CHAPTER 4

PROLINE IS A PROTEIN-COMPATIBLE HYDROTROPE
4.0. INTRODUCTION:

Certain low molecular weight compounds, both inorganic ions and small organic molecules, known collectively as osmolytes, get accumulated under stress, be it a heat stress, salt stress, cold stress, or water stress (Yancey et al., 1982). Out of a variety of osmolytes, some of the amino acids and their derivatives appear to have an effect on the structure and function of different enzymes and proteins. Some of these are known as compatible solutes (Borowitzka and Brown, 1974; Yancey et al., 1982). The term “compatible solute” is used to describe a low-molecular weight solute which accumulates to a high intracellular concentration and which, by virtue of being a poor enzyme inhibitor, protects enzymes against the inhibition which would otherwise occur in solutions of low water availability (Brown and Simpson, 1972). Proline is one such compatible solute and has been shown to have an ability to enhance the solubility of sparingly soluble proteins several-fold in aqueous solutions (Schobert and Tschesche, 1978). Interaction of proline with M₄ lactate dehydrogenase appears to stabilize its structure and conformation against cold-induced denaturation (Rajendrakumar et al., 1994). Also, proline is considered to have a biological role in several higher plants which, under water stress conditions, accumulates in the cytoplasm of the plant cell and acts as a vehicle, which balances the concentration differences between the cytoplasm and the central vacuole of the plant cells (Stewart and Lee, 1974; Treichel, 1975).

Proline is an imino acid and its solubility in water is remarkably high, as much as 7 M at ambient temperatures. Its crystal structure has been determined by Kayushina and Vainshtein (1965) and the results suggest an orderly packing or layering of the pyrrolidine rings. One might expect to see such intermolecular interactions beginning to manifest at high concentrations in solution. Indeed, such a suggestion has been made earlier by Schobert and Tschesche (1978).
Considering these properties of proline and also its ability to function as a compatible solute (Schobert, 1977), it would be of value to understand its mechanism of action from the physical chemistry point of view and also its ability to function as a hydrotrope.

4.1. MATERIALS AND METHODS:

L-proline, 4-hydroxyproline, choline and glycine betaine of the highest available purity, were obtained from Sigma Chemical Company. The purity of L-proline was checked by HPLC and amino acid analysis and was found to have no impurities. All other chemicals of the highest purity were obtained from other commercial sources.

4.1.1. Solubilization Studies: Fluorescein diacetate (FDA), pyrene and the hormones progesterone and estradiol were used as the solubilizates. We studied their solubility behaviour in aqueous proline using the same experimental procedure as described in chapter 2.

4.1.2. Surface Tension Measurements: These were made at room temperature (ca 25°C) using a White Fisher Tensiometer. Interfacial tensions were measured using water and carbon tetrachloride as the two liquids.

4.1.3. Fluorescence Studies: ANS and DPH were used as the fluorescent probes and the procedure followed for measuring various microenvironmental features has been described in chapter 2. Fluorescence quenching studies were done, using perylene as the fluorophore and Cs⁺ as the quencher. Micromolar concentration of perylene was solubilized in 0.5 M and 5.0 M proline solutions and also in 2.0 M hydroxyproline solution and the fluorescent intensity of the emission band maximum at 470 nm was measured, with the excitation wavelength fixed at 435 nm. To this solution, increasing amounts of CsCl was added and the drop in the fluorescent intensity signal of perylene was monitored and the analysis was done using the Stern-Volmer equation.

4.1.4. Thermal Denaturation Studies: These were carried out using α-chymotrypsin as the
representative protein. α-chymotrypsin was dissolved in a citrate buffer (pH 5.0), both in the presence and absence of the additives. These solutions were excited at 280 nm and the tryptophan fluorescence was measured in the 300-400 nm region, and the shift in the emission band maximum was followed as a function of increasing temperature.

4.2. RESULTS AND DISCUSSION

4.2.1. Solubilization Experiments: Figure 4.1 shows the enhancement of the solubility of the dye FDA in water, brought about by increasing concentrations of L-proline. It is evident that up to a concentration of 1 M proline, there is no significant increase in the solubility of FDA. Upon further addition of proline, the solubility of FDA gradually increases and reaches a plateau at 4.5 M proline and beyond. When pyrene was tried as the solubilizate, its solubility was seen to be enhanced by a factor of ten in 4.5 M proline compared to its value in water. The solubilizing ability of proline towards progesterone and estradiol was also looked at and the results indicate that there is 4-fold enhancement in their solubilities at 25°C.

The above results show that proline enhances the solubility of diverse hydrophobic substances in water. In this behaviour, proline is as efficient as the hydrotropes sodium salicylate (NaS), p-toluenesulfonate (NaPTS), cumenesulfonate (NaCS), and the linear chain hydrotrope butylmonoglycolsulfate (NaBMGS). However, the solubilization curve displayed by proline (Figure 4.1) is less steep and somewhat more shallow than those of NaS and NaPTS and comparable to that of NaBMGS (Balasubramanian et al., 1989). This might be a reflection of the point that aromatic hydrotrope molecules might be able to pack better through the planar benzene rings. This cannot be visualized in the case of proline because it is a five membered saturated ring system as opposed to that of the six membered unsaturated ring system found in the case of most of the typical hydrotropes, and the planarity of the pyrrolidine ring is somewhat less. Figure 4.1 suggests that solubilization becomes significant above a proline concentration
Figure 4.1. Enhancement in the solubility of FDA in aqueous proline solutions at room temperature, as measured by the optical density (OD) values at 480 nm.
of 2.5 M or so (halfway point).

