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INTERACTIONS OF SOME NATURALLY OCCURRING CATIONS WITH PHENYLALANINE AND INITIATOR tRNA FROM YEAST AS REFLECTED BY THEIR THERMAL STABILITY

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The thermal unfolding of phenylalanine and initiator tRNA from yeast was investigated over a broad range of solution conditions by differential ultraviolet absorption at 260 nm. Under most conditions, the initiator tRNA exhibits two clearly separated transitions in its differential melting curve which were assigned to unfolding of tertiary and secondary structure elements, respectively. The tertiary transition of this tRNA and the overall transition observed for $tRNA^{Phe}$ do not show a maximum in a curve of T_m values plotted as a function of $[Na^+]$. Such a maximum is usually observed for other nucleic acids at about 1 M Na^+. In the presence of 5 mM of the divalent cation Mg^{2+} (or Ca^{2+}), an overall destabilization of the tRNAs is observed when increasing the sodium concentration. The largest fall in T_m ($\approx 15°C$) is observed for the tertiary transition of the initiator tRNA. Among various cations tested the following efficiency in the overall stabilization of $tRNA^{Phe}$ is observed: spermine > spermidine > putrescine > Na^+ ($\approx NH_4^+$). Mg^{2+} is most efficient at concentrations above 5 mM, but below this concentration spermine and spermidine appear to be more efficient. The same hierarchy in stabilizing power of the polyamines and Na^+ is observed for both transitions of the initiator tRNA. However, when compared with Mg^{2+}, the polyamines are far less capable of stabilizing the tertiary structure. In contrast, spermine and spermidine are slightly better than Mg^{2+} in stabilizing the secondary structure. At increasing concentrations of the polyvalent cations (at fixed $[Na^+]$) the T_m values of the tRNAs attain a constant value.

1. Introduction

Various biological processes, in which tRNA molecules participate, can be activated or repressed in vitro by altering the cation composition of the solution. A well known example is the aminoacylation of tRNA; it has been found that the yield and/or rate of transfer of the amino acid from the synthetase aminoacyl adenylate complex to the tRNA is sensitive to changes in the cationic composition of the buffers used [1–3]. Of relevance from a biological point of view is the observation that the (in vitro detected) error frequencies connected with aminoacylation and with incorporation of amino acids into polypeptides can be reduced (to levels found in vivo) by addition to the assay buffers of carefully balanced mixtures of naturally occurring cations [4,5].

The effects of cations on tRNA functioning may be mediated by interaction of these ions with the tRNA itself or by interaction with its partner proteins. Because of the polyanionic nature of tRNA molecules and because it is known that cations affect the conformation and dynamics of tRNA [6], including its solvent and counterion shell [7,8], one might anticipate that a particular, cation-dependent, molecular shape and flexibility is decisive for the proper functioning of tRNA at various stages of protein synthesis. However, in

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most cases it has not been possible to deduce a clear correlation between cation content of solutions, tRNA structure and biological activity. The situation is rendered even more complex by the fact that the influence of cations on such activities is often found to be rather diverse with respect to the tRNA species and the kind of the cation examined. This is typically so for the aminoacylation of tRNAs by their cognate synthetase [1–3].

In studies on the nature of cation interaction with tRNA most efforts have been devoted to investigations of the mode of binding of divalent ions like Mg^{2+}, a cation recognized for its importance in many cellular processes [9–11]. tRNA molecules have long been thought to contain a limited number of high-affinity sites for divalent metal ions, but it is now realized that the apparent strong binding observed in solution studies can be accounted for by electrostatic effects and that the binding affinity for these ions is probably not very different among the various available sites on the tRNA [12–14].

Recent studies on yeast $tRNA^{Phe}$ indicate that the overall structure of this molecule is not very sensitive to the absence or presence of Mg^{2+} [7,15]. Nevertheless, subtle but unique changes are observed for its structure and conformational stability as a result of Mg^{2+} binding and these may well be of importance for the biological functioning of the molecule [6,15].

In contrast to the knowledge acquired on Mg^{2+} binding, far less is known about the effect of other important naturally occurring cations, such as Ca^{2+}, spermine, spermidine and putrescine, on the structure and dynamics of tRNA. Also, little attention has been paid to the simultaneous effects of mono-, di- and polyvalent cation interaction with tRNA, a situation which is expected to occur at physiological conditions.

