A CALORIMETRIC AND INFRARED SPECTROSCOPIC STUDY OF THE STABILIZING SOLUTE PROLINE

ALAN S. RUDOLPH AND JOHN H. CROWE
Department of Zoology, University of California Davis, California 95616

ABSTRACT We have studied the calorimetric and infrared spectroscopic properties of the amino acid proline which has been implicated in the stabilization of biomacromolecules during reduced water states. It has been suggested that the ability of this molecule to protect biomacromolecules during these stress states may be related to the formation of polymeric aggregates of proline monomers in solution. The structure of this aggregate is thought to be an alternates stack, forming a hydrophilic colloid-like polymer which is thought to interact with hydrophobic moieties of biomacromolecules, reducing the exposed hydrophobic area during reduced water conditions. Calorimetric data presented in this work show that in increasing concentration of proline in solution the enthalpy associated with the melting of bulk water is greatly reduced, indicating strong hydrogen bonding character of proline in aqueous solution. Proline shows two eutectic phase separations at moderate concentrations and one of these eutectics may be the proposed intermolecular state. A partial phase diagram for proline is presented. Fourier-transform infrared spectroscopic data indicate that the COO^- asymmetric stretch of proline shows marked splitting with increasing proline concentration. This suggests that the carboxylate is in different environments, with the high energy vibrations representing COO^- groups which are participating in the hydrogen bonding pattern associated with the formation of the intermolecular stack. Changes in the CH_2 asymmetric and symmetric stretches of the pyrrolidine rings of proline are consistent with the proposed stack structure. We also suggest a possible mechanism by which these intermolecular associations may be important in the protection of biomacromolecules during reduced water states.

INTRODUCTION

The amino acid proline, along with other di- and trisubstituted amines (betaine, prolylbetaine) have been shown to accumulate in a variety of plants and animals during dehydration and low temperature stress (3, 19, 22, 24, 28, 32, 34). In addition to providing osmotic relief from the loss of water from cells and tissues, the accumulation of these compounds is thought to stabilize biomacromolecules (by a yet unknown mechanism) during the removal of bulk water (25–30). Our recent focus has been determining the mechanism by which these compounds stabilize biomacromolecules, particularly phospholipid bilayers during the removal of bulk water by dehydration or freezing (6, 7, 25, 26). We have previously studied the effect of three stabilizing agents, proline, betaine, and the disaccharide trehalose, on membrane phospholipids and all three agents have been shown to effect phospholipid dynamics (6, 25, 26). Proline and trehalose inhibit freezing induced fusion in small unilamellar vesicles of phospholipid, and preserve the structure and function of frozen biological membranes (sarcoplasmic reticulum) (25). These agents maintain unilamellar vesicles of phospholipid or biological membrane vesicles in solution during reduced water states, and prevent multilamellar vesicle formation or vesicle size growth as a result of freezing induced fusion (25). These compounds also depress the liquid-crystalline to gel phase transition in a variety of phospholipid small unilamellar vesicles and decrease the packing density of these same lipids in phospholipid monolayers by increasing their molecular spacing (26). The result of these effects may be the alteration of the gel phase of phospholipids, preventing deleterious phase separations and the aggregation of membrane proteins during dehydration and freezing (6, 7, 25, 26).

In addition, proline has been shown to stabilize proteins in solution, rendering sparingly soluble proteins more soluble in solutions of reduced water states (27–30). Protein denaturation is also reduced in the presence of moderate concentration of proline (29). The proposed mechanism of such action has been the alteration of the entropic component of the driving force for biomacromolecule destabilization during reduced-water states. Conditions under which particular biomacromolecules are destabilized (such as protein denaturation or membrane cracking) may be entropically driven which results in a net negative free energy for the destabilizing event. This driving force occurs as the result of the exposure of hydrophobic regions

Please address all correspondence to Dr. Rudolph. His present address is the Bio/Molecular Engineering Branch, Code 6190, Naval Research Laboratory, Washington, D.C. 20375-5000.

on proteins or phospholipids during reduced hydration states. These groups, when exposed, require structured water and statistically more water than the exposure of hydrophilic moieties. Agents such as proline alter the entropic contribution of this process, reducing the negative free energy of destabilization (27). This is presumably due to the alteration of water structure associated with exposed hydrophobic regions of biomacromolecules during destabilizing stress events such as dehydration or low temperature stress. This alteration may arise as a result of proline-water interactions and the interaction of proline with exposed hydrophobic regions of biomacromolecules during destabilization.

Proline is quite soluble at room temperature (we have measured 7.5 M at 25°C, which is 86% proline by weight) and is the most soluble of all the amino acids (12). It is thought to participate in intermolecular alternate stacking at moderate concentrations in solution (29). These proposed stacks are thought to be arranged so that the association forms a polymer-like hydrophilic colloid; with the hydrophobic moiety of the pyrrolidine rings stacked and the carboxylic group that is extended from the ring hydrogen bonded to the disubstituted amine of the next adjacent monomer via a water bridge (see Fig. 1). This stacking might result in the removal of clathrate water associated with the hydrophobic region of the pyrrolidine ring. The charge distribution is such that the disubstituted amine group in the pyrrolidine ring is alternated from one monomer to the next monomer in the stack. The amine is also proposed to be involved in the hydrogen bonding pattern, as it is thought to be hydrogen bonded to the carboxylic group of the adjacent monomer via associated water (29). Near IR, NMR, and viscosity studies indicate that proline strongly hydrogen bonds water in solution (29, 30).

