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Thermodynamics of denaturation of ribonuclease A in aqueous amino acid solutions

Swarita Gopal and J.C. Ahluwalia*

Department of Chemistry, Indian Institute of Technology, Hauz Khas, New Delhi 110 016, India.

Abstract -Differential scanning calorimetry has been used to investigate the effects of amino acids (osmoregulatory solutes) on the thermodynamics of denaturation of ribonuclease A. The transition temperature, heat capacity and the enthalpy of denaturation of RNase A in aqueous amino acids'solutions at pH 6.00 has been determined by the least squares fit of the excess heat capacity data to the two state model. The amino acids increase the thermal stability of RNase A in the order:leucine < valine < proline < α-alanine < serine <γ aminobutyric acid < β -alanine < glycine. The results have been explained on the basis of the cosphere overlap model.

INTRODUCTION

The structural stability of proteins is extensively controlled by the interactions between the protein and surrounding solvent molecules (ref.1). Solvent additives can affect macromolecular structure by direct interaction with the macromolecule or by indirect action through effects on the structure and properties of the solvent or by a combination of both these mechanisms (ref.2). Solute macromolecule interaction is important in cellular function and evolution of marine organisms. Most of the marine organisms are isosmotic or slightly hyperosmotic to sea water and to achieve such a condition, these organisms accumulate a variety of inorganic and organic osmolyte solutes in high concentrations (ref.3). The major osmolytes are polyhydric alcohols (polyols), free amino acids and amino acid derivatives, and urea and methylamines (ref.3). Amino acids and their derivatives are the dominant solutes in halophytes, marine invertebrates, hagfishes and salt-tolerant bacteria (ref.4), which play an important role in the osmotic balance of the intracellular fluids. These osmolytes are found to be widely compatible with protein function, without major inhibiting or activating effects on the enzyme activity and avoid the need for special protein adaptations other than those concerned with regulation of free amino acid concentrations (ref.5).

The thermodynamic studies of the effects of osmolytes on the unfolding process of proteins can give useful information about the involvement of hydrophobic interactions and electrostatic forces in the conformational stability of the macromolecules. The aim of this investigation is to obtain a quantitative estimate of the degree of stabilization of ribonuclease A by amino acids using the scanning calorimetric technique and to correlate the results with the various interactions stabilizing the complex structure of protein. In the present study, various thermodynamic parameters: transition temperature T_d, enthalpy ΔH_d, entropy ΔS_d, and free-energy of denaturation ΔG_d, have been determined in the presence and absence of aqueous amino acid solutions.

The amino acids chosen are glycine, α-alanine, L-valine, L-leucine, to study the effect of increasing hydrophobicity, β-alanine and γ-amino butyric acid to investigate the effect of the position of the amino group in the side chains, L-serine to measure the effect of substitution of H by OH group, in comparison to α-alanine and L-proline to study the effect of a cyclic amino acid on the thermodynamic parameters of denaturation of RNase A.

EXPERIMENTAL

Amino acids : glycine, β- and α-alanine, γ-aminobutyric acid, L-serine, L-proline, L-valine, L-leucine were procured from Sigma Chemical Co. All these compounds were of best available purity grade (99%). These amino acids were dried over P_2O_5 in vacuum dessicator for nearly 72 hours and were used as such, for making solutions. Bovine pancreatic ribonuclease A (type R4875, lot No. 93F-0498), also procured from Sigma Chemical Co., was deionized further by exhaustive dialysis against distilled water at 277 K for over 24 hours and was lyophilized before use. Millimolar solutions of protein were prepared by dissolving dried protein sample in 0.025 M NaCl/NaOH solution buffered at pH 6.00.

Protein concentration was determined spectrophotometrically on a Perkin Elmer Lambda 3B spectrophotometer. For the UV spectroscopic analysis, the protein solutions were diluted to a concentration of about 0.1 mass % with a measured volume of 0.2 M Na_2HPO_4-NaH_2PO_4 buffer at pH 7.00. The optical density was measured at 278 nm for RNase A and the concentration was determined using a value of the optical density of 0.738 for 0.1 mass % RNase A slution of at 278 nm (ref.6). Solutions of the °amino acid and protein' were prepared by dissolving a known mass of amino acid in a sample of the protein solution of RNase A.