In the present paper we address these two aspects of cation interactions insofar as these are reflected in the thermal stability of tRNA which, in this study, is followed by recording ultraviolet differential melting curves. Because we also wish to improve our understanding of the nature of cation interaction, the ions were tested at concentrations well beyond the physiological range. Two species of tRNA were studied: $tRNA^{Phe}$ and the initiator tRNA from yeast. The latter tRNA is shown to exhibit two clearly separated transitions in its melting curve over a wide variety of cation mixtures, in contrast to $tRNA^{Phe}$ which shows only one transition under most buffer conditions. This allowed us to draw more specific conclusions with regard to the effects of cations on the stability of the initiator tRNA.

2. Materials and methods

Unfractionated baker's yeast tRNA was prepared by the method of Holley [16]. Starting with approx. 3 g of this material the initiator and phenylalanine tRNA were purified by sequential chromatography: first on BD-cellulose [17] and then on DEAE Sephacel (instead of the more commonly employed DEAE-Sephadex A50 [18]). Final purification of the initiator tRNA was obtained on a 2.7 × 90 cm Sepharose 4B column [19] developed with a 2 × 1.5 l linear gradient between 1.5 and 1.2 M ammonium sulfate in 10 mM sodium acetate and 10 mM $MgCl_2$, pH 4.5.

The purity and integrity of the tRNAs were established by electrophoresis using a 12% polyacrylamide gel in 7 M urea buffer and by ^{31}P-NMR from which it is possible to detect cuts in the polynucleotide chain, which are monitored by resonances of cyclic phosphate groups [20]. From these analyses it was concluded that the tRNAs were pure and at least 90% intact. Employing a crude yeast aminoacyl synthetase preparation, the amino acid acceptance of the tRNAs was found to be between 1250 and 1500 pmol/A_{260} following an acceptor assay essentially according to ref. 19. For ^3H-labelled amino acids used in this assay, the application of glass fiber filters (GF/A from Whatmann) and a solubilizer (Solulyte from J.T. Baker) in the scintillation liquid appeared to be necessary to avoid severe overestimations of charging figures. Corrections were made for degradation of the radioactive amino acids, which was estimated by thin-layer chromatography. 90% of the initiator tRNA could be formylated by a crude *Escherichia coli* transformylase preparation (with respect to the fraction of this tRNA that was aminoacylated). tRNA samples obtained by the

isolation procedure described above were completely free of polyamine contaminations as observed by ^{1}H-NMR; polyamines, which are deliberately added, can be removed by two ethanol precipitations with 0.8 M NaCl in a buffer at pH 7.0.

tRNA samples free of divalent cations (such as Mg^{2+}) were prepared by the following procedure. 3–5 mg tRNA were dissolved in 300 μl of a buffer containing 10 mM sodium cacodylate, 25 mM EDTA and 500 mM NaCl, pH 7.3, and extensively dialysed against the same buffer in a microdialysis cell. The dialysis was repeated several times with successive smaller concentrations of EDTA and NaCl. The final dialysis buffer contained 1 mM sodium cacodylate and 0.5 mM EDTA, pH 7.0. All glassware used was acid-washed to avoid contamination with polyvalent ions; also for this reason suprapur NaCl (Merck, Darmstadt) and doubly distilled water were used. From atomic absorption measurements it followed that after application of this procedure the Mg^{2+} content of the sample was negligibly small.

The dialysed tRNA solution was divided into portions of 1 *A* unit and lyophilized. For each thermal denaturation experiment such a portion was dissolved in 1 ml buffer containing 5–30 mM sodium cacodylate (pH 7.0), 0.1 mM EDTA and other ions as indicated in the figure legends, after which it was transferred to a 1 cm quartz cuvette. The 'polymix' buffer [5], used in a few experiments, contained 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 30 mM sodium cacodylate, 95 mM KCl, 5 mM NH_4Cl, 1 mM DTE, 0.1 mM EDTA, 8 mM putrescine and 1 mM spermidine at pH 7.6.

Melting curves were recorded on a Cary 118 C spectrophotometer interfaced to a PDP 11/23-computer as described in ref. 21 at a wavelength of 260 nm. The samples were heated at a rate of approx. 1°C/min. For both tRNAs investigated the total change in absorbance upon complete denaturation was found to be between 25 and 40% depending on buffer conditions. Differential melting curves were obtained by fitting overlapping segments of the melting curves with a third degree polynomial and calculating the derivative of the polynomial in the center of the segment. Transition midpoints (melting temperature: T_m) were directly obtained from the differential curves. These values were found to be reproducible to within 0.5°C. Experiments performed with the initiator tRNA dissolved in a solution with more than 1.0 M Na^+ only resulted in reproducible T_m values if the tRNA was kept for 12 h at room temperature or for 10 min at 50°C before starting the melting experiments. The incubation did not effect T_m values obtained from tRNA with less than 1.0 M Na^+ in solution.

