Research Papers published from the present investigation:

1. **Chadalavada S.V. Rajendrakumar,** Boojala V.B. Reddy & Arjula R. Reddy *(1994).* Proline-protein interactions: Protection of structural and functional integrity of **M4-Lactate Dehydrogenase.** ***Biochemical and Biophysical Research Communications*** **201** (2) 957-963.

2. **Chadalavada S.V. Rajendrakumar**, B. Karunasree, & Arjula R. Reddy *(1996)* Molecular approaches to abiotic stress tolerance. pp. 112-121. In the ***5th National Rice Biotechnology Network (The Rockefeller Foundation National Grantees Meet),*** ICGEB, New Delhi, Nov-13-16,

3 **Chadalavada S.V. Rajendrakumar,** Tangirala Suryanarayana & Arjula R. Reddy *(1997).* DNA helix destabilization by proline and betaine: Possible role in the salinity tolerance process. *FEBS Letters,* 410, (2-3) 201-205

Research papers under communication/preparation :

- Abscisic acid mediated salinity and chilling tolerance in rice requires the induction of stress responsive polypeptides
  Chadalavada S.V. Rajendrakumar B. Karunasree & Arjula R. Reddy
  (Under preparation to *Plant Science)*

- Identification of a developmentally regulated and ABA responsive aldose reductase related protein in rice *(Oryza saliva L)*
  B. Karunasree, Rajendrakumar, CSV. & Arjula R Reddy
  (Under preparation to *Plant Physiol.*)

Abstracts presented at the Conferences **from the present investigation:**

- **Rajendrakumar,** C.S.V., B.V.B. Reddy & A.R. Reddy (1994). Biochemical and bio physical study of protection of enzyme's structural and functional integrity of proline. **At the *Second Asia Pacific Conference on Agricultural Biotechnology,*** 6-10 **March.** Madras, India.
- Reddy A.R.., Rao A.H.K., Joanny K., Reddy V.S., Radha Rani., **Rajendrakumar C.S.V.** Garg A.K., Sree B.K., Madhuri G., Sailaza & Reddy **K.R.K** (1992). ***National Rice Biotechnology Network. Second annual meet.*** **D.R.R. Hyderabad, INDIA.**
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- Karunasree, B., Rajendrakumar, CSV and Arjula R Reddy Identification of a developmentally regulated and ABA responsive aldose reductase related protein in rice *(Oryza sativa* L) accepted in Fourth Asia Pacific Conference on Agricultural Biotechnology to be held in Darwin, Australia, 13-17, July, 1998.

Practical course work/ Workshops attended during the tenure as a doctoral fellow:

1. Attended the practical course work on "Plant Transformation" during Nov.-Dec. 1994 held at Int. Center for Genetic Engineering and Biotechnology (ICGEB) conducted by UNIDO at New Delhi
2. Attended the practical course work on "Fluorescence in situ hybridization using non radioactive nucleic acid probes "(FISH) held at the Indian Institute of Science, Bangalore between 22 July to 2 Aug, 1995 (Prof. 11. Sarat Chandra's Lab)
3. Attended the Mahabaleswar seminar seris on modern biology "Nucleic acid protein interactions" held at TIFR centre Ooty in December 1995
4. Attended the Indo-Japan workshop on "DNA and protein foot-printing" held at Centre for Cellular and Molecular Biology, Sponsored by JSPS-DST in March, 1997

Symposia & Conferences attended during the doctoral investigation:

1. Annual Meeting of the Society of Biological Chemists (India) held at Centre for Cellular and Molecular Biology, Hyderabad 1992
2. Attended the Second Asia Pacific Conference on Agricultural Biotechnology held at Madras, India, between 6-10 March-1994
3. International Symposium on Plant Molecular Biology & Biotechnology held at International Centre for Genetic Engineering and Biotechnology in December 1994 at New Delhi. November 1995
4. Pot poury meet of Society of Biological Chemists (Hyderabad chapter) at Centre for Cellular and Molecular Biology Hyderabad March 21 1995
5. II International Crop Science Congress, New Delhi, Nov. 17-23

Membership in professional scientific organizations:

- Member, Society of Biological Chemists, INDIA
- Life member, Society for Plant Biochemistry and Biotechnology, IARI, New Delhi

Vol. 201, No. 2, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1994 Pages 957-963

PROLINE - PROTEIN INTERACTIONS: PROTECTION OF STRUCTURAL AND FUNCTIONAL INTEGRITY OF M_4 LACTATE DEHYDROGENASE

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Received April 28, 1994

Summary: A well defined labile isozyme, rabbit muscle M4 - lactate dehydrogenase was denatured under freeze-thaw, heat and GuHCl treatment in the presence and absence of proline, and the corresponding structural changes of the enzyme were monitored through fluorescence and CD spectral studies. The data reveal that proline confers protection to the structural integrity of the enzyme, thereby protecting its activity. This was attributed to its property of forming hydrophilic colloids in aqueous media with a hydrophobic backbone interacting with protein. Unlike other osmolytes, proline is proposed to act on the enzyme stability not only by inducing preferential hydration of proteins but also through the interactions of its multimeric hydrophobic backbone with the solvent-accessible hydrophobic regions of the enzyme. © 1994 Academic Press, Inc.