Figure 4.1 also shows the solubilizing ability of other compatible solutes such as choline, glycine betaine, and hydroxyproline. Despite the fact that choline and glycine betaine are highly soluble in water, no significant solubilizing ability is displayed by them. This might probably suggest that these molecules do not self-aggregate in water or do not form a host system that can sequester the hydrophobic molecules. The molecule 4-hydroxyproline has a limited solubility in water, ca. 2.5 M at 27°C. The low solubility of hydroxyproline in water could be because of the hydroxyl group which might pose steric problems. Even at the limiting concentration of 2.5 M, hydroxyproline does not solubilize any appreciable amount of FDA. This confirms the earlier results discussed in chapter 3 and also in (Srinivas et al., 1991), wherein it was shown that the molecular structure of the hydrotrope is crucial in determining its hydrotropic efficiency.

4.2.2. Surface Tension Measurements: As mentioned in the earlier chapters, conventional hydrotropes self-aggregate in water beyond their respective MHC values, to produce loose noncovalent assemblies of lowered polarity. When surface tension measurements were carried out in water, with increasing concentrations of proline, up to a concentration of 5 M, no significant drop in the surface tension value from that of water is observed. This shows that proline is not surface active at the air-water interface. However, it is able to alter the liquid: liquid interfacial tension of the water/CCl₄ interface. The variation of the interfacial tension of this binary liquid system with increasing concentrations of proline is shown in Figure 4.2. The decrease in interfacial tension occurs in a biphasic fashion, trailing off beyond 3 M or so. This shows that, under conditions wherein the molecules are forced to be near an interface by virtue of their amphiphilic nature, proline exhibits interfacial activity. From this interfacial behaviour it is clear that proline is only modestly surface active; however, this would act as a cohesive force that aids its self-assembly and hydrotropy.

It is of interest to compare the behaviour of other compatible solutes with that of proline. Figure 4.2 shows that 4-hydroxyproline is even weaker than proline in terms of its interfacial
Figure 4.2. Variation in the interfacial tension of the $\text{H}_2\text{O} : \text{Ccl}_4$ system at room temperature with concentration of proline and hydroxyproline.
activity, at a concentration up to its saturation limit of 2.5 M in water. Figure 4.1 shows that up to this limit, hydroxyproline is not able to solubilize hydrophobic compounds in water; its hydrotropy appears to be limited by its solubility in water. A similar situation was encountered with the other compatible solutes choline and glycine betaine. These two compounds do not display significant interfacial activity or any solubilizing ability even at the highest concentrations. The remarkably high solubility of proline and its interfacial activity thus appear to be important factors in its hydrotropic action.

4.2.3. Microenvironmental features: Assemblies of hydrotropes have been shown to offer a relatively less polar interior where the solubilizate can be incorporated. The polarity features of such microenvironments are readily monitored using fluorescent probes such as ANS, the emission wavelength (and intensity) of which shifts significantly with the polarity of the medium (Stryer, 1968). Another useful probe used in these type of studies is pyrene, for which the Ham effect ratio of the first and third vibronic bands in its fluorescence spectrum is altered by a change in the medium polarity (Nakajima, 1976; Dong and Winnick, 1982). The results that were obtained with proline are surprising. The pyrene Ham ratio, \( I_3/I_1 \), has a value of 0.6 in water, and was found to shift very little even as the proline concentration was raised to 5 M in water, suggesting that the polarity offered by proline even beyond its MHC is not notably different from that of water. Sequestration of the solubilizate (FDA or pyrene) appears to occur so that solubilization is effected, but the microenvironment offered to the solubilizate by the proline molecular assembly is not significantly less polar than water.

Similar results were obtained when ANS was used as the probe. The emission spectrum of 10 μM ANS in water was seen around 522 nm, and in 5M proline it moved to near 510 nm. This very modest blueshift indicates that the polarity of the proline solution is only slightly lower than that of water and is comparable to that of 20% methanol in water (v/v). Figure 4.3a shows the variation of ANS fluorescence as the concentration of proline is increased in aqueous solution. Here again, the transition is observed in the emission wavelength values of the probe.
at a concentration of around 2 M proline. The emission intensity, another polarity sensitive factor, also increases at high concentrations of proline. The supramolecular assembly of proline does not seem, however, to offer a microenvironment that is as nonpolar as that of any other hydrotrope. It was shown earlier (Balasubramanian et al., 1989) that NaBMGS blue shifts ANS fluorescence to 460 nm, and increases its intensity by almost by a factor of 40.