Spermine added to a tRNA solution, containing 5 mM Mg^{2+} and 15 mM Na^+, resulted in precipitation of the nucleic acid. Under all other solution conditions employed, polyamines could be added up to a concentration of 50 mM without such precipitation (spermidine and putrescine even up to 150 mM).

3. Results

3.1. Effects of monovalent cations on the thermal denaturation of tRNA in the absence of Mg^{2+}

Differential melting curves of the initiator tRNA from yeast reveal that the melting proceeds in two steps. The first step comprises 30–40% and the second 60–70% of the total change in absorbance at 260 nm. In fig. 1 differential melting curves are shown in the presence of monovalent cations with different ionic radii. Urbanke et al. [22] have demonstrated that melting of a tertiary structure is much more sensitive to the ionic radius of monovalent cations than melting of a secondary structure. As can be seen in fig. 1 the first melting transition is clearly the most sensitive to the ionic radius of the monovalent cations and we therefore assume that this transition reflects the disruption of the tertiary structure of the initiator tRNA. This also seems the most straightforward interpretation when considering the high content of GC base-pairs in the secondary structure [23] and the large difference in T_m between both transitions (more than 25°C below 1 M NaCl).

Semilogarithmic plots of the T_m values of both transitions as a function of NaCl concentration are given in fig. 2. For comparison, T_m values obtained from melting profiles of yeast $tRNA^{Phe}$

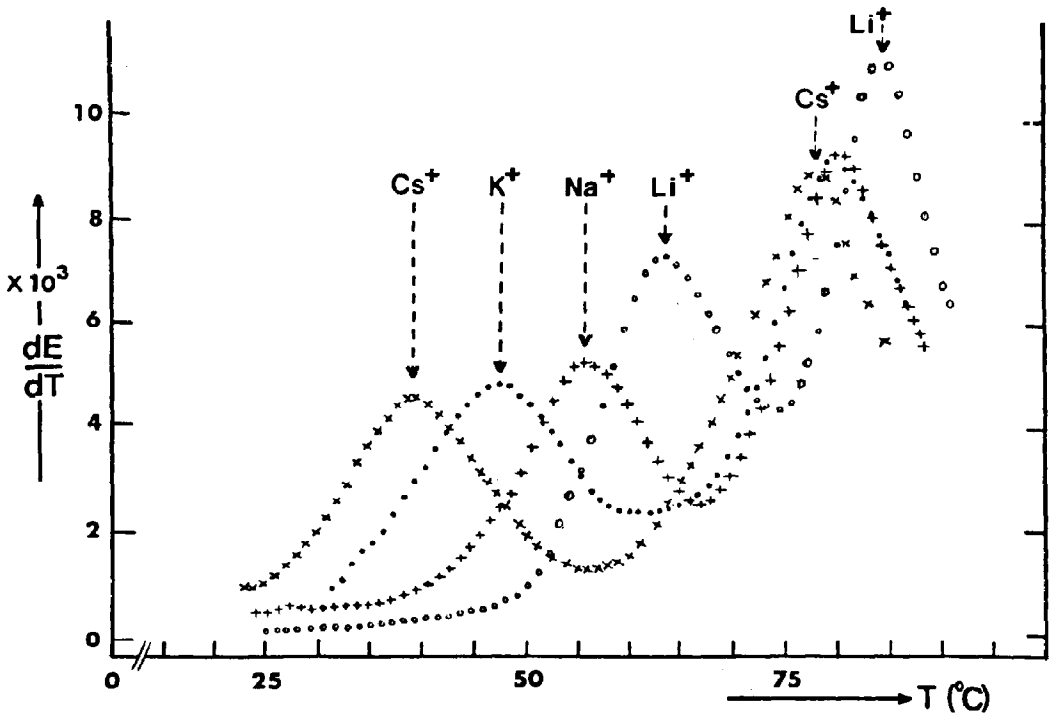

Fig. 1. Ultraviolet differential melting profiles for the initiator tRNA of yeast in the presence of 1 M CsCl (×), 1 M KCl (●), 1 M NaCl (+) or 1 M LiCl (○). E, absorbance at 260 nm. The cations employed in each experiment are indicated. For the highest temperature transition only those obtained in the presence of Cs^+ and Li^+ are marked.

(which exhibits only one melting transition at NaCl concentrations exceeding about 0.1 M [24]) are also included. Up to 4 M NaCl the T_m values of the latter transition and of the tertiary transition of the initiator tRNA are virtually a linear function of log[Na^+], while for the secondary transition of the initiator tRNA this is only so up to 1 M NaCl. The T_m values of the tertiary transition depend most strongly on the ionic strength ($dT_m/d\log[Na^+] = 24$), as has also been found for other tRNAs [24].

