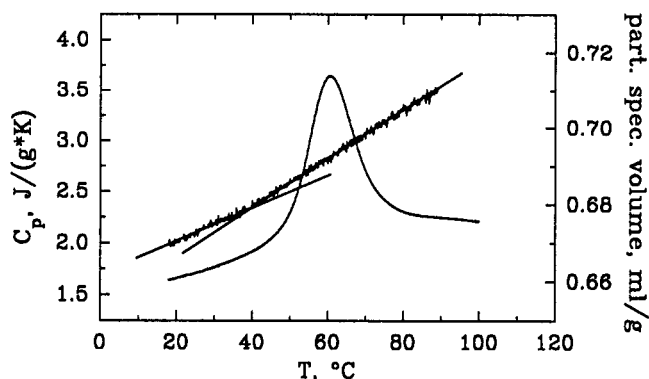


Fig. 2. Temperature dependence of C_p and \bar{v} of cytochrome c ([protein] = 16.1 mg/ml; 100 mM potassium phosphate, pH 3.2; heating rate = 0.5 K/min).

A model in accordance with these DSD results is displayed in Fig. 3. The compact protein undergoes a structural change at a temperature close to the commencement of the calorimetrically observed melting transition that renders it indiscriminate with regard to the expansion coefficient from the hydrated unfolded polypeptide chain. The intermediate, hydrated species of the protein bears some formal resemblance with a molten globule structure. It is, however, different from a molten globule in that it still unfolds cooperatively as the heat capacity curve demonstrates. Though the model is rather speculative at this stage it is consistent with the experimental data. The temperature course of the partial specific volume reveals facets of the protein structure that cannot be assigned uniquely to either a crystalline or a liquid like nature.

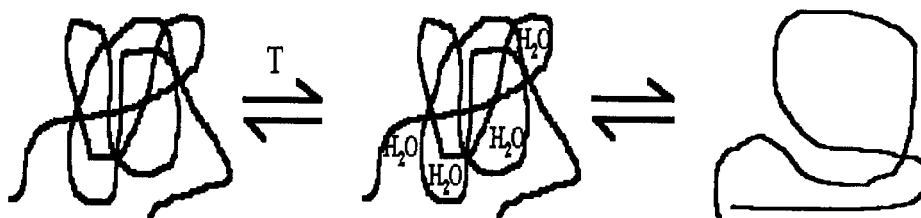


Fig. 3. Schematic view of the unfolding transition implied by the temperature dependence of the partial specific volume.

DUE TO THE COOPERATIVE NATURE OF INTERACTIONS IT IS DIFFICULT TO DISSECT THE GLOBAL THERMODYNAMIC PARAMETERS INTO GROUP CONTRIBUTIONS

Intrinsic contributions to protein stability arise from i) covalent bonds (disulfide bridges), and ii) non-covalent interactions such as hydrogen bonds, hydrophobic effects, van der Waals (packing) forces, hydration differences of polar groups, and - in the case of helix-helix interactions - macrodipole forces. Extrinsic interactions such as ligand or solvent binding that can raise or decrease stability are not considered here. They are superbly covered in a recent article by Timasheff (ref. 3).

DISULFIDE BRIDGES MAY STABILIZE ENTHALPICALLY OR ENTROPICALLY

While crosslinks of inert polymers increase stability entropically by decreasing the entropy gain on unfolding or dissociation, disulfide bonds in proteins can increase stability also by increasing the transition enthalpy. This can be tested by comparing transition parameters of proteins, whose number of disulfide bridges is reduced by chemical or genetic modification with those of the native protein. Figure 4 shows two proteins having two different types of disulfide bridges. Native Tendamistat has two disulfide bonds of the sequential type, while BPTI has cross-linked bonds, that bridge e.g. the ends of the polypeptide chain. Doig and Williams (ref. 4) and Shirley et al. (ref. 5) derived an average stabilization per disulfide group of $\Delta G_D \approx 13$ kJ/(mole of disulfide group). Studies on mutated BPTI and Tendamistat show, however, that this number may not be a universal parameter but is dependent on the individual protein (Table 1). Due to the small number of systematic investigations more definitive numbers cannot be provided yet.

