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

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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.

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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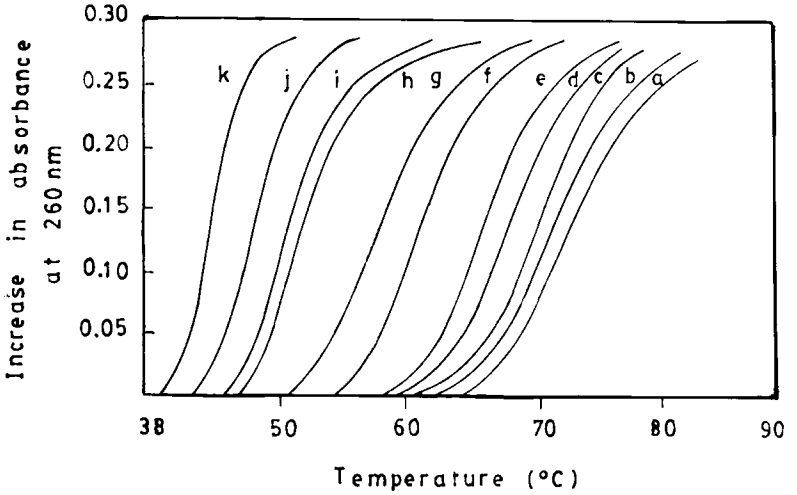

Fig. 1. Effect of increasing concentrations of proline on the T_m of calf thymus DNA: (a) Control DNA (without proline), (b) 0.06 M, (c) 0.25 M, (d) 0.5 M, (e) 1.0 M, (f) 2.0 M, (g) 3.0 M, (h) 4.0 M, (i) 4.5 M, (j) 5.0, (k) 5.5 M.

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 an

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	60.0
+2.0 M glycine	76.0
+2.0 M serine	79.0
+1.0 M alanine	72.0
+0.25 M valine	71.0
+0.1 M leucine	71.0
+2.0 M hydroxy proline	63.0
+0.5 M glycyl glycine	82.0
+2.0 M sarcosine	72.0
+1.0 M glucose	71.0
+1.0 M betaine	67.0
+1.0 M proline+1.0 M betaine	63.0
+10 mM spermidine	93.0
+0.5 M NaCl	96.0
+10 mM spermidine+1.0 M proline	85.0
+0.5 M NaCl+1.0 M proline	90.0
+0.5 M NaCl+2.0 M proline	86.0
+0.5 M NaCl+2.0 M glycine	94.0

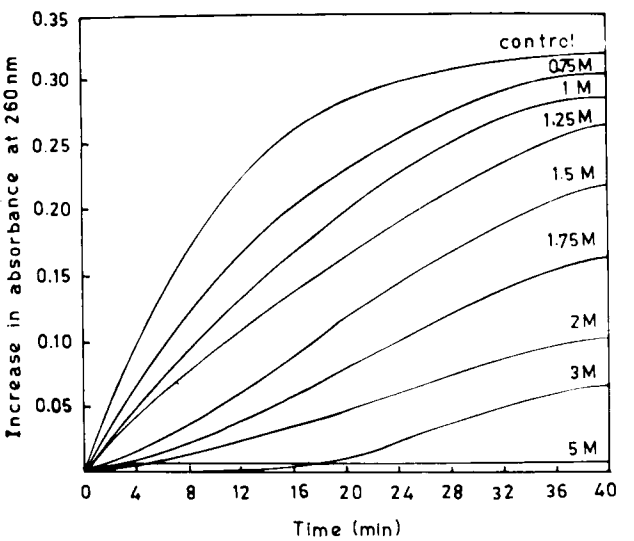

Fig. 2. DNase I sensitivity of calf thymus DNA in the presence of increasing concentrations of proline.

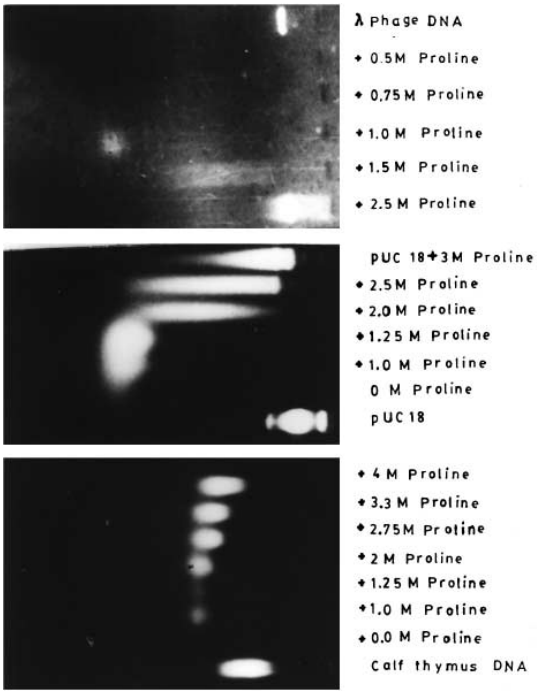

Fig. 3. DNase I sensitivity of λ phage, pUC 18 and calf thymus DNA in the presence of different concentrations of proline.

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.