Also, even saturated solutions of other compatible solutes such as hydroxy proline, choline, glycine betaine, do not significantly shift the emission band of ANS from their respective aqueous solution values. In the case of hydroxyproline, its solubility in water appears to be the limiting factor.

Heavy metal ion quenching studies often prove to be quite informative in terms of determining the organizational abilities of supramolecular assemblies and the polarities involved there in. Figure 4.3b shows the efficiency with which the heavy metal ion Cs+ quenches the fluorescence of perylene that is solubilized in aqueous solution of proline. Proline at 5 M is able to protect or shield the fluorophore from quenching by Cs+ better than at a concentration of 0.5 M. This is consistent with the interpretation that proline forms self aggregates beyond MHC and sequesters the solubilizate more effectively. Here too, the inefficiency of 2 M hydroxyproline to inhibit the Cs+-mediated quenching effectively is suggestive of its inability to form a hydrotropic assembly.

The contrasting behaviour of proline and hydroxyproline leads to the conclusion that the aggregation of proline molecules occurs via their hydrophobic pyrrolidine rings. This point has already been noted by Schobert and Tschesche (1978). Since the hydrophobicity of the pyrrolidine rings is not as pronounced as that of the benzene ring, amphiphilicity and surface activity are weaker and self association should occur in a less efficient and less cooperative fashion than in the case of either conventional hydrotropes or detergents (Mukherjee, 1974). The MHC value of proline is thus high and perhaps represents not the onset of a micelle-like compact assembly but step-wise oligomerization. That the self aggregation of proline is loosely organized...
Figure 4.3. (A) Variation in the wavelength and intensity ($I_0$) of the fluorescence spectral band of the probe ANS as a function of the concentration of proline and hydroxyproline. ANS concentration, 10 μM; excitation at 365 nm. (B) Quenching of the fluorescence of perylene solubilized in 0.5 M proline, 5 M proline, and 2 M hydroxyproline in water by added CsCl. Perylene concentration, 1 μM; excitation at 435 nm and emission at 470 nm. $F_0$ and $F$ are the fluorescence intensities in the absence and presence of the quencher.
is apparent from the above fluorescence results. In the case of hydroxyproline, the self-association of molecules seems to be influenced by the presence of the hydroxyl group.

To support the above arguments, measurements were made to determine the microviscosity values offered by the proline molecular aggregate. These values were determined by measuring the polarization and anisotropy of the fluorescence of diphenylhexatriene solubilized in proline solutions (Schinitzky and Barenholz, 1978). The microviscosity value measured by DPH in 0.5 M proline was 0.45 P and it increased to 1.14 P in 5 M proline, indicative of a more viscous environment in the latter case. It is notable in this connection that the bulk viscosity of the aqueous proline solution increases steeply beyond 2-3 M concentrations (Schobert and Tschesche, 1978).

4.2.4. Interaction with proteins: The above mentioned results suggest that proline is able to self-aggregate and sequester or include guest-molecules within, a phenomenon exhibited by conventional hydrotropes. In this sense, proline may be considered as a hydrotrope. But the microenvironment that a proline assembly offers is more polar, closer to that of water than of nonpolar media. This polarity feature displayed by proline would be of immense value in its interaction with biopolymers which need to maintain their native, active conformation. In this light, the interaction of proline with proteins is of particular interest considering its role as a “compatible solute” and as “enzyme protector”.

Figure 4.4 shows the effect of 5 M proline on the thermal denaturation profiles of globular protein, α-chymotrypsin in the pH 5 region. Proline is seen to increase the denaturation temperature from 56°C to 66°C, thus offering the protein structural stability and protecting it from thermal denaturation. In contrast, the conventional hydrotropes, NaBMGS and NaCS, destabilize the protein somewhat, by decreasing the Tm value and precipitating the protein from solution. Therefore, proline appears to stabilize the protein structure whereas conventional hydrotropes and surfactants denature the proteins. It is interesting to note in this connection that proline is also able to stabilize the structure and activity of lactate dehydrogenase.
Figure 4.4. Variation in the wavelength maximum of tryptophan emission band of α-chymotrypsin with temperature. Excitation wavelength, 295 nm; protein concentration, 0.2 mg/mL.
Hydrotropes are known to be important in industrial and pharmacological applications. If one looks for such an application in biology, proline may be considered as the appropriate molecule. Its hydration is high (Kayushina and Vainshtein, 1965; Schobert, 1977), which aids compatible solutes to preserve the necessary content of water demanded when cells and molecules are water stressed. While choline, glycine betaine and hydroxyproline share this property, proline has an additional feature of relevance; it is able to self-aggregate and provide a host system which can solubilize guest molecules. The dual property of high hydration and selfassembly makes the microenvironment of the proline system close to or comparable to that of water and thus far more benign than those of conventional hydrotropes or detergents. It is this feature that might help proline maintain the native conformation of proteins and keep them functional.

Proline thus appears to have a dual role, namely (1) it solubilizes hydrophobic substances and (2) it interacts with biomolecules and stabilizes their structure and conformation. Thus, proline could be considered as a “protein-compatible hydrotrope”.

(Rajendrakumar et al., 1994) and cytochrome c (Taneja and Ahmed, 1994).