TABLE 1. Stability parameters of native and disulfide bridge deficient proteins. The buffer for BPTI was 10 mM sodium phosphate, 10 mM sodium sulphate, pH 3. The buffer for Tendamistat was 10 mM sodium phosphate, 10 mM sodium sulphate, pH 7.

protein		T _m °C	ΔC _p kJ/(mol·K)	ΔH kJ/mol	ΔG _D ⁰ (25°C) kJ/mol	Δ(ΔG _D ⁰) kJ/mol
BPTI:	WT	94.7	1.2	309	50.1	
	Ala ¹⁴ /Ala ³⁸	67.9	2.5	211	19.5	-30.6
Tendamistat:	WT	81.6	2.9	274	29.9	
	Ala ⁴⁵ /Ala ⁷³	59.0	2.8	213	16.8	-13.1

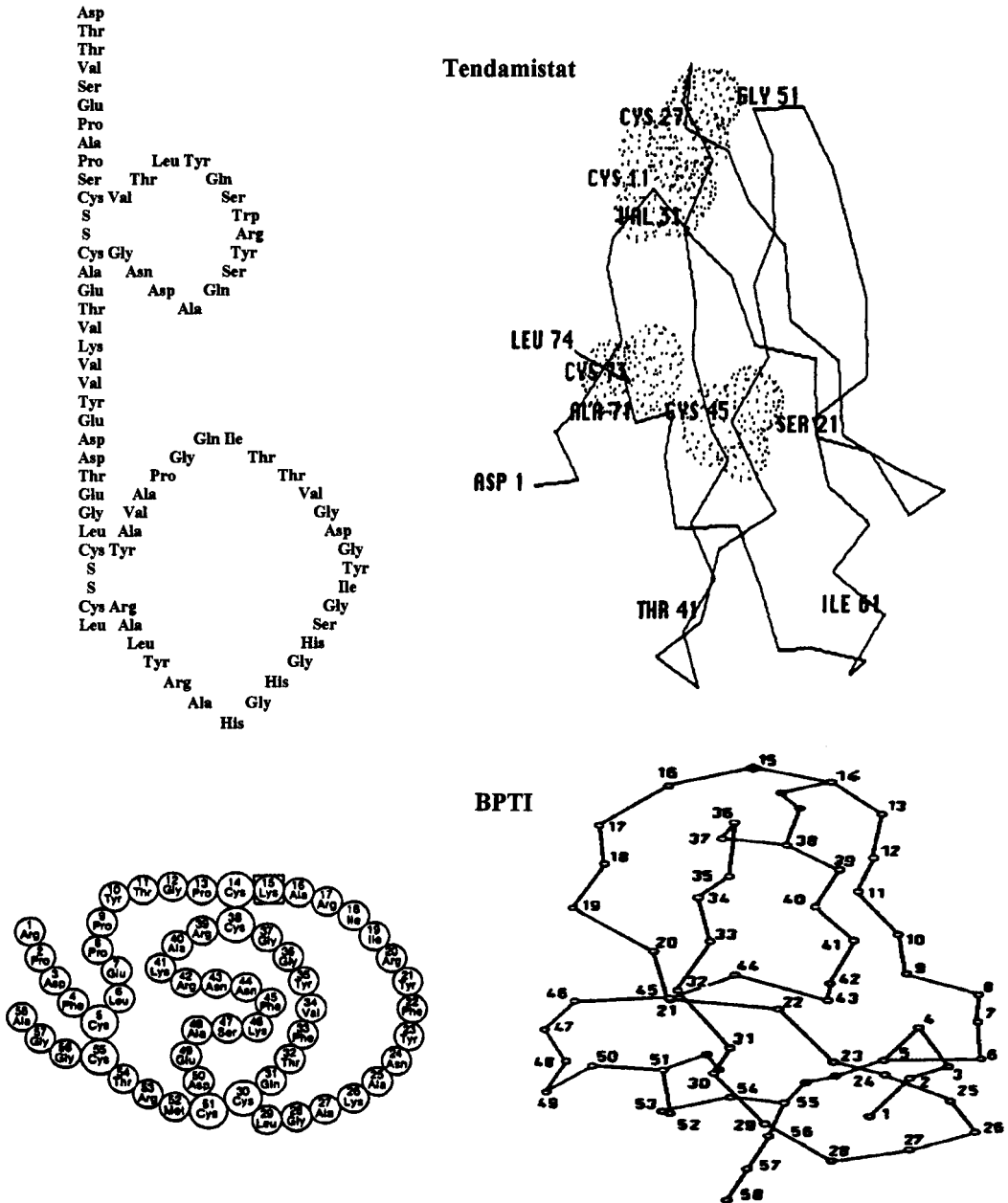


Fig. 4. Primary and tertiary structure of Tendamistat (top) and BPTI (bottom).

HYDROGEN BONDS START CATCHING UP WITH THE HYDROPHOBIC EFFECT AS FUNDAMENTAL SOURCE OF PROTEIN STABILITY

The results of dimerisation studies of small molecules such as urea or lactams (refs. 4 and 6) and unfolding studies of native and mutated ribonuclease T1 (ref. 5) reiterate the significance of hydrogen bonds in stabilizing the native structure of proteins. For decades any significant involvement of hydrogen bonds in stabilizing protein- and nucleic acid structures has been excluded on the basis of the argument that hydrogen bonds to the solvent water are energetically equivalent to intrinsic hydrogen bonds, leaving no favourable ΔG_D^0 contribution to this type of interaction. Recent studies demonstrate a significant role of hydrogen bonds in the maintenance of the native structure of proteins. The 86 hydrogen bonds in ribonuclease T1 are reported to contribute as much as 460 kJ/(mole of enzyme) to the stability of the native state which amounts roughly to the same value as provided by the hydrophobic effect (ref. 5). A new interpretation of the role of water in maintaining protein stability is also going to change our view on the significance of hydrogen bonds (ref. 3, Privalov (1992), personal communication).

INDIRECT PROOF FOR THE IMPORTANCE OF HYDROGEN BONDS CAN BE DELINEATED FROM STABILITY STUDIES ON DRY PROTEINS

Figure 5a shows transition curves of glucose oxidase (GOD) at various water contents measured in (g water) / (g protein). Figure 5b exhibits the dependence of the temperature of maximal excess heat capacity, T_{max} , and the maximal transition width, ΔT_{max} , as a function of water content. The significant result is the tremendous gain in stability with decreasing water content. Internal hydrogen bonds can no longer be replaced by partially equivalent bonds with the solvent water. As a result the protein becomes significantly more stable.