There have been reports on the protection of enzyme activity, organelle systems and free cells by proline from the deleterious effects of heat, pH, salt and chemicals (1-4). In all these cases, a significant concentration dependent protection was observed against a range of stressful conditions. It was reasoned that any proline-induced decrease in enzyme precipitation would reflect a lessening of the thermodynamically unfavourable conditions leading to precipitation, which in turn could result in enhanced stability of an enzyme to remain in the solution (5 - 7). In aqueous solutions, proline forms aggregates by a step-wise stacking and hydrophobic interactions of the pyrrolidine ring, forming multimers which contain a hydrophobic backbone with hydrophilic groups on the surface (8). Thus, proline was proposed to increase the solubility of sparingly soluble proteins by its hydrophobic interaction with the hydrophobic surface residues of the protein. To our knowledge, however, there is no experimental evidence for proline-protein interactions in the maintenance of structural and functional integrity of any enzyme under different stress conditions. To this end, we provide

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here such an evidence by making use of a labile enzyme, M_4 lactate dehydrogenase and show that proline confers protection to its structure and function under severe freeze-thaw, high temperature and chemical denaturation. A possible mechanism involved is discussed.

Materials and Methods

Rabbit muscle L-lactate dehydrogenase-5(M4) isozyme type VS (EC 1.1.1.27), proline, hydroxy-proline, guanidinium hydrochloride, NADH, sodium pyruvate were purchased from Sigma. All other chemicals used were of analytical grade and purchased locally.

Denaturation studies. The enzyme was denatured by three different methods, namely freeze-thaw cycles, heat denaturation and chemical denaturation by GuHCl (0.5M). $LDH-M_4$ at a concentration of $60\,\mu g/ml$ dissolved in 10 mM tris buffer ($pH\,7.5$) was frozen in liquid nitrogen for 60 seconds and thawed at room temperature for five minutes. Such cycles were repeated for a minimum of three times with or without proline. For a comparative analysis of cryoprotection conferred by different osmolytes, enzyme at a concentration of 60 $\mu g/ml$ dissolved in 10 mM tris buffer (pH 7.5) was freeze-thawed, with different osmolytes (1M each) added separately. Aliquotes were used for fluorescence measurements. For the heat denaturation studies, the enzyme at a concentration of $15\mu g/ml$ was incubated at different temperatures for 15 minutes with or without proline (1.0 M). These were left at 4^0C for six hours. Aliquots were used for fluorescence measurements as well as enzyme assays. GuHCl (0.5 M) was added to the enzyme at a concentration of $90\,\mu g/ml$ containing different concentrations of proline and incubated for an hour at 27° C. Aliquots from the above samples were taken for fluorescence studies and enzyme assay. The assay was followed essentially as described by Tarniya *et al.*, (1985) (9).

Fluorescence spectral studies. The intrinsic fluorescence of tryptophan was monitored to record its contribution to the emission intensity of the enzyme at an excitation wave length 295 nm using Hitachi Spectrofluorimeter. The relative fluorescence and $\lambda_{emission\,max}$ were determined under different denaturing conditions in the presence and absence of proline and certain other osmolytes have been recorded (Fig. 1 and 2). The fluorescence was recorded at room temperature, with a band pass of 5 nm.

Circular Dichroic spectral studies. ("I) spectra were recorded with Jasco 20 Spectropolarimeter at room temperature. Sample solutions contained enzyme (3.74 μM) dissolved in 10 mM tris buffer $pH\,7.5$The CD values were computed **for** molar elipticities *(6)* using the method of Rao and Kumar (1991) (10). Each spectrum is an average of three scans. The spectra could not be recorded in the presence of high concentrations of proline as it interferes with the CD spectrum of enzyme at such concentrations. Therefore, the CD spectra were recorded with 10 mM proline which was later corrected **for its** contribution to the CD signal of the enzyme,

Results

[illegible] intensities of the enzyme freeze-thawed with different osmolytes revealed that [illegible] a stabilizing effect on the enzyme's structure in comparison with the other [illegible] tested (Fig. 1). Proline-mediated cryoprotection of enzyme becomes evident at as [illegible] concentrations as 10mM and appeared to saturate at 250 mM (Fig. 2a). Similarly [illegible] temperatures, proline (1M) maintains both fluorescence intensity as well as [illegible] (Fig. 2b). However, proline was found to be ineffective beyond $45°C$. Fur[illegible] the protection of enzyme by proline under the denaturing influence [illegible] was known to dissociate the tetrameric form into inactive monomers

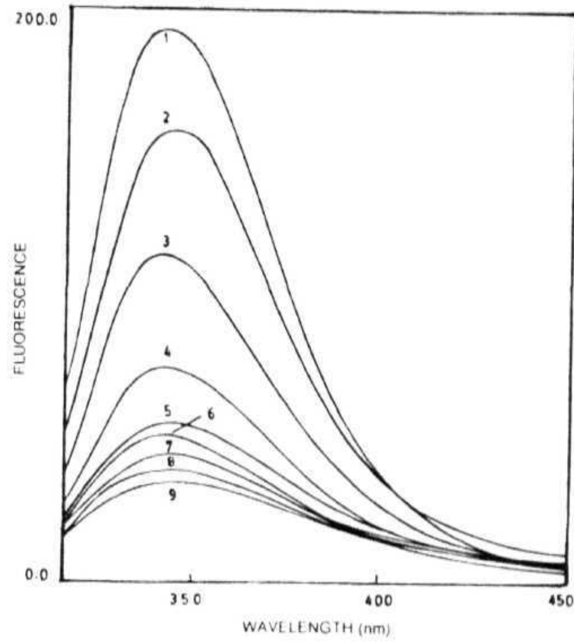


Figure 1. Fluorescence emission spectra of $LDH-M_4$ (1) control, freeze-thawed with 1.0 M each of (2) proline, (3) Sucrose, (4) Mannitol, (5) Glycine, (6) Hydroxy-proline, (7) Glucose, (8) Valine and (9) Freeze-thawed enzyme.