The involvement of such agents as proline in the alteration of water structure has led us to study the solution properties of proline in bulk aqueous solution as they could have important bearing on the ability of proline to protect biomacromolecules from dehydration and freezing damage. In the present work we examine the bulk solution properties of proline using Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry. FTIR is a useful method for studying intermolecular associations in aqueous solution since functional groups that may participate in such interactions have been assigned for many molecules of biological interest (31). Particular functional groups that may be good indicators of intermolecular hydrogen bonding, considering the proposed intermolecular association of proline in bulk solution are the ionized carboxylic asymmetric vibration at 1,620 cm^{-1}, and the N—H stretching vibration at 3,350 cm^{-1} (5, 16, 23, 31). In addition, stacking of the pyrrolidine rings might be manifest in changes in C—H stretch region of proline.

Differential scanning calorimetry provides a method of probing the effect of proline on bulk water characteristics. Amphipathic solutes such as proline often shown unusual solution thermodynamics because they have characteristics that are considered both structure making and structure breaking with regard to solvent water (8, 9, 11, 12). The intermolecular associations of proline are proposed to involve associated water and the binding of this water in the suggested alternate stacks could be manifest in unusual changes in the thermal properties of bulk water. Since construction of phase diagrams is often helpful in determining the solution properties and phase characteristics of mixed solutes (9, 20), we have constructed a partial phase diagram for proline to examine these properties.

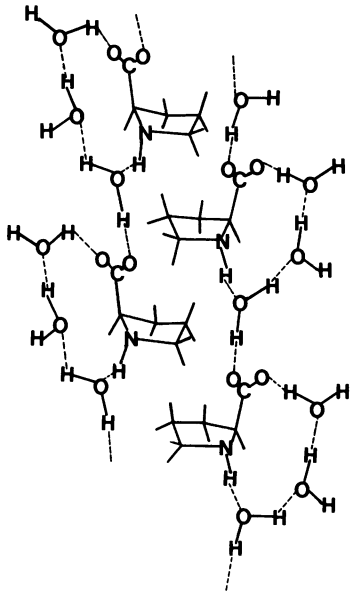

FIGURE 1 Diagrammatic scheme of the proposed alternate stack of proline monomers during reduced water states (29). The hydrophilic colloid formed may involve hydrogen bonding between the carboxylic groups and the disubstituted amine on the pyrrolidine ring via associated water.

Based on the evidence that proline alters phospholipid dynamics and the existing data on the solution properties of proline, we have put forth two mechanisms by which proline may stabilize membranes during dehydration or freezing stress (26). One of these mechanisms involves the coordination of proline monomers, or alternatively small stacks of proline monomers to polar residues of phospholipids. The mechanism of this interaction could be hydrogen bonding, although the amphipathic nature of proline does not preclude the possibility of a hydrophobic interaction by intercalation between head groups and slight insertion between hydrocarbon chains. This action would result in the stabilization of membrane components during dehydration and low temperature stress with proline acting as a molecular "spacer," preventing deleterious lateral phase separation of membrane components. Recent IR studies with the protectant trehalose indicate that it may hydrogen bond to the phosphate region of the polar head group. This

action may be responsible for its ability to preserve membranes in a dry state (6–7). The strong hydrogen bonding character of proline might suggest this as a potential mechanism for its action. An alternative hypothesis we have put forth for the action of proline in stabilizing phospholipids is the alteration of the long range order of water at the head group site (26). This may involve both the hydration shell of the polar head group and the hydrophobic regions of the bilayer. The present work is directed toward understanding the effect of proline on water structure and suggests what role this effect may have in the stabilization of biomacromolecules during reduced water states.

MATERIALS AND METHODS

L-proline, trans-4-hydroxy-L-proline, and glycine (free base) were obtained from Sigma Chemical Co. (St. Louis, MO). Water used to make solutions for IR and DSC experiments was passed through a Barnstead deionizing column and a Barnstead organics column for purification and provided resistivity of at least 15 M ohms-cm. This water was then passed through a millipore (Bedford, MA) filtration system (0.2 μm nylon filter) to remove any residual particles and used immediately in the preparation of the desired solutions. D_2O was also obtained from Sigma. Before each experiment, the purity of both the water and D_2O were examined by taking an IR spectra.

Calorimetry scans were recorded on a Perkin-Elmer DSC-2C differential scanning calorimeter, assisted by a Perkin-Elmer 3600 data station. The scanning rate for all of the heating scans presented was 2.5 K/min. Samples were cooled at 5°C/min before the heating scans were recorded. The initial temperature of all of the heating scans was 200°C. Twenty microliter samples were loaded in weighed small volume aluminum pans. Following the scans, the pans were punctured and placed in a 105°C oven overnight. The pans were then reweighed and the sample weight determined. Digitized data of the scans were then normalized and plotted.