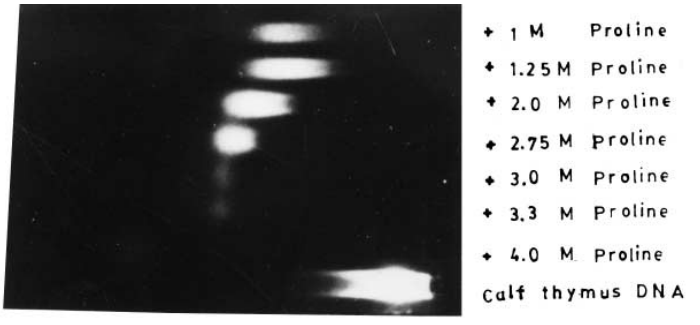

Fig. 4. S1 nuclease sensitivity of calf thymus DNA in the presence of different concentrations of proline.

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.

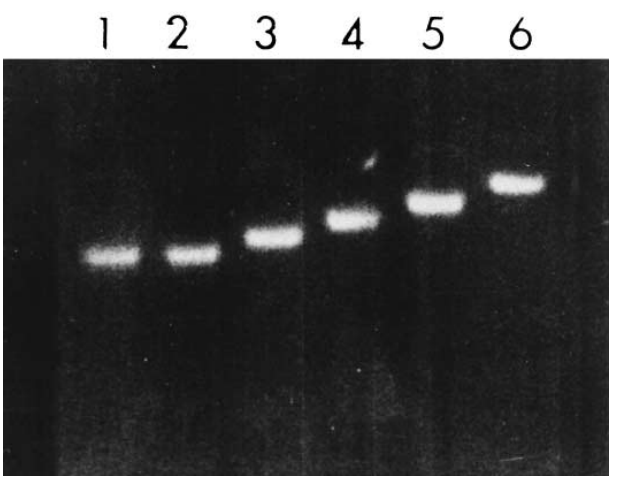

Fig. 5. Gel mobility shift assay of λ phage DNA in the absence and presence of 3.0 M proline with increasing concentrations of ssb protein. Lane 1, λ phage DNA alone. Lane 2, λ phage DNA+12 μg of ssbp. Lanes 3–6, λ phage DNA in the presence of 3.0 M proline with increasing concentrations of ssbp as follows: 3, 3 μg of ssbp; 4, 6 μg of ssbp; 5, 9 μg of ssbp; 6, 12 μg of ssbp.

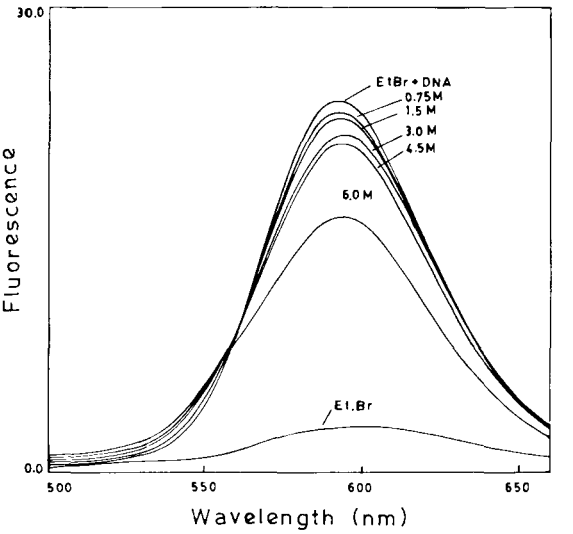

Fig. 6. Fluorescence emission spectra of ethidium bromide in free and DNA-bound form: effect of proline in displacing the DNA-bound ethidium bromide.

Several studies indicate that both the in vitro binding affinities and rate of binding of certain transcriptional regulatory proteins to their target sites on DNA are extremely sensitive to the electrolyte concentrations of the buffers used [29]. Since DNA at physiological pH exists as a highly charged anion, it is expected to be surrounded by cations which have a natural binding affinity. Moreover, the salts which accumulate during salinity stress may also unduly stabilize the double helix which could adversely inhibit the DNA function in replication and transcription [3]. Presumably, proline and betaine play an important role in partially alleviating such an effect. In fact, *E. coli* cells grown at very high salinity conditions (1 M NaCl) were found to actively concentrate glycine betaine as much as 10^5 times that of the medium [12]. It was further envisaged that during severe stress conditions in bacteria, cellular constituents may completely be bathed in osmoprotectants that reach concentrations above 1 M and interact with biomacromolecules [12]. Similarly, the presence of high internal concentrations of betaine under the stress-adapted conditions was found to reverse the effects of salinity mediated osmotic stress on DNA replication and cell division in *E. coli* which supports the role of osmoprotectants in alleviating the stress effects on DNA function [30]. Thus, the selective accumulation of these two osmolytes in a wide range of organisms under the salt stress appears to be a conserved adaptive measure rather than a mere coincidence. While such an adaptive value of betaine/proline-DNA interactions can be envisaged in prokaryotes where a direct access for osmoprotectants to DNA exists, the same can not yet be ascertained with respect to eukaryotes with a distinct nuclear membrane barrier. However, such interactions could logically be possible during certain stages of cell division where the nuclear membrane barrier transiently disappears. Though a direct interaction in vivo of proline or betaine with DNA is yet to be established, these osmolytes are the likely biological choices to counteract the effect of accumulated salts on DNA.

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