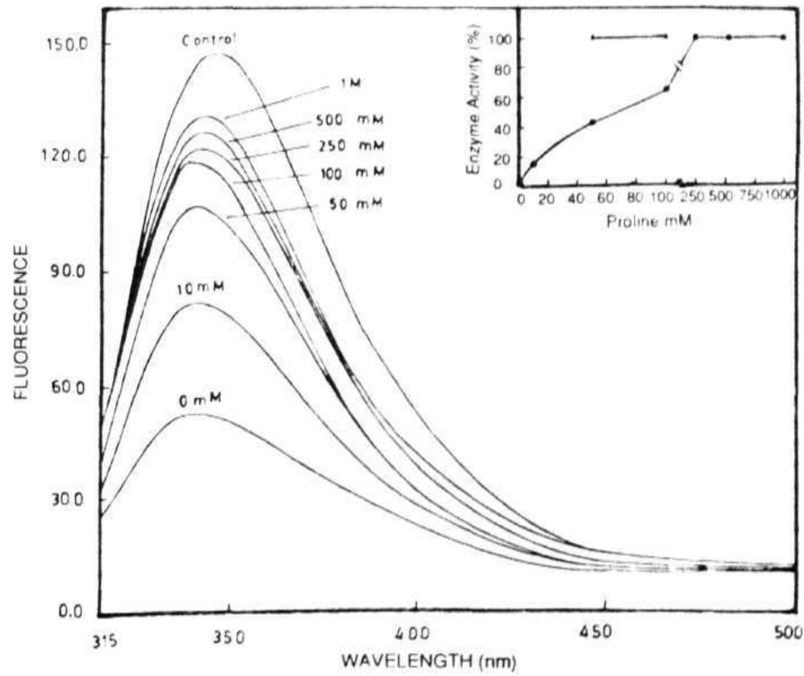


Figure 2A. Fluorescence emission spectra of $LDH-M_4$ control and freeze-thawed with different concentrations of proline as indicated. Enzyme activity shown in the inset, control (▲) and freeze-thawed (●) with proline.

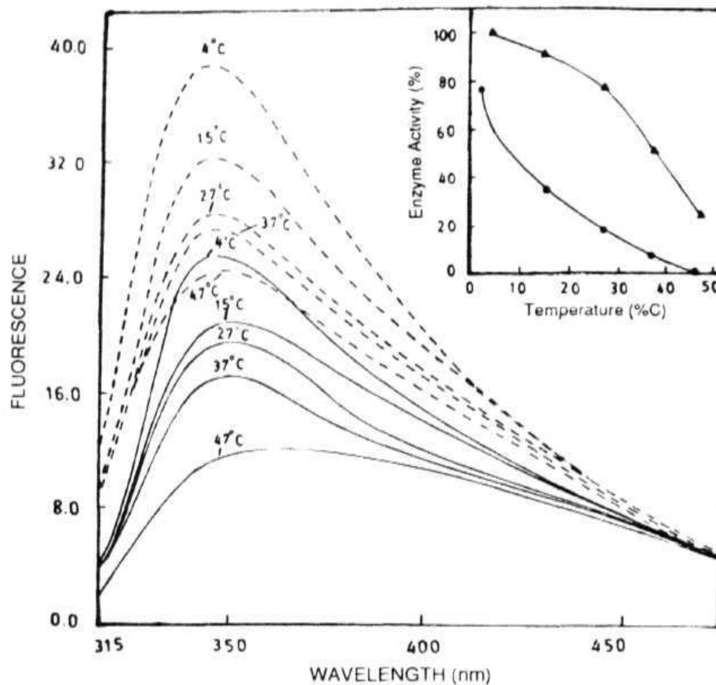
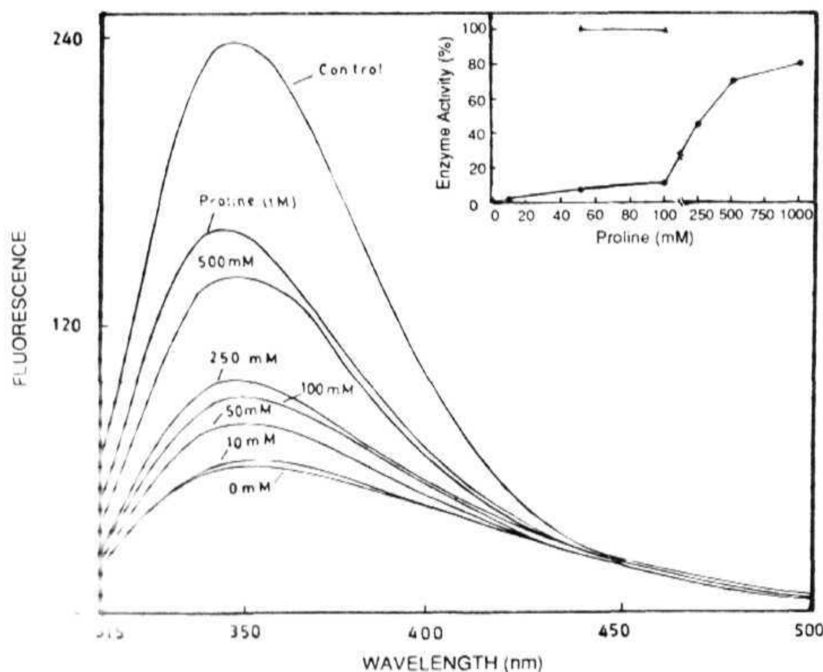


Figure 2B. Fluorescence emission spectra of heat denatured $LDH-M_4$ in the absence (—) and presence (- - - -) of proline (1.0 M). Enzyme activity shown in the inset, with (▲) and without (●) proline.