Infrared spectra were taken on a Perkin-Elmer Fourier Transform Infrared Spectrometer (Model 1550 and 1700) and data were acquired with a Perkin-Elmer 7500 data station. Each spectrum is a result of 16 co-added spectra, and each of three samples was scanned three times. BaF windows with a Teflon 0.015 mm spacer were used in a sealed demountable cell. The data were collected over the IR region 4,000 cm^{-1} to 800 cm^{-1}, with trapezoidal apodization. The particular region of interest was then copied out from this large region, converted to absorbance and put through an abscissa expansion software routine to bring out the significant peaks. This routine expands the spectra so that relative heights of the bands are maintained while the most intense band is expanded to a predetermined absorbance. Any other data manipulations such as spectral enhancement or smoothing were avoided unless otherwise indicated in the results.

RESULTS

Calorimetry of Proline Solutions

Fig. 2 shows heating scans from calorimetry experiments on varying concentration of proline in solution. Three major endotherms are observed with the heating of these solutions. The two endotherms at 246 K and 252 K are associated with temperature invariant eutectic melt of proline. The enthaplies associated with these eutectic melts increase with increasing proline concentration, but the transition temperature of these events do not vary more than 1 K. The enthalpy of the eutectic at 252 K increases

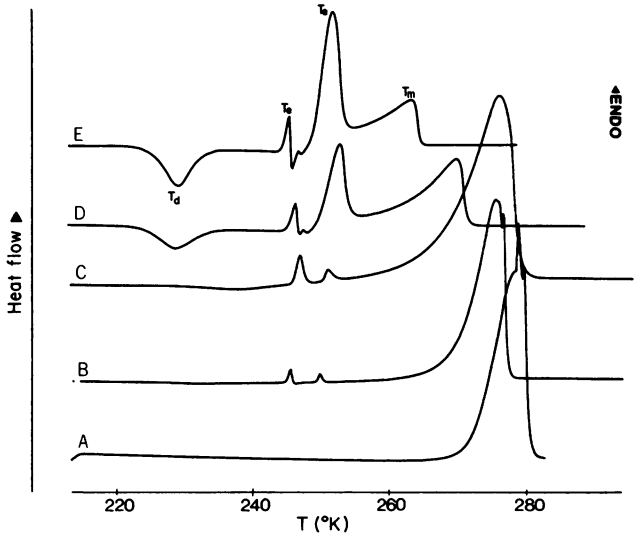

FIGURE 2 Normalized heating scans of increasing concentration of proline in solution. T_d, devitrification temperature; T_e, eutectic melts of proline; T_m, melting of bulk water. (*A*) 0.1 M, (*B*) 0.5 M, (*C*) 1 M, (*D*) 2 M, (*E*) 3 M proline. All scans were run at 2.5°C/min after cooling at 5°C/min. Enthalpies and temperatures of transitions are found in text.

significantly more than the enthalpy of the eutectic at 246 K with increasing proline concentration. The enthalpy of the eutectic at 246 K increases only slightly from 7.15 cal/g in 0.1 M proline, to 8.29 cal/g in 5 M, while the enthalpy associated with the eutectic melt at 252 K increases from 7.02 cal/g to 58.84 cal/g in these same concentrations. In addition, as the concentration of proline increases, the endotherm associated with the melting of bulk water is reduced and the melting temperature decreased, until at the highest concentration it is almost completely absent (Fig. 3). The onset temperature of this transition is also reduced and the range of transition

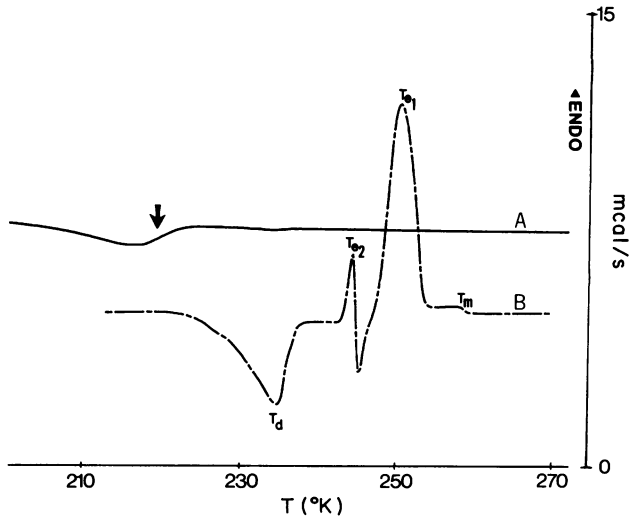

FIGURE 3 Cooling (*A*) and heating (*B*) scan of 5 M proline. Note glass transition in cooling scan (*arrow*), and the almost complete absence of the endotherm associated with the melting of bulk water (T_m, in *B*).

increased indicating that this transition is broadened. The enthalpy of the melting of water in 0.1 M proline is 78.81 cal/g, compared to 17.58 cal/g in 3 M proline and the onset temperature is reduced from 269.75 K to 256.80 K in these concentrations. Cooling scans of the proline solutions in Fig. 2 indicate that there is significant supercooling of water, until at the highest concentrations examined, the exotherm associated with the freezing of bulk water (T_m, Fig.3) is eliminated and a glass transition is seen at 220 K. Devitrification (T_d) of water (between 225 and 235 K) is seen in the heating of proline solutions above 0.5 M. The amount of water that devitrifies does not appear to vary greatly in the concentrations of proline. Fig. 4 shows a partial phase diagram constructed form these plots (Fig. 2 and 3).