3.2. Effect of monovalent cations in the presence of Mg^{2+} or Ca^{2+}

T_m values of melting transitions of $tRNA^{Phe}$ and the initiator tRNA from yeast for the case in which NaCl concentration is increased in the presence of a fixed level of Mg^{2+} (5 mM) are shown in fig. 3. The importance of Mg^{2+}, as compared to NaCl, for the stability of tRNA is clearly demonstrated. When only 5 mM Mg^{2+} and 5 mM Na^+ are present in solution, the T_m values of all three transitions are higher than at any other Na^+ concentration up to 4 M. When the concentration of Na^+ is raised, in the presence of 5 mM Mg^{2+}, a destabilization of the tRNA structure is observed. The T_m values of the transitions decrease to a minimum and then increase at still higher Na^+ concentrations. The destabilization is most marked for the tertiary transition of the initiator tRNA; its T_m drops by about 15°C when the Na^+ concentration is raised to 0.75 M. For the secondary transition T_m only decreases about 5°C and the minimum of the T_m curve is found at a lower Na^+ concentration (0.4 M). For the melting transition of $tRNA^{Phe}$ an intermediate situation is observed.

For comparison, in fig. 3 the T_m values mea-

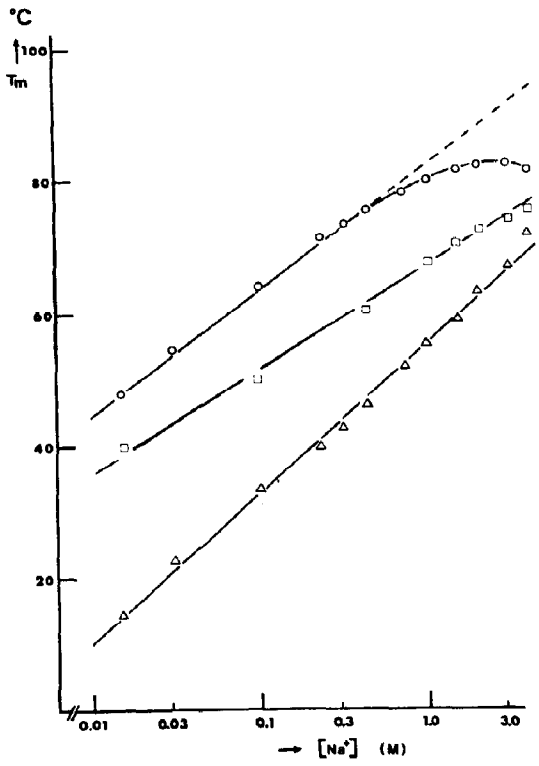

Fig. 2. Semi-logarithmic plots of T_m values of transitions in the melting curves of phenylalanine and initiator tRNA of yeast (recorded at 260 nm) as a function of Na^+ concentration. T_m values: secondary transition of the initiator tRNA (○); its tertiary transition (△); transition of $tRNA^{Phe}$ (□). At Na^+ concentrations of less than about 130 mM the latter two melting conversions show multiphasic behavior. The T_m values indicated refer to the main part of each of these two transitions.

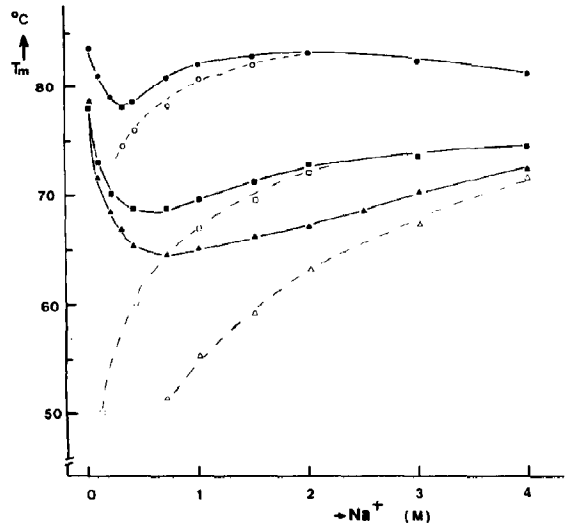

Fig. 3. T_m values of transitions in the melting curves of phenylalanine and initiator tRNA of yeast as a function of Na^+ concentration in the presence of 5 mM Mg^{2+} (filled symbols) and absence of Mg^{2+} (open symbols). Transitions are labeled as in fig. 2.

sured as a function of Na^+ concentration in the absence of Mg^{2+} are also included. Between 1.5 and 2 M NaCl, the T_m curves for the melting transition of $tRNA^{Phe}$ and the secondary transition of the initiator tRNA start to coincide with the T_m curves obtained in the presence of 5 mM Mg^{2+}. For the tertiary transition of the initiator tRNA this only happens at about 4 M NaCl, indicating that Mg^{2+} binds to tRNA even at this high concentration of NaCl (see section 4). The antagonistic behavior of Mg^{2+} and Na^+, as described here, has previously been observed for the T_m of DNA [25,26].