Fluorescene emission spectra of $LDH-M_4$, control (without GuHCl and ... GuHCl (0.5 M) denatured enzyme with different concentrations of proline ... The inset shows the enzyme activity in the presence (●) and absence of ...

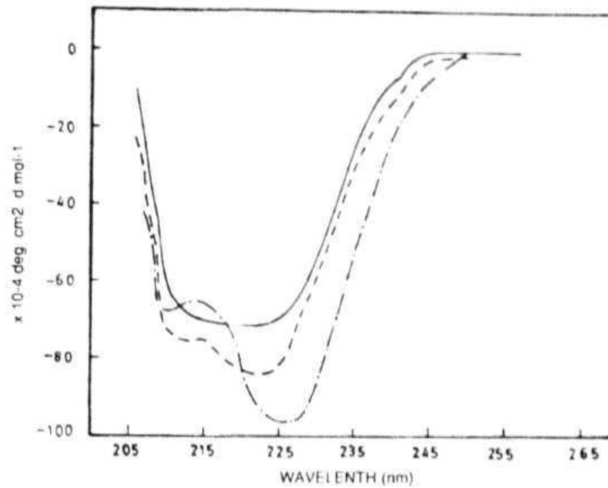


Figure 3. CD spectra of native $LDH - M_4$ (- - - -), freeze-thawed enzyme (——) and freeze-thawed enzyme in the presence of 10 mM proline (— · — · —).

(11,12). The results revealed that proline,in a concentration dependent manner, exhibited a stabilizing effect on the fluorescence intensity of the enzyme. This was also supported by the enzyme activity study, wherein the structure and function of the tetrameric form was evidently stabilized under the denaturing conditions of GuHCl (Fig. 2c).

The CD spectra of the native and proline-protected enzymes show typical characteristics of a protein with good helical content (Fig. 3). The native enzyme showed minima at 212 nm and 222 nm, while the proline protected enzyme, under freeze-thaw, showed a minima at 210 and 225 nm. However, the freeze-thawed enzyme without proline showed a single minimum from 215 run to 224 nm indicating the disturbed secondary structure. The α helicity of the proline-protected enzyme was found to be increased marginally when compared to the native form. On the contrary, the freeze-thawed enzyme exhibited a decrease in α helicity.

Discussion

The stabilization of native protein structure as a result of preferential hydration, induced by certain osmolytes, has been reported previously (13-15). However, such an increase in preferential hydration of a protein can not fully explain its stability under stress. For instance, both proline and valine induce preferential hydration of lysozyme, although valine is ineffective in conferring protection to the enzyme (16). Proline has been implicated in the structural stabilization of a range of enzymes. Present study reveal that proline is a better protective agent of $LDH — M_4$ when compared to other tested amino acids and sugars. This could be attributed to the formation of proline aggregates in aqueous media (8). Thus, proline behaves like a hydrophilic colloid with a hydrophobic backbone thereby inducing

is somewhat analogous to the chaperone function wherein a similar kind of hydrophobic interaction prevents the enzymes from denaturation (21).

Acknowledgments: This work is supported by EEC and Rockefeller Foundation grants to ARR. CHRK acknowledges CSIR, India for the fellowship.

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FEBS Letters 410 (1997) 201–205

DNA helix destabilization by proline and betaine: possible role in the salinity tolerance process

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Received 4 February 1997; revised version received 14 April 1997

Abstract **Evidence is provided for the ability of proline, a salinity induced osmoprotectant, to destabilize the double helix and lower the T_m of DNA in a concentration dependent manner. At the reported salinity-adaptive bio-accumulation of 1 M and above, proline could considerably decrease the T_m and partially counteract the effect of sodium chloride and spermidine on DNA stability. On the contrary, several other amino acids tested did not show any such destabilizing effect on DNA helix. Enhanced susceptibility to S1 nuclease and insensitivity to DNase I in presence of increasing proline concentrations have further suggested a clear destabilization of the double helix. Such an effect is somewhat reminiscent of the interaction between betaine, another salinity induced osmolyte, and DNA resulting in decreased T_m values. These interactions may be significant in view of the abundance of such osmolytes in cells under salinity stress-adapted conditions, with many a bacterial mutant accumulating them exhibiting improved tolerance to salinity.**

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Key words: Proline; Betaine; DNA helix; T_m curves; Salinity stress

1. Introduction

Proline and betaine are the two known major osmoprotectants which accumulate in plants, bacteria, algae and marine invertebrates in response to an array of abiotic stresses, most prominent being the salinity stress [1–4]. More often, this accumulation is the result of an adaptive de novo synthesis in cells contributing a major share among osmolytes [5–8]. Such accumulations were found to reach up to 1 M internal concentration in certain halophytes and bacteria, accounting for as much as 10–20% of the dry weight [9–12]. These two osmolytes were also reported as efficient stabilizers of proteins, lipid membranes, organelles and cells under severe stress conditions without being inhibitory to cellular functions [13–19]. Further, genetically engineered hyper-accumulation of proline was reported to confer salinity tolerance in tobacco seedlings under laboratory conditions [20]. Many plant and bacterial mutants accumulating proline and betaine have also been found to exhibit an increased tolerance to salinity stress [11,12,21–24].