As controls we have studied the solution properties of other amino acids with similar molecular weights and structure to proline (hydroxyproline and glycine). These two amino acids do not stabilize biomacromolecules during dehydration or low temperature stress (14, 15, 33). The calorimetric properties of these two amino acids in solution are examined in Fig. 5. Neither glycine or hydroxyproline show the same effect as proline on the melting endotherm of water. The enthalpy of the melting transition of water is 83.63 cal/g in 1 M glycine, 82.34 cal/g in 1 M hydroxyproline, and 59.2 cal/g in 1 M proline. Furthermore, neither glycine or hydroxyproline show eutectic phase separation at low temperatures as observed in solutions of proline.

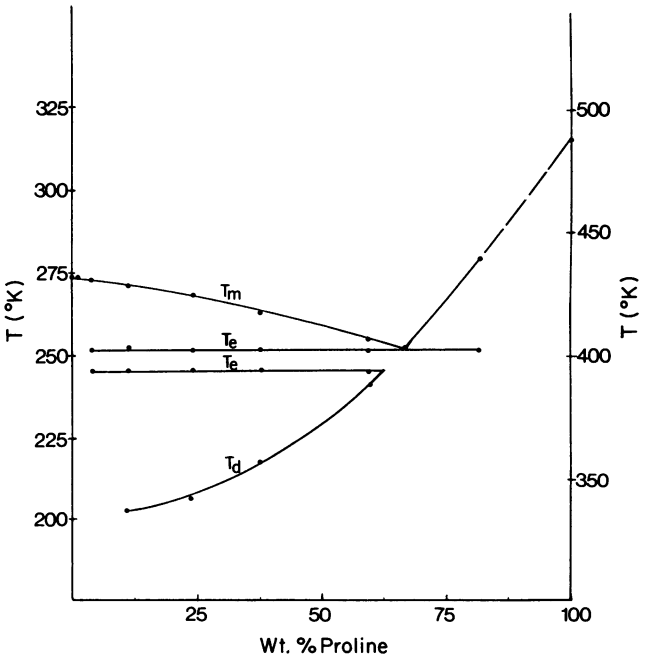

FIGURE 4 A partial diagram of proline. This diagram is constructed from the data presented in Figs. 2 and 3. The right-hand scale is for the 100% proline data point.

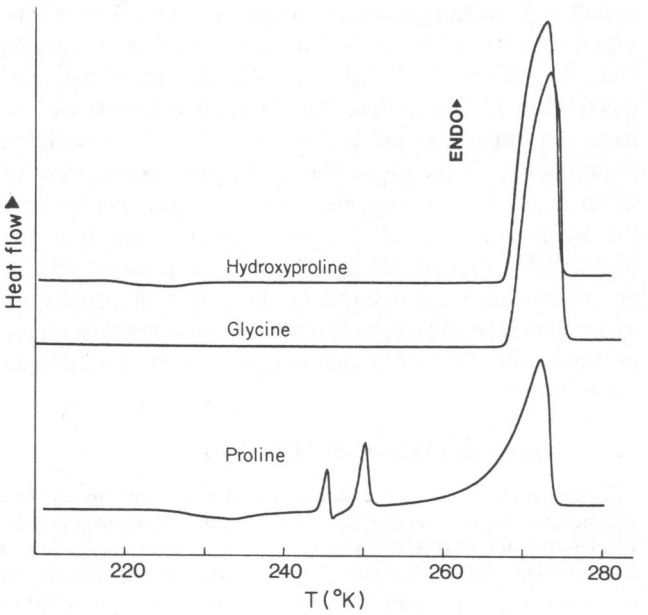

FIGURE 5 Heating scans of 1 M hydroxyproline, glycine, and proline. Note proline gently reduces the enthalpy associated with the melting of water. The onset of this transition is also reduced, resulting in a broadened transition. Glycine and hydroxyproline do not show the eutectic phase separation that proline exhibits.