In addition, another divalent metal ion, known to be present under physiological conditions, was investigated, viz. Ca^{2+}. At a constant concentration of 5 mM Ca^{2+}, T_m values were measured as a function of Na^+ concentration and the results obtained were similar to those presented in fig. 3 for Mg^{2+}. Compared to the curves obtained for Mg^{2+} only, at low ionic strength (< 0.4 M NaCl) a small decrease (2–5°C) was recorded for the T_m values of the secondary transition of the initiator tRNA (data not shown) and the transition of $tRNA^{Phe}$ [15].

3.3. *Effect of polyvalent cations*

We have also investigated how the stability of tRNAs is affected by the important polyamines spermine, spermidine and putrescine and compared the results with the stabilizing effect of Mg^{2+}. At pH 7, polyamines have positively charged amino groups and electrostatic forces have been shown to provide an important contribution

to their interaction with nucleic acids [27,28]. As expected for such interactions, the ability of polyamines to stabilize tRNA depends strongly on the number of (charged) amino groups. This is demonstrated in fig. 4, which shows T_m values for $tRNA^{Phe}$ as a function of polyamine concentration in the absence of Mg^{2+} (at 125 mM Na^+ and pH 7). The concentrations of polyamines in fig. 4 are expressed in equivalences of positive charge, emphasizing the effect per amino group or positive charge. From this figure we observe that a T_m elevation of 14°C is reached at a positive charge concentration of 0.5 mM spermine, while 2.7 mM spermidine and 100 mM putrescine are needed to obtain this T_m increase. More than 300 mM NH_4^+ is needed for the same T_m increase (data not shown).

Fig. 4 also displays a plot of T_m as a function of the Mg^{2+} concentration, obtained under the same conditions as for the polyamines. The rise of the curves is steeper for spermine and spermidine than that for Mg^{2+} and far more so than for putrescine (see also inset to fig. 4) which might be attributed to a higher affinity of these two polyamines for