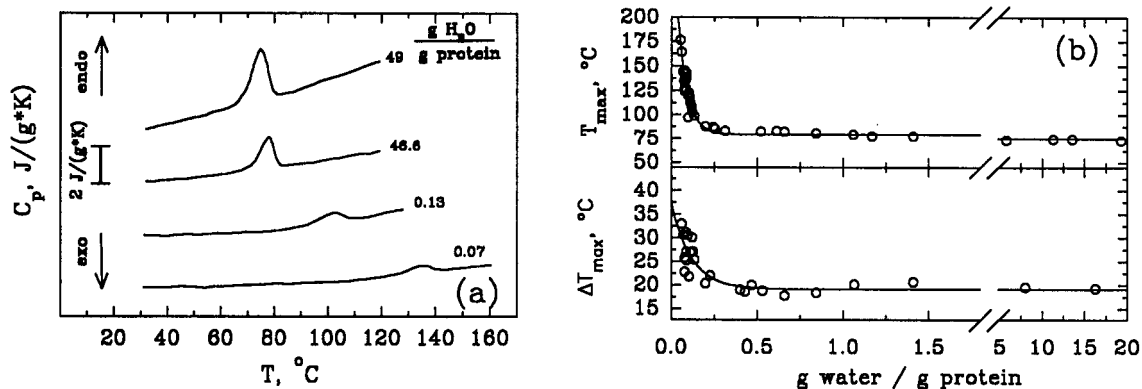


Fig. 5. Influence of hydration on the stability of GOD. (a) transition curves at various water contents. (b) transition temperature, T_{max} , and transition widths, ΔT_{max} , as a function of hydration.

SIGNIFICANT INVOLVEMENT OF HYDROGEN BONDS IN PROTEIN STABILIZATION RENDERS IT EASIER TO UNDERSTAND THE EXISTENCE OF MOLTEN GLOBULES

The characteristics of molten globule structure are: presence of large amounts of secondary structure (revealed by the circular dichroism at 222 nm), the lack of tertiary structure, a high degree of hydration of core residues, occurrence of rapid fluctuations between several conformations (apparent in NMR spectra and amide proton exchange studies) and the absence of cooperative phase transitions (ref. 7). The latter property is illustrated in Fig. 6 for the unfolding of the F2-fragment of *E. coli* tryptophan synthase which exhibits all the above criteria for molten globules (ref. 8). Since hydrophobic or van der Waals interactions between groups of the protein should no longer exist due to hydration and lack of tertiary structure, hydrogen bonds maintaining secondary structure are good candidates for the residual transition enthalpy of 17 kJ/mol associated with the uncooperative unfolding shown in Fig. 6.

THE IMPORTANCE OF VAN DER WAALS (PACKING) INTERACTIONS IS REVEALED BY STUDIES ON CAVITY MUTATIONS

The densely packed core of proteins is particularly sensitive to alterations in short range van der Waals interactions. This can be probed by replacing the large amino acid side chain of leucine by the smaller

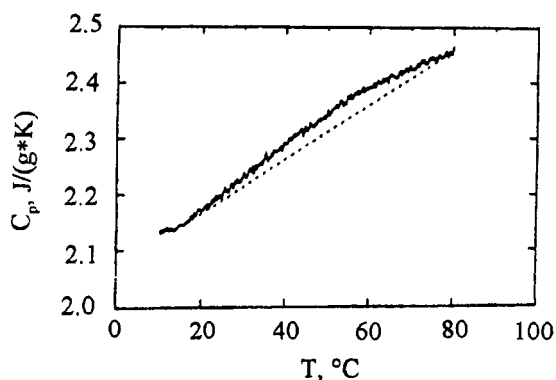


Fig. 6. Transition curve of the molten globule structure of the F₂-fragment of tryptophan synthase (see ref. 8 for solvent conditions).

TABLE 2. Stability parameters for wild type (WT) and mutated ROP protein. The buffer used was 10 mM sodium phosphate, 10 mM sodium sulphate, pH 6.

protein	T _m °C	ΔH kJ/mol	ΔG _D ⁰ (25°C) kJ/mol	Δ(ΔG _D ⁰) kJ/mol
ROP: WT	71.0	580	71.7	
L ⁴⁸ A	43.4	238	36.4	-35.3

groups of valine or alanine. Table 2 shows thermodynamic parameters obtained with native and mutated ROP protein (ref. 9). They illustrate the dramatic decrease in enthalpy and Gibbs energy resulting from such replacements that create a cavity in the packing-sensitive interior of a protein. Stability of native ROP protein is decreased by 50% as a result of the exchange of two residues per dimer. These representative results clearly demonstrate for ROP protein that the cooperative nature of the interactions involved in maintaining protein structure may be an obstacle in arriving at handy additivity schemes of side chain contributions.

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