Infrared Spectroscopy of Proline Solutions

The IR spectra of the COO^- asymmetric stretch of varying concentrations of proline in D_2O is shown in Fig. 6. In 0.1 M proline, there is a single band at 1,617 cm^{-1}. This band broadens as the concentration is increased toward 0.5 M, until at 0.5 M significant band splitting is observed, with bands at 1,623, 1,609, and 1,604 cm^{-1} in 0.5 M proline, and 1,634, 1,626, 1,612, 1,604, 1,596 cm^{-1} in 1 M solution (and 2 M, data not shown). Fig. 7 shows the COO^- asymmetric stretch of glycine in D_2O. In 0.1 M glycine there is a single peak at 1,618 cm^{-1}. At 0.5 M two new peaks arise at 1,622 and 1,614 cm^{-1}. In 1 M glycine, three peaks are observed at 1,625, 1,627, and 1,608 cm^{-1}.

Fig. 8 shows the CH_2 region of proline in increasing concentration of proline in solution. The vibrational frequencies for C—H groups in the pyrrolidine ring are higher than in straight chain alkanes, as vibrational motions of these groups are often restricted in small cyclic structures due to angle distortions (5, 31). The C—H stretch intensity seen at 2,928 cm^{-1} is decreased with increasing concentration. The C—H stretch frequency at 2,958 cm^{5Z1} increases with increasing proline concentration at 1.25 M proline the frequency is shifted by 5 cm^{-1}s higher than the frequency observed at 0.25 M. This region can also be seen in Fig. 9, which shows band broadening of the disubstituted amine of the pyrrolidine ring as proline concentration is increased. The pH of proline solutions in this concentration range is 6.4–6.6. This band broadening of the disubstituted N-H group is indicative of intermo-

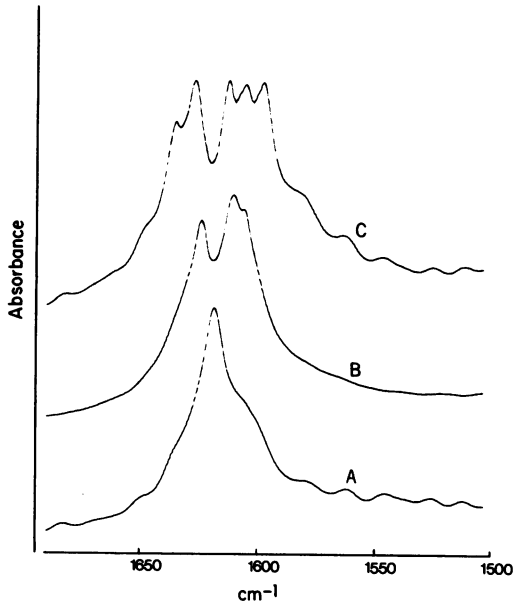

FIGURE 6 COO^- asymmetric stretch of increasing concentrations of proline in D_2O. (*A*) 0.1 M (peak at 1,617 cm^{-1}, (*B*) 0.5 M (peaks at 1,623, 1,609, 1,604 cm^{-1}), (*C*) 1 M (peaks at 1,634, 1,626, 1,612, 1,604, 1,596 cm^{-1}). Note increased splitting with increase in concentration. This may indicate that this group is involved in intermolecular associations.

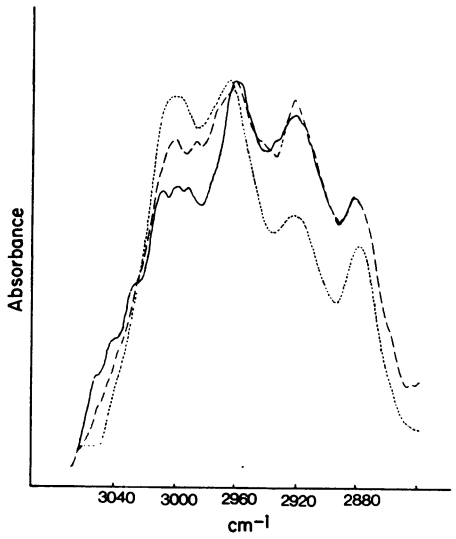

FIGURE 8 C-H stretch region of increasing concentrations of proline in D_2O. Solid line = 0.25 M, broken line = 0.6 M, dotted line = 1.25 M. Note frequency increase in band at 2,958 cm^{-1} and the reduction in intensity of the bands at 2,926 and 2,880 cm^{-1} with increasing concentration. These changes may reflect the stacking of pyrrolidine rings of proline and the establishment of a hydrophobic core of the polymer as the rings stack. Other frequency assignments can be found in the text.

lecular hydrogen bonding (5, 31). These results will be discussed in relation to the proposed solution properties of proline.

DISCUSSION

The calorimetric data indicate that the unusual solution properties of proline may be due to unique intermolecular associations at moderate concentrations of proline in solution. This association may involve the alternate stack of proline monomers and data presented in this paper support this hypothesis. The alternate stack of proline may play an important role in the mechanism by which proline protects