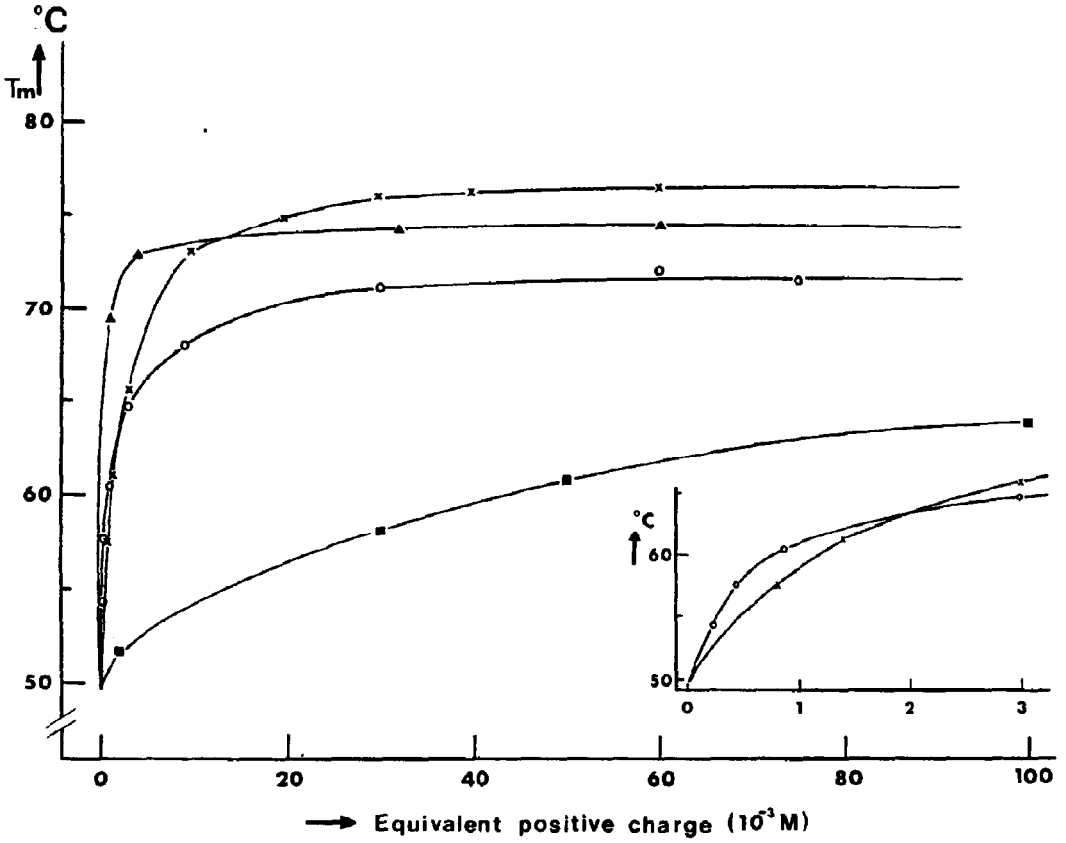

Fig. 4. T_m values of the melting transitions of $tRNA^{Phe}$ from yeast as a function of the concentration of polyamine or Mg^{2+}. All samples contained 95 mM NaCl and 30 mM sodium cacodylate at pH 7. Concentrations are expressed in equivalences of positive charge: i.e., putrescine is assumed to have 2, spermidine 3 and spermine 4 positive charges at this pH. In the case of the Mg^{2+} titration a correction was made for the small amount of EDTA (0.1 mM) also present in solution, assuming that 1 mol EDTA binds 1 mol Mg^{2+}. (■) Putrescine, (○) spermidine, (▲) spermine, (×) Mg^{2+}. Inset: expanded part of the T_m curves as a function of Mg^{2+} and spermidine.

tRNA [29]. With the polyamines spermine and spermidine as well as with Mg^{2+} T_m appears to become constant after sufficient ions have been added. For Mg^{2+} the plateau value is 76.5°C, for spermine 74°C and for spermidine 71°C. For spermidine the value of 71°C remained constant up to 150 mM (450 mM in positive charge), the highest concentration of this polyamine studied. Apparently, Mg^{2+} at sufficiently high concentration is most efficient in the overall stabilization of $tRNA^{Phe}$.

It should be noted that the experiments were performed at pH 7. The pK values of polyamines decrease with increasing temperature. In particular, for spermine, which has one amino group with a pK value of 8 at 30°C and about 7 at 70°C [30], the choice of pH 7 in these experiments might affect the plateau value. However, this does not appear to be the case. Control experiments with spermine (or Mg^{2+}) did not show any effect on the T_m of the plateau between pH 6.5 and 7.6 (within the error of measurement).

For spermidine we also obtained T_m values in the presence of Mg^{2+} and 125 mM Na^+. It is observed (see fig. 5) that the higher the Mg^{2+} content, the less is the effect exerted on the T_m by spermidine. When the tRNA is nearly saturated with Mg^{2+} (upper curve in fig. 5) the addition of