DSC measurements were performed with Setaram micro-batch calorimeter. The calorimeter was operated at a scan rate of 0.5 K min^{-1} from 298 to 371 K with sample mass in the range of 0.8 to 0.85 g and protein concentration of 0.24 mM. For each protein concentration, at least three experiments were performed. To analyze a DSC experiment, the instrumental baseline determined with buffer in both cells was subtracted from the results obtained with the sample. All the excess power thermal scans were converted to excess heat capacity vs. temperature scans, following the procedure described by Schwarz and Kirchhoff (ref.8). Thermodynamic functions of protein denaturation, the transition temperature T_d, heat capacity ΔCp_d and the enthalpy ΔH_d of denaturation of RNase A in buffer solution (0.025 M NaCl/NaOH, pH 6.00) and amino acid solutions (0-1 m) were determined by the least squares fit of the excess heat capacity data to the two-state model (ref.7).

The two state model provides a functional dependence of the measured heat capacity on temperature of the following form

$$Cp_s = a + b\ (T - T_d) + \alpha[\Delta a + \Delta b\ (T-T_d)]$$

where $a + b\ (T - T_d)$ is the linear fit of the pretransition baseline to T, and $\Delta a + \Delta b(T-T_d)$ is the difference between linear extrapolation of the pre- and post- transitional baselines at T. Cp_s is the sigmoidal baseline extrapolated under the transition curve and α is the fractional area under the transition curve at T. The transition enthalpy ΔH_d is determined from the area under the transition curve and from the number of moles of RNase A in the cell.

RESULTS

The thermal scans of RNase A in aqueous amino acid solutions (1.0 m) exhibit single symmetrical peaks for the denaturation transition. The values of T_d, ΔH_d and ΔCp_d, for RNase A in aqueous amino acid solutions are presented in Table 1. The T_d values are also illustrated in Fig. 1.Each value represents an average of three to four measurements and the uncertainty represents the standard error of the mean. The T_d values have experimental error of ± 0.1 K and the ΔH_d values have maximum expected error of of ± 5% including errors in sample preparation, calibration constant and reproducibility The cooperativity η , which is the ratio of transition enthalpy to van't Hoff enthalpy for each measurement was determined by the approximate relation $\eta = \Delta H_d^2/4RT_d^2C_d$, where C_d is the height of the transition peak per mole of protein at T_d. The value of η was found to be 0.987 ± 0.02.

TABLE 1. Thermodynamic parameters obtained from DSC scans on RNase A in the presence of varying concentrations of amino acids at pH 6.00

Conc./m	T_d/K	ΔT_d/K	ΔH_d/kJ mol^{-1}	ΔCp_d/kJ K^{-1} mol^{-1}
Glycine				
0	335.5	-	444	4.8±0.5
0.151	336.3	0.8	450	5.1±0.3
0.505	337.7	2.2	469	4.8±0.9
1.002	339.6	4.1	484	4.7±0.7
β-Alanine				
0.153	336.2	0.7	448	4.7±0.7
0.503	337.5	2.0	468	4.8±0.6
1.082	339.3	3.8	483	4.7±0.9
γ-Aminobutyric acid				
0.147	336.1	0.6	452	4.3±0.7
0.502	337.4	1.9	467	4.9±0.5
1.004	339.0	3.5	482	5.5±0.7
α-Alanine				
0.152	335.9	0.4	450	5.3±0.2
0.504	336.9	1.4	462	4.7±0.6
1.009	338.6	3.1	487	4.9±0.3
L-Valine				
0.152	335.6	0.1	455	5.9±0.3
0.504	335.7	0.2	460	5.1±0.9
L-Leucine				
0.150	335.2	-0.3	444	4.8±0.3
L-Serine				
1.002	338.7	3.2	482	4.7±1.2
L-Proline				
1.003	336.4	0.9	449	5.0±0.4