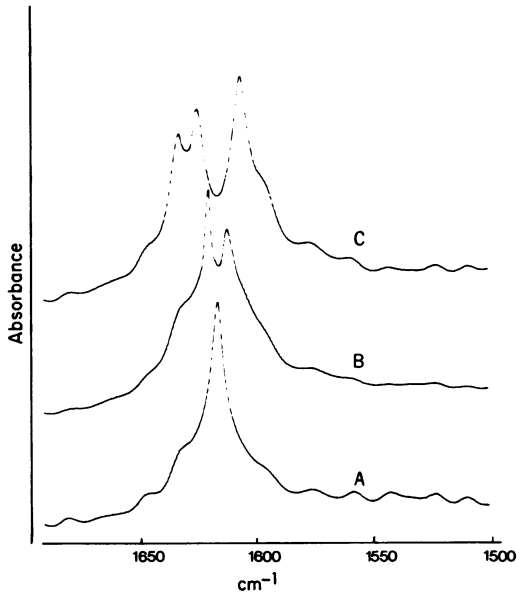

FIGURE 7 COO^- asymmetric stretch of increasing concentrations of glycine in D_2O. (*A*) 0.1 M (peak at 1,618 cm^{-1}, (*B*) 0.5 M (peaks at 1,622 and 1,614 cm^{-1}), (*C*) 1 M (peaks at 1,635, 1,628, 1,608 cm^{-1}).

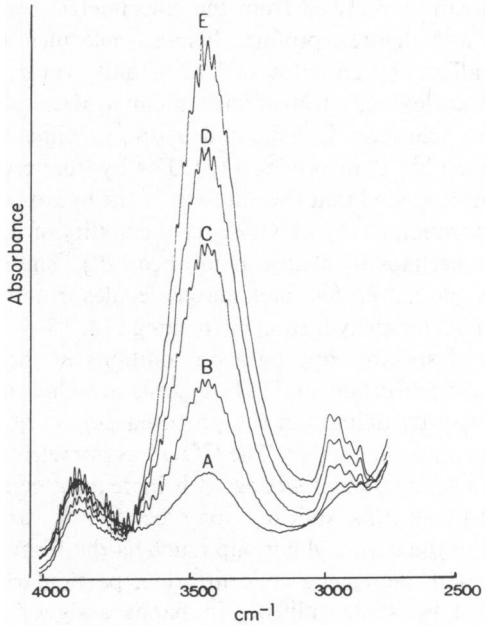

FIGURE 9 N-H stretch region of increasing concentration of proline in D_2O at pH 6.4. (*A*) 0.1 M, (*B*) 0.25 M, (*C*) 0.5 M, (*D*) 1 M, (*E*) 2 M. The broadening and increase in intensity of this band may indicate that the N-H group is involved in intermolecular hydrogen bonding.

biomacromolecules during reduced water states. The calorimetry data suggest that proline strongly hydrogen bonds water as the phase properties of water are altered with increasing concentration of proline. This is evident in the reduction in the enthalpy associated with the melting of bulk water in moderate concentrations of proline (1 M and greater), and the marked decrease in the onset temperature of this transition at these concentrations. In addition, the eutectic phase separation shown at relatively low concentrations of proline indicate that intermolecular associations are likely to occur. While these data do not specifically confirm the existence of an alternate stack of proline monomers, they are consistent with this hypothesis as they indicate the formation of a polymeric aggregate. It is important to point out that formation of these stacks involves the inclusion of water in a hydrogen bonding pattern, and this might be responsible for the alteration of bulk water characteristics that is observed at moderate proline concentrations. With increasing proline concentration, the eutectic at 252 K increases significantly more than the eutectic at 246 K and it is possible that this eutectic melt is associated with the melting of the proposed stack of proline monomers. Further evidence for the polymer-like association of proline is observed at the highest concentrations of proline; the enthaply associated with the melting of bulk water is greatly reduced, with a concomitant rise in the enthalpy associated with the eutectic melt of proline at 252 K. Cooling of these solutions at slow cooling rates shows only a glass-like transition, which is common for polymeric materials (8, 9, 20).

Further evidence that the phase behavior of proline is unusual can be realized from the calorimetric results for glycine and hydroxyproline. These molecules do not greatly affect the enthalpy or Tm of bulk water, nor do they possess eutectic transitions similar to those observed in proline solutions. Glycine and hydroxyproline are also far less soluble than proline (12). For hydroxyproline, it has been suggested that the addition of the hydroxyl group to the pyrrolidine ring may disrupt the ability of the rings to stack, perhaps by stearic hindrance (29). These molecules also do not protect biomacromolecules from damage incurred during dehydration or freezing (14, 15).

Infrared spectroscopy data on solutions of increasing proline concentration in D_2O suggests which functional groups may participate in the proposed polymeric aggregate of proline monomers. The COO^- asymmetric stretch region shows marked splitting with increasing concentration. Some of this splitting may be due to structural changes in the carboxylic group (such as the formation of dimers) with increasing concentration, particularly since glycine shows some splitting in bands assigned to this group. The identification of bands that arise from the observed splitting is a nontrivial exercise. However, the more complex splitting in proline solutions (compare Fig. 6 with Fig. 7) in otherwise similar functional groups suggests that the intermolecular associations of proline in solution are different from those of glycine. Stacking of proline monomers would place the COO^- group in a hydrogen bonding pattern within the stack, which might be expected to give rise to fundamental changes in the vibrational frequency of this group. The additional bands that arise in the splitting of this functional group are higher energy vibrations at 1,605 and 1,595 cm^{-1}, which would be expected if these groups are participating in a hydrogen bonding pattern such as the alternate stack.