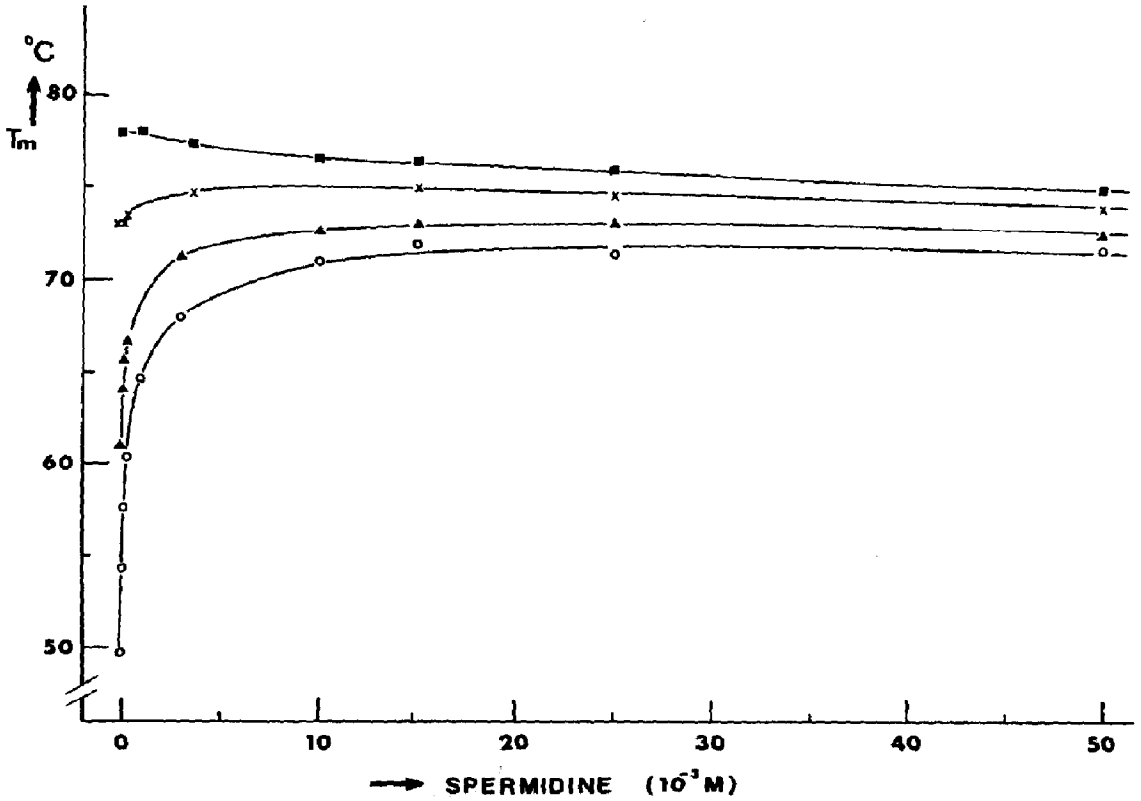

Fig. 5. T_m values of the melting transition of $tRNA^{Phe}$ from yeast as a function of spermidine and Mg^{2+} concentration. (○) No $MgCl_2$, (▲) 1 mM $MgCl_2$, (×) 5 mM $MgCl_2$. All these T_m values were obtained from samples with 95 mM NaCl and 30 mm sodium cacodylate (pH 7) in solution. (■) T_m values obtained from samples with 15 mM sodium cacodylate (pH 7) and 5 mM $MgCl_2$.

spermidine may even cause a destabilization. The T_m drops 3°C, with respect to the situation without polyamine, when 50 mM spermidine is added. Furthermore, T_m values were obtained for $tRNA^{Phe}$ dissolved in the polymix buffer [5] (see section 2) without and with polyamines (8 mM putrescine + 1 mM spermidine). The T_m values recorded were 73 and 73.5°C, respectively.

Finally, the effect of polyamines on the stability of the initiator tRNA was investigated. Fig. 6 shows T_m values of this tRNA as a function of polycation concentration. From the shifts of the T_m values of both transitions we find the same order in stabilizing power among the polyamines as found for $tRNA^{Phe}$. Also, plateau values in the T_m curves are observed for spermine and spermidine.

A priori, it is not clear whether Mg^{2+} and polyamines have the same hiearchy of stabilizing power for the secondary and tertiary structure, respectively, in the initiator tRNA from yeast. Therefore, we performed the experiment depicted in fig. 7 : Fig. 7A shows the differential melting curve of the initiator tRNA in the presence of 125 mM Na^+. The melting temperatures are 30 and 65°C for the tertiary and secondary transitions, respectively. Fig. 7B depicts the differential melting curve in the presence of 125 mM Na^+ plus 5 mM spermine. It can be seen that the addition of 5 mM spermine increases the melting temperature for the tertiary transition by 30°C and that of the secondary transition by 22°C. However, as shown in fig. 7C, addition of 5 mM $MgCl_2$ instead of 5 mM spermidine results in an increase of 43°C in the melting temperature of the tertiary transition (so, 13°C more as compared with 5 mM spermine) and only 15°C for the secondary transition (i.e., 7°C less as compared with 5 mM spermine). When more polyamine or Mg^{2+} is added to the sample

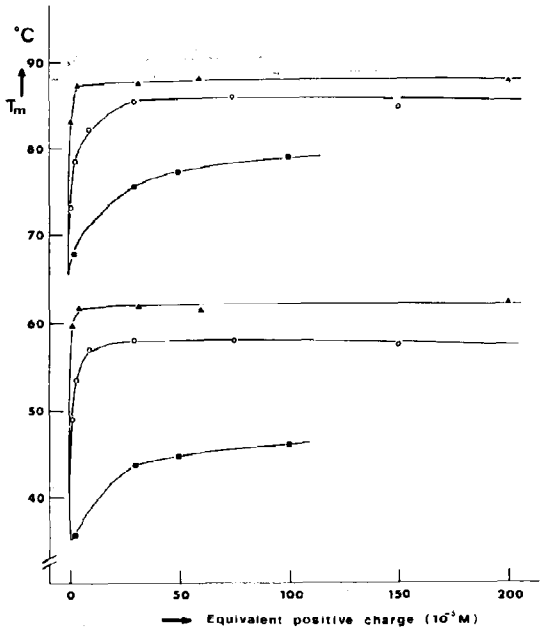

Fig. 6. T_m values of the two melting transitions of the initiator tRNA of yeast as a function of the polyamine concentration, expressed in equivalences of positive charge (see fig. 4). All samples contained 95 mM NaCl, 30 mM sodium cacodylate (pH 7). (■) Putrescine, (○) spermidine, (▲) spermine. Lower three curves, tertiary transition; upper three curves, secondary transition.