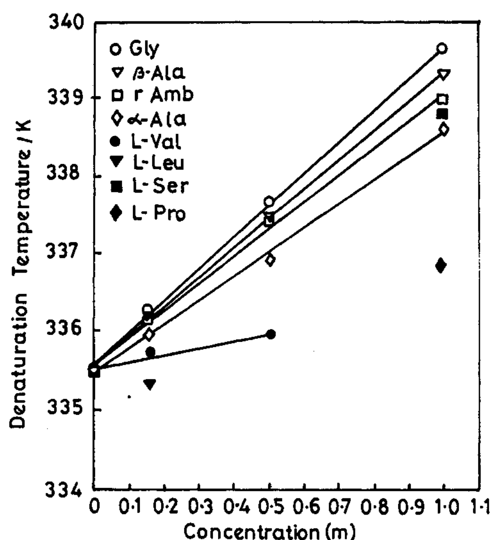

Fig.1 The effect of amino acids on the thermal denaturation of RNase A

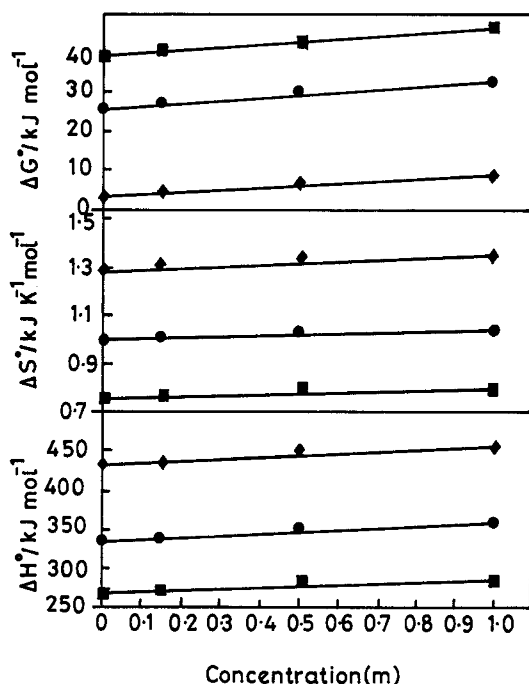

Fig.2 Variation of standard thermodynamic parameters of denaturation of RNase A at (■)298,(●) 313 and (♦) 333 K with glycine concentration

The difference in thermodynamic functions of the protein in the native and denatured states, $\Delta G°$, $\Delta S°$ and $\Delta H°$ are calculated at different temperatures for the transition of RNase A in amino acid solutions at pH 6.00 using equations 1-3. The values of these parameters in aqueous glycine solutions as a function of concentration of glycine and temperature are illustrated in Fig. 2.

$$\Delta H°(T) = \Delta H_d - (T_d - T)\ \Delta Cp_d \tag{1}$$

$$\Delta S°(T) = \Delta H_d/T_d - \Delta Cp_d \ln T_d/T \tag{2}$$

$$\Delta G°(T) = (T_d - T/T_d)\ \Delta H_d - (T_d - T)\ \Delta Cp_d + T\ \Delta Cp_d \ln T_d/T \tag{3}$$

The ΔCp_d values of RNase A in amino acid solutions are assumed to be identical to those in aqueous solution at pH 6.00 i.e.4.8 kJ K^{-1} mol^{-1}. This assumption will not cause any significant error in standard thermodynamic parameters of denaturation when T is close to T_d, (ref.8) since ΔCp_d does not seem to be largely affected by addition of amino acids.

DISCUSSION

The cooperativities of RNase A in buffer and amino acid solutions are within the experimental error of 1.0 (Table 1), which indicate that thermal denaturation in these solutions is a two-state transition with a stoichiometry of one. The reversibility for the denaturation of RNase A in amino acid solution was checked by reheating the protein solution after rapidly cooling from the first scan. No significant variation in the position of transition peak and the peak area was observed on reheating, indicating that the denaturation of RNase A is reversible in amino acid solutions. It is thus permissible to apply equilibrium thermodynamics for the evaluation of thermodynamic parameters as functions of temperature and amino acid concentration. The T_d and ΔH_d values for RNase A in aqueous buffer solution at pH 6.00, are in agreement with those reported (ref.9).

On comparing the thermodynamic parameters obtained for RNase A in the presence and absence of amino acids it is observed that the amino acids (except L-leucine) increase the thermal unfolding temperature T_d. Transition temperature, T_d, of RNase A increases in the order : L-leucine < L-valine < L-proline < α-alanine < L-serine < γ-aminobutyric acid < β-alanine < glycine i.e. increasing hydrophobic character of the amino acid decreases the thermal stability of the protein. Glycine, β- and α- alanine, γ-amino butyric acid appear to act as stabilizers, while L-valine seems to have little effect on the stability, L-leucine on the other hand decreases the thermal stability of the protein. L-proline has small stabilising effect. L-serine is better stabilizer than α-alanine. The free energy of stabilization(calculated using equation 3) increases in the presence of amino acids e.g. it is 5.8 kJ mol^{-1} in the presence of 1.0 m glycine.

Enhancement of the denaturation temperature of proteins by amino acids has been reported earlier for BSA (ref.10), Lysozyme (ref.11) and RNase A (ref.4,10) from spectroscopic studies of thermal denaturation. These workers have observed trends similar to the ones described here. Increase in the transition temperature T_d with increasing amino acid concentration and lowering of T_d with increasing alkyl chain length of the amino acid have been interpreted by Timasheff and coworkers (ref.2,10,11) in terms of the preferential exclusion of the solvent (amino acid + water) from the protein-surface/domain. It seems possible to explain the thermodynamic functions T_d and ΔH_d observed for the denaturation of RNase A in amino acid solutions, in terms of the overlap of hydration co-spheres of the amino acids and the exposed nonpolar groups (upon denaturation). The region occupied by the solvent that is markedly affected by the presence of solute molecules is termed as cosphere. According to cosphere overlap model (ref.12,13), properties of water molecules in the hydration cosphere depend on the nature of the solute species. When two solute particles come close enough together so that their cospheres overlap, some of the cosphere material is displaced, and this is accompanied by change in the thermodynamic parameters (ref.14).

In the case of glycine and β-alanine, the charged head groups of COO^- and NH_3^+ dominate the interactions. The hydration spheres of glycine and β-alanine are mainly composed of charge-dipole forces. Therefore, an overlap of cospheres of glycine or β-alanine with that of exposed nonpolar residues (of the unfolded protein) would squeeze out some water mainly from the hydrophobic hydration spheres of these nonpolar groups. This would result in a net decrease in the hydrogen-bonded structure of water. But this effect is overcome by the dominating hydrogen bonding or dipolar interactions between the zwitterions and the surface of the native protein. In the higher homologues such as L-valine and L-leucine the hydrophobic effect of the alkyl side chain dominates over the electrostatic effects of the zwitterionic head groups.The overlap of cosphere of nonpolar groups of protein (exposed upon unfolding) with those of amino acids, releases some water molecules from the cosphere of the side chains resulting enhanced hydrophobic interactions between the solvent (amino acid + water) and the nonpolar groups of the unfolded protein, and thus relatively favouring denatured state.

The higher T_d of RNase A in β-alanine solution as compared to that in α-alanine solution indicates that β-alanine has greater stabilizing effect than α-alanine.It has been observed (ref. 15,16) that due to greater charge separation of NH_3^+ and COO^- in β-alanine as compared to α-alanine, β-alanine electrostricts water to a greater extent and thus has less hydrophobic hydration effect than α-alanine which explains the greater stabilizing effect of β-alanine. In case of α-alanine and L-serine, the magnitude of stability T_d, is more in case of L-serine. It shows that replacing H in α-alanine by -OH group leads to the more favourable native state. L-Proline has much less effect on the increase in T_d of ribonuclease A as compared to α-alanine at 1.0 m.

The surface of a protein is regarded as a mosaic of regions of different degree of polarity and hydrophobicity. RNase A is one of the most "polar" globular protein and should interact favourably with amino acids essentially in direct proportion to amino acid concentration, as is evident from the observation that the temperature of denaturation of RNase A increases with the increase in the concentration of the amino acids (Table 1). This increase becomes less pronounced with increasing alkyl length of amino acids.

ΔH_d and ΔS_d (= $\Delta H_d/T_d$) of RNase A increases with increasing amino acid concentrations. No particular clear trend is observed for the ΔH_d values with change in alkyl length of amino acids. The effect on ΔH_d of RNase A may be too small to be detected within experimental errors.

Thermodynamic functions $\Delta G°$, $\Delta H°$ and $\Delta S°$ (Fig. 2) calculated at different temperatures 298.15, 313.15 and 333.15 K for the thermal unfolding of RNase A increase with increasing amino acid concentration. $\Delta H°$ and $\Delta S°$ are more positive than those for the native protein indicating that the enthalpy and entropy of transfer of the denatured protein from water to amino acid are larger than those of the native protein.

CONCLUSIONS

In conclusion, our experimental results have shown that thermal denaturation of RNase A in amino acid solutions is a reversible, two state transition between thermodynamically defined states. The amino acids except L-leucine impart thermal stability to the protein structure. Increasing hydrophobic character of the amino acid added, decreases the thermal stability of the protein. The results on the thermodynamics of denaturation of RNase A in amino acid solutions can be explained on the basis of the cosphere overlap model.

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