Examination of the changes in the CH_2 region of proline (2,800–3,100 cm^{-1}) with increasing concentration in D_2O show an increase in one of the C—H stretch frequencies (from 2,958 to 2,964 cm^{-1}) and a reduction in the intensity of the stretch at 2,926 cm^{-1}. This change may arise as clathrate water associated with the hydrophobic region of the pyrrolidine rings is removed and the rings stack, creating a more fluid hydrocarbon environment for the C—H vibrational motions. Stacking of the pyrrolidine rings might be expected to give rise to an increase in the disorder of these groups as they are now able to exist in more random conformations. This may result in the broadening and decrease in intensity observed in some of the C—H stretch frequencies. Reflections of the order-disorder in the C—H region with changes in the environment of biomacromolecules has been elucidated in the examination of the CH_2 groups in the alkyl chain region of phospholipids (1, 2, 10). As these groups are found in a more fluid environment, the disorder of the system is reflected by an increase in the wavenumber and decrease in the intensity of the C—H stretch frequencies. This indicates the increased number of gauche conformers in the chain region and results in the observed rotomer broadening. This kind of analysis might be helpful in interpreting the changes that occur in this region with the induction of the aggregation of proline monomers into the proposed alternate stack. The stacking of the pyrrolidine rings could account for the observed changes in the C—H stretch region in increasing concentrations of proline in solution.

Other changes in this region occur in the disubstituted amine in the pyrrolidine ring. This group is thought to participate in the hydrogen bonding pattern in the alternate stack. This group may exchange in D_2O, but the rate of exchange at the pH of these solutions (6.4–6.6) may be slow. As the concentration of proline increases, there is a large intensity increase and a slight broadening in this group, which is indicative of intermolecular hydrogen bonding (5, 31).

The concentration at which the polymeric association of proline occurs according to the spectroscopic and calorimetric data is 1–2 M. The extreme solubility of proline may result in the maintenance of proline in solution as water is removed by dehydration or freezing. Considering these unusual properties, it may be possible that as water is removed, the concentrations that result in the formation of these aggregates may be realized. The existence of vicinal layers associated with surfaces of biomacromolecules

might create microenvironments where the formation of these aggregations could take place (4). During more severe water deficits such as freezing and dehydration, the eutectic phase separation and its apparent alteration of water structure by the formation of the aggregate, may be important in the stabilization of biomacromolecules during these events. An additional component of the stabilizing action of proline may be more direct interactions with biomacromolecules. The unusual solution properties of proline investigated in this paper may also play an important role in this direct interaction.

CONCLUSIONS

We conclude from this data that there is good evidence for the intermolecular associations of proline in moderate concentrations in solution. The calorimetric data indicate that proline shows eutectic phase separation at moderate concentration, and this is consistent with strong hydrogen bonding characteristics. The changes in the enthalpies associated with the thermal events during the warming of slowly cooled proline solutions also indicates that the eutectic melt at 252 K may be associated with the melting of an aggregate form of proline. Infrared spectroscopic data confirm the strong hydrogen bonding character of this amphipathic solute, and provide good evidence for the proposed alternate stack of proline monomers. Using this technique we have identified changes in functional groups that are likely to be involved in the polymeric aggregate. In particular, examination of the changes in the CH_2 region and the COO^- asymmetric stretch indicate that this polymeric aggregate may involve stacking of proline monomers in solution.

Received for publication 26 November 1985 and in final form 1 April 1986.