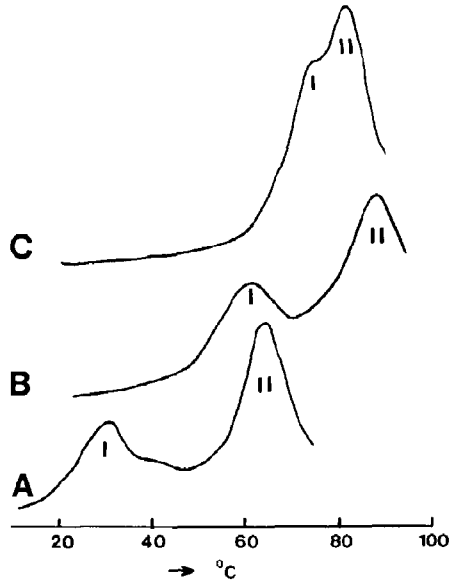

Fig. 7. Comparison of differential melting curves of the initiator tRNA of yeast (recorded at 260 nm) under different solution conditions. (A) In the presence of 95 mM NaCl and 30 mM sodium cacodylate (pH 7). (B) As in panel A but with 5 mM spermine. (C) As in panel A but with 5 mM $MgCl_2$. The low- and high-temperature transitions are indicated by I and II, respectively.

the plateau values can be obtained. For spermidine these are 58°C for the first and 85°C for the second transition, and for spermine we find 62 and 88°C, respectively. For Mg^{2+} these values are 79 and 84°C, respectively. We conclude that polyamines, especially spermine, are more effective than Mg^{2+} in stabilizing the secondary structure. In contrast, the tertiary structure is much more sensitive to Mg^{2+} as compared with polyamines.

4. Discussion

Previous melting studies were aimed mainly at a deeper understanding of the sequential melting of tRNAs. In the present paper the melting properties were studied for concentration ranges of different counterions significantly extended with respect to the earlier studies to obtain more information on the interaction between cations and tRNA. This led to the observation of some interesting effects which had not been previously demonstrated. It turns out that (a) upon increasing Na^+ concentrations at a constant Mg^{2+} level the stability of tRNA may go through a minimum, (b) at increasing concentrations of Mg^{2+} or the polyamines ($[Na^+]$ constant) the T_m values of tRNA reach a constant value. A physical explanation for these observations will be given below; the underlying theory will be presented in a separate paper.

4.1. Melting of the initiator tRNA from yeast

The differential melting curves of the initiator tRNA from yeast are presented here for the first time. In these melting curves, obtained at 260 nm, two transitions are discernible. The lowest temperature transition reflects unfolding of the tertiary structure and the highest temperature transition unfolding of the secondary structure. Biphasic melting curves have also been observed for the initiator tRNA of *E. coli* [31,32] but this behavior is clearly different from that of the initiator tRNA of yeast. While two transitions are observable in the melting curve of the latter tRNA over a broad range of solution conditions (even when the molecule is nearly saturated with Mg^{2+}), biphasic melting for the initiator tRNA of *E. coli* is only observed under particular ionic conditions and cooperative unfolding of the molecule is seen when 3 mM Mg^{2+} is present in solution. This variance in melting behavior of these tRNAs may be due to differences in the composition of the secondary structure (e.g., an AU base-pair in the D stem and a GU base-pair in the T stem of the *E. coli* tRNA at positions where the yeast tRNA has a GC and AU base-pair, respectively) and/or the tertiary structure (e.g., instead of the purine-pyrimidine AT base-pair in the TψC loop the yeast initiator tRNA has a purine-purine interaction).

4.2. Influence of the concentration of Na^+ in the absence of Mg^{2+}

The stabilizing effect of NaCl (in the absence of Mg^{2+}) on helical structures of nucleic acids is well known [33]. The main reason is that Na^+ interacts more strongly with helix forms than with coil forms. Hence, upon melting there is a net release of Na^+. Theoretically, it has been shown that the combination of the change in electrostatic free energy, accompanying the melting, and the net release of Na^+ results in a linear relation between T_m and $\log[Na^+]$ [26,34]. Thus far, melting