We have investigated the interaction of these osmolytes with DNA, since their access, even transiently, to DNA in vivo under the stress adapted conditions can not be ruled out due to their abundance. In fact, betaine was proved recently to considerably destabilize DNA [25]. We report here that proline destabilizes DNA and partially counteracts the effect of sodium chloride and spermidine on the stability of the double helix within the adaptive bio-accumulated concentrations. The present study indicates a possible role of these osmolytes in salinity tolerance process by negating the undesirable effect of NaCl on DNA stability.

2. Materials and methods

L-Proline, hydroxy proline, glycine, alanine, valine, leucine, serine; betaine, D-glucose, sarcosine, calf thymus DNA, Tris, EDTA, spermidine, NaCl, agarose and λ phage DNA were purchased from Sigma (St. Louis, MO, USA). *E. coli* single strand DNA binding protein (ssb protein) and pUC 18 plasmid were procured from Bangalore Genei (Bangalore, India). DNase I was procured from Boehringer-Mannheim (Mannheim, Germany) and the S1 nuclease from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade purchased locally.

2.1. DNA melting studies

DNA melting studies were conducted in a buffer (1 ml) containing 10 mM Tris-HCl (pH 7.5) and 2 mM EDTA and the indicated concentrations of NaCl and additives. Calf thymus DNA (1.0 A_{260}) in the above buffer, with or without the additives, was taken in a 1 cm path teflon-stoppered quartz cell and incubated at the initial assay temperature for 5 min. The increase in absorbance at 260 nm was monitored in a Hitachi spectrophotometer attached to a temperature programmer KPC-6 and temperature controller SPR-7. Both the sample and reference cells were heated together at a rate of 1°C/min, and the net absorbance was recorded after every 1°C increase. The T_m of DNA was determined graphically from the transition mid-point of the absorbance versus temperature profile.

2.2. DNase I sensitivity assay

The sensitivity of DNA to DNase I digestion was studied spectrophotometrically (Hitachi) by measuring the increase in absorbance at 260 nm at 37°C in presence of different concentrations of proline. DNase I (1 μg) was added to double stranded calf thymus DNA (1.0 A_{260}) in a buffer (1 ml) containing 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. The enzyme was diluted to required concentration in 10 mM Tris-HCl (pH 7.8) and 50% (v/v) glycerol. DNase I sensitivity of DNA was also analysed by agarose gel electrophoresis. Calf thymus DNA, λ phage DNA, or pUC 18 DNA (1 μg each) in the DNase I assay buffer (30 μl) was incubated at 37°C for 10 min with 25 ng of DNase I in the presence of different concentrations of proline and the digestion products were separated on a 0.8% agarose gel.

2.3. S1 nuclease sensitivity assay

The S1 nuclease reaction mixture (30 μl) contained calf thymus DNA (0.5 μg), buffer (5 mM sodium acetate (pH 4.7), 15 mM sodium chloride, 0.1 mM $ZnCl_2$) and proline. DNA samples in presence of increasing concentrations of proline were heated at 65°C for five minutes and quickly chilled on ice. Reaction was started by adding S1 nuclease (1 unit) and incubated at 37°C for 15 min. The digestion was stopped by adding EDTA and SDS to a final concentration of 50 mM and 1%, respectively, and the products were separated on a 0.8% agarose gel.

2.4. Single strand binding protein gel shift assay

The λ phage DNA (0.5 μg) in 30 μl buffer containing 10 mM Tris-

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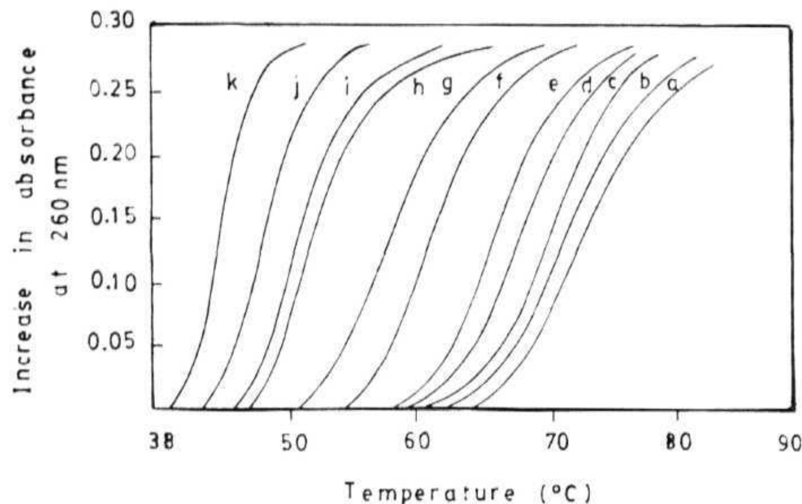

Fig. 1. Effect of increasing concentrations of proline on the T_m of calf thymus DNA: (a) Control DNA (without proline), (b) 0.06 M, (c) 0.25 M, (d) 0.5 M, (e) 1.0 M, (f) 2.0 M, (g) 3.0 M, (h) 4.0 M, (i) 4.5 M, (j) 5.0, (k) 5.5 M.

HCl (pH 8.1), 1 mM EDTA and 20 mM NaCl, was heated at 65°C in the presence or absence of 3.0 M proline for 5 min and quickly chilled on ice. Increasing concentrations of ssb protein was added and after incubation at room temperature for 5 min, the samples were electrophoresed on a 0.7% agarose gel.

2.5. Displacement of DNA bound ethidium bromide by proline

Ethidium bromide (0.4 μg) in the buffer (10 mM Tris-HCl (pH 7.5) and 50 mM NaCl) was excited at 480 nm and the emission was recorded between 500–660 nm in a Hitachi spectrofluorimeter. Later, calf thymus DNA (0.5 μg) was added to it to record the enhancement in fluorescence emission intensity. Similarly, the emission spectra were recorded with the addition of increasing concentrations of proline to the above mixture after incubating at room temperature for 15 min.

3. Results

Destabilization of DNA double helix by proline was analysed by various methods. Proline was found to significantly lower the melting temperature of calf thymus DNA in a concentration dependent manner. Though such an effect found at 60 mM was marginal, an appreciable decrease in T_m was observed consistently (Fig. 1) at concentrations ranging from 250 mM to 1 M, which are widely reported to be biologically relevant [9–12]. In order to know whether the effects shown by proline are specific, several other amino acids were tested as controls. The results reveal (Table 1) that none of the amino acids tested could induce a similar effect even at high concentrations. While glycine, glycyl glycine, and serine were found to significantly stabilize the double helix and increase the T_m, alanine, valine, leucine and sarcosine could not greatly alter the T_m. However, hydroxy proline at its maximum aqueous solubility point (2.0 M), could reduce the T_m by 8°C. Proline, unlike its hydroxylated analogue, with a high aqueous solubility (6.0 M) due to the reported anomalous solution properties [18], was found to destabilize DNA even beyond such a concentration (Fig. 1). However, the differential aqueous solubility of tested solutes prevented an ideal comparison between them in their interaction with DNA.

Proline and betaine (1 M each) were found to have a[r]

Table 1
Effect of proline and other amino acids on the T_m of calf thymus DNA in the presence and absence of additives

Concentration	T_m of DNA ± 1.0°C
DNA	71.0
+1.0 M proline	65.0
+2.0 M proline	[illegible]
+2.0 M glycine	[illegible]
+2.0 M serine	[illegible]
+1.0 M alanine	[illegible]
+0.25 M valine	[illegible]
+0.1 M leucine	[illegible]
+2.0 M hydroxy pro[illegible]	[illegible]
+0.5 M glycyl glycin[illegible]	[illegible]
+2.0 M sarcosine	[illegible]
+1.0 M glucose	[illegible]
+1.0 M betaine	[illegible]
+1.0 M proline+1.0 M [illegible]	[illegible]
+10 mM spermidine	[illegible]
+0.5 M NaCl	[illegible]
+10 mM spermidine [illegible]	[illegible]
+0.5 M NaCl+1.0 M [illegible]	[illegible]
+0.5 M NaCl+2.0 M [illegible]	[illegible]
+0.5 M NaCl+2.0 M [illegible]	[illegible]

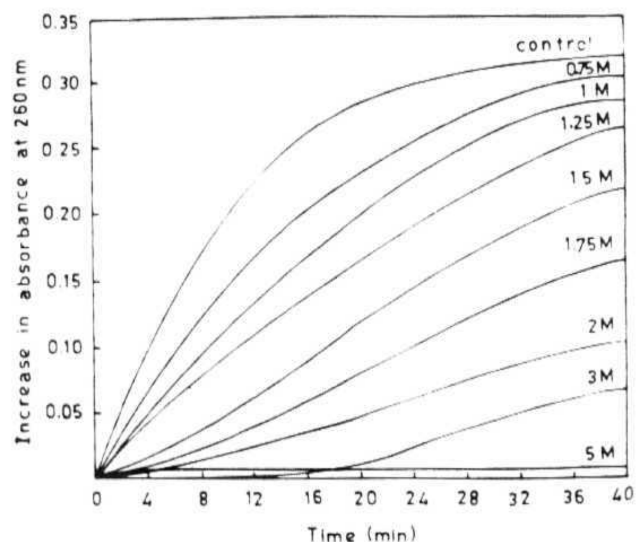

Fig. 2. DNase I sensitivity of calf thymus DNA in the presence of increasing concentrations of proline.

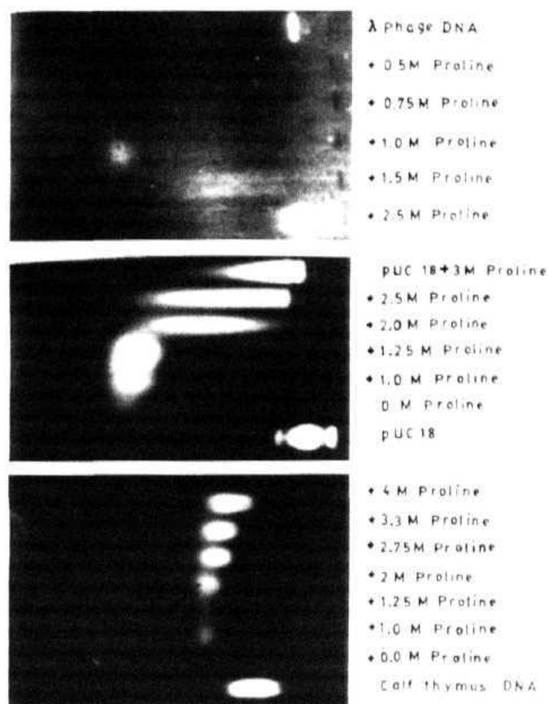

Fig. 3. DNase I sensitivity of λ phage, pUC 18 and calf thymus DNA in the presence of different concentrations of proline.

additive effect in the reduction of T_m (Table 1). Moreover, proline (1 M) was found to individually reduce the effect of NaCl (0.5 M) and spermidine (10 mM) on DNA stability as indicated by the decrease in T_m by 6°C and 8°C, respectively. On the contrary, the co-addition of glycine (2 M) with sodium chloride (0.5 M) did not influence the effect of the latter on DNA indicating the ineffectiveness of glycine in counteracting the salt effect (Table 1).

The helix destabilization was further confirmed with the DNase I and S1 nuclease sensitivity assays. In the spectrophotometric analysis of DNase I digestion, increased proline concentrations were found to progressively protect the calf thymus DNA against the digestion, with a near complete protection observed at higher than 3.0 M (Fig. 2). This was further demonstrated by gel electrophoresis profile of DNase I digested samples of λ phage, plasmid and calf thymus DNA (Fig. 3). Rice and barley DNA did show a similar pattern of resistance to DNase I activity in the presence of proline (data not shown). This effect is either due to a decreased binding of DNase I to DNA or destabilization of the double helix. The former is less likely because proline does not affect the binding properties of proteins which interact with DNA (see below). In contrast, proline at increasing concentrations was found to make the double stranded calf thymus DNA more susceptible to S1 nuclease digestion (Fig. 4). In the gel retardation assay, binding of increasing amounts of ssb protein to λ DNA in presence of 3.0 M proline was found to retard the mobility of the DNA-protein complexes which was clearly absent in the control λ DNA with the addition of 12 μg of ssb protein (Fig. 5). These results indicate the non-interference of proline in interactions between such proteins and DNA. Finally, the ability of proline in replacing the ethidium bromide bound to double stranded calf thymus DNA was tested and the fluorescence emission data (Fig. 6) revealed a marginal displacement which is expected of compounds that destabilize the double helix.

4. Discussion

Proline was found to bring down the T_m in a concentration dependent manner (Fig. 1), somewhat similar to betaine which was reported to lower the T_m and partly reduce the impact of KCl on DNA stability [25]. While 1 M proline could reduce the T_m of calf thymus DNA by 6°C (Table 1), betaine at a similar concentration could reduce the T_m of poly (dG-dC) by 5°C and the bacterial DNA by 4°C [25]. The results are significant in view of the reported hyper bio-accumulation of these osmolytes under salinity stress. Such an effect was not found with other tested amino acids, of which, glycine, glycyl glycine and serine were in fact found to considerably stabilize the DNA. Interestingly, with the addition of methyl group(s) on the glycine structure, alanine, valine and leucine have correspondingly lost both the aqueous solubility and the stabilizing effect on the DNA. Similarly, *N,N,N*-trimethylglycine (betaine) was found to be helix destabilizing when compared to glycine and sarcosine (Table 1) [25]. In one such related attempt to test the influence of methyl groups on the potency of osmoprotection, it was demonstrated that, contrary to glycine and sarcosine, compounds of betaine series, with trimethyl groups on the nitrogen were found to ameliorate the effect of high salinity (0.8 M) on the growth of *E. coli* [12]. Similarly, the observed inability of glycine in counteracting the effect of NaCl on DNA (Table 1) could probably be accounted as one of the reasons for its failure to protect *E. coli* from high salinity (0.8 M NaCl) stress [12]. Though preliminary, these results apparently establish a correlation between the reported capability of these osmolytes to protect the organism from salinity stress with their ability to negate the salt effect on DNA stability.

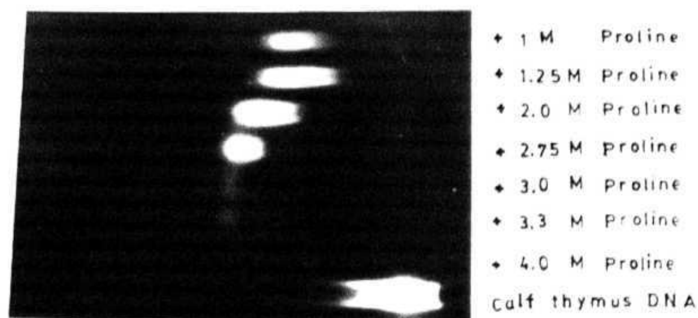

Fig. 4. S1 nuclease sensitivity of calf thymus DNA in the presence of different concentrations of proline.

However, the destabilizing effect shown by hydroxy proline is biologically insignificant as it is not known to accumulate in cells under the stress-adapted conditions. Proline, on the contrary, is a widely reported osmoprotectant, known to stabilize proteins somewhat analogous to chaperones [13] and act as a protein compatible hydrotrope [26]. Further, the antagonistic effect of proline to that of NaCl on DNA stability in vitro possibly suggests a similar interaction in vivo where proline could counteract the effect of high concentration of salt and cations accumulating under stress conditions. Presumably, DNA surrounded by a high concentration of salts is biologically less active than that is surrounded by both salts and their counteracting osmolytes such as proline and betaine. Moreover, proline and betaine were shown to have an additive effect on DNA stability (Table 1), and when present together could account for effective concentrations in vivo. Apart from the suggested effect on DNA, these osmolytes are known to be highly bio-compatible with a proven role in the stabilization of proteins, organelles and cells [13–19] which can not be ascertained with other amino acids. Interestingly, upon increase in salinity of the growth medium, *Lactobacillus plantarum* cells were found to instantaneously accumulate betaine and proline in preference to alanine as an adaptive measure [27]. Similarly, of the 150 compounds tested, only proline and betaine series were found to effectively protect *E. coli* from the severe salinity stress suggesting the versatility of these osmolytes in comparison to other solutes [12].

DNA destabilization by proline in our study was further confirmed by the observed resistance of DNA to DNase I in the presence of high concentrations of proline. In fact, it is known that the activity of this enzyme on a stable double helix is 5000 times higher than that on a destabilized helix [28]. Further, this could not be due to structural changes in the enzyme induced by proline as there are evidences that proline, even at high concentrations, does not substantially affect the structure and function of proteins [13–18]. On the other hand, proline was found to confer structural stability to DNase I at higher temperatures (data not shown). Increased resistance to DNase I digestion and susceptibility to S1 nuclease in the presence of increasing proline concentrations suggest that the destabilized DNA structures could exist at physiological temperatures under stress adapted conditions.

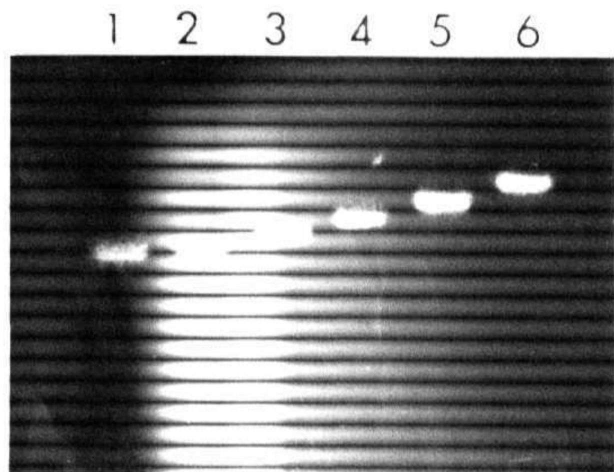

Fig. 5. Gel mobi[illegible] phage DNA in the absence and presence of 3.0 M [illegible] increasing concentrations of ssb protein. Lane 1, λ pha[illegible] Lane 2, λ phage DNA+12 μg of ssbp. Lanes 3–6, [illegible] in the presence of 3.0 M proline with increasing co[illegible] follows: 3, 3 μg of ssbp; 4, 6 μg of ssbp; [illegible] of ssbp.

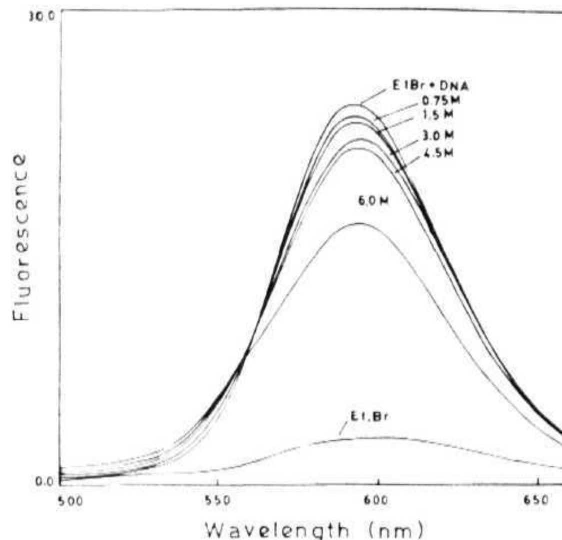

Fig. 6. Fluorescence emission spectra of ethidium bromide in f[illegible] and DNA-bound form: effect of proline in displacing the DN[illegible] bound ethidium bromide.

Several studies indicate that both the in vitro binding affi[illegible]ities and rate of binding of certain transcriptional regulato[illegible] proteins to their target sites on DNA are extremely sensiti[illegible] to the electrolyte concentrations of the buffers used [29]. Sin[illegible] DNA at physiological pH exists as a highly charged anion, [illegible] is expected to be surrounded by cations which have a natur[illegible] binding affinity. Moreover, the salts which accumulate durin[illegible] salinity stress may also unduly stabilize the double helix whi[illegible] could adversely inhibit the DNA function in replication an[illegible] transcription [3]. Presumably, proline and betaine play a[illegible] important role in partially alleviating such an effect. In fa[illegible] *E. coli* cells grown at very high salinity conditions (1 M NaC[illegible] were found to actively concentrate glycine betaine as much [illegible] 10^{5} times that of the medium [12]. It was further envisage[illegible] that during severe stress conditions in bacteria, cellular co[illegible]stituents may completely be bathed in osmoprotectants th[illegible] reach concentrations above 1 M and interact with biomacr[illegible]molecules [12]. Similarly, the presence of high internal conce[illegible]trations of betaine under the stress-adapted conditions wa[illegible] found to reverse the effects of salinity mediated osmotic stre[illegible] on DNA replication and cell division in *E. coli* which suppo[illegible] the role of osmoprotectants in alleviating the stress effects o[illegible] DNA function [30]. Thus, the selective accumulation of the[illegible] two osmolytes in a wide range of organisms under the sa[illegible] stress appears to be a conserved adaptive measure rather tha[illegible] a mere coincidence. While such an adaptive value of betai[illegible] proline-DNA interactions can be envisaged in prokaryot[illegible] where a direct access for osmoprotectants to DNA exi[illegible] the same can not yet be ascertained with respect to eukaryo[illegible] with a distinct nuclear membrane barrier. However, such [illegible]teractions could logically be possible during certain stages [illegible] cell division where the nuclear membrane barrier transie[illegible] disappears. Though a direct interaction in vivo of proline [illegible] betaine with DNA is yet to be established, these osmolytes [illegible] the likely biological choices to counteract the effect of ac[illegible]mulated salts on DNA.

Acknowledgements: This work was supported by The Rockefeller Foundation as a part of International Rice Biotechnology Program to A.R.R. C.R.K. acknowledges Council of Scientific and Industrial Research, India for Fellowship.

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