REFERENCES

1. Casal, H. L., and H. H. Mantsch. 1983. The thermotropic phase behavior of N-methylated dipalmitoyl phosphatidylethanolamine. *Biochem. Biophys. Acta.* 735:387–396.
2. Casal, H. L., and H. H. Mantsch. 1984. Polymorphic phase behavior of phospholipid membranes studied by infrared spectroscopy. *Biochem. Biophys. Acta.* 779:381–401.
3. Chu, T. M., M. Jusaitis, D. Aspinall, and L. G. Paleg. 1978. Accumulation of free proline at low temperatures. *Physiol. Plant.* 43:254–260.
4. Clegg, J. S. 1978. Hydration-dependent metabolic transitions and the state of cellular water in *Artemia*. *In* Dry Biological Systems. J. Crowe and J. Clegg, editors. Academic Press, Inc., NY. 117–155.
5. Conley, R. T. 1972. Infrared Spectroscopy. Allyn and Bacon, Boston. 119–146.
6. Crowe, J. H., L. M. Crowe, and D. Chapman. 1984. Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. *Arch. Biochem. Biophys.* 231:400–407.
7. Crowe, J. H., and L. M. Crowe. 1984. Effects of dehydration on membranes and membrane stabilization at low water activities. *In* Biological Membranes. D. Chapman, editor. Vol. 5. Academic Press, London. 58–98.
8. Franks, F. 1983. Solute-water interactions: do polyhydroxy compounds alter the properties of water? *Cryobiology.* 20:335–345.
9. Franks, F., and D. S. Reid. 1973. Thermodynamic studies. *In* Water: A Comprehensive Treatise. F. Franks, editor. Vol. 2. Plenum Publishing Corp., NY. 323–380.
10. Gaber, B. P., and W. L. Peticolas. 1977. On the quantitative interpretation of biomembrane structure by raman spectroscopy. *Biochim. Biophys. Acta.* 465:260–274.
11. Gilles, R. 1979. Intracellular organic osmotic effectors. *In* Mechanisms of Osmoregulation in Animals. R. Gilles, edtior. John Wiley & Sons. NY. 111–157.
12. Greenstein, J. P., and M. Winitz. 1961. Chemistry of the Amino Acids. John Wiley & Sons, NY. 523–566.
13. Gurskaya, G. V. 1968. The Molecular Structure of Amino Acids, Determination by X-ray Diffraction Analysis. Consultants Bureau, NY. 93–99.
14. Heber, U., L. Tyankova, and K. A. Santarius. 1971. Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. *Biochim. Biophys. Acta.* 241:578–592.
15. Hellergren, J., and P. H. Li. 1981. Survival of *Solanum tuberosum* suspension culture to −14°C: the mode of action of proline. *Physiol. Plant.* 52:449–453.
16. Herlinger, A. W., and T. V. Long. 1970. Laser-raman and infrared spectra of amino acids and their metal complexes. III. Proline and bisprolinato complexes. *J. Am. Chem. Soc.* 92:6481–6486.
17. Le Rudulier, D., T. Bernard, G. Goas, and J. Hamelin. 1984. Osmoregulation in *Klebsiella pneumoniae*; enchancement of anaerobic growth and nitrogen fixation under stress by proline betaine,butyro-betaine, and other related compounds. *Can. J. Microbiol.* 30:299–308.
18. Le Rudulier, D., A. R. Strom, A. M. Dandekar, L. T. Smith, and R. C. Valentine. 1984. Molecular biology of osmoregulation. *Science (Wash. DC).* 224:1064–1068.
19. Levy, D. 1983. Water deficit enhancement of proline and amino nitrogen accumulation in potato plants and its association with susceptibility to drought. *Physiol. Plant.* 57:169–173.
20. Luyet, B. J. 1970. The Frozen Cell. P. Mazur, editor. Academic Press, Inc., NY. 27–47.
21. Maggio, B., and J. Lucy. 1978. Interactions of water soluble fusogens with phospholipids in monolayers. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 94:301.
22. Pahlich, E., R. Kerres, and H. Jager. 1983. Influence of water stress on the vacuole/extravacuole distribution of proline in protoplasts of *Micotiana rustica*. *Plant Physiol.* 72:590–591.
23. Parker, F. S. 1971. Applications of infrared spectroscopy in biochemistry, biology, and medicine. Plenum Publishing Corp., NY. 165–188.
24. Reynoso, G. T., and B. A. DeGamboa. 1982. Salt tolerance in freshwater algae *Chlamydomonas reinhardii*; the effect of proline and taurine. *Comp. Biochem. Physiol.* 73A:95–99.
25. Rudolph, A. S., and J. H. Crowe. 1985. Membrane stabilization during freezing: the role of two natural cryoprotectants, proline and trehalose. *Cryobiology.* 22:367–377.
26. Rudolph, A. S., J. H. Crowe, and L. M. Crowe. 1986. The effects of three stabilizing agents: proline, betaine, and trehalose on membrane phospholipids. *Arch. Biochem. Biophys.* 245:134–143.
27. Schobert, B. 1982. Evidence for a protein stabilizing mechanism in plant cells under water stress. *In* Biophysics of Water. F. Franks, editor. John Wiley & Sons, NY. 309–312.
28. Schobert, B. 1977. Is there an osmotic regulatory mechanism in algae and higher plants? *J. Theor. Biol.* 68:17–26.
29. Schobert, B. 1978. Unusual solution properties of proline and its interactions with proteins. *Biochem. Biophys. Acta.* 541:270–277.
30. Schobert, B. 1979. A near-infrared spectroscopic investigation of

water in solution with proline, glycinebetaine, glycerol, and albumin. *Z. Naturforsch.* 34C:699–703.

31. Silverstein, R. M., G. C. Bassier, and T. C. Morrill. 1974. Spectrometric identification of organic compounds. 3rd ed. 87–113.
32. Stewart, G. R., and J. A. Lee. 1974. The role of proline accumulation in halophytes. *Planta.* 120:279–289.
33. Tyankova, L. 1972. The effect of amino acids on thylakoid membranes during freezing as influenced by side chain and position on the amino group. *Biochim. Biophys. Acta.* 274:75–82.
34. Treichel, S., E. Brinckmann, B. Scheitien, and D. J. von Willert. 1984. Occurrence and changes in proline content in plants in the southern namib desert. *Planta.* 162:236–242.
35. Withers, L. A., and P. I. King. 1979. Proline: A novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays*. *Plant. Physiol.* 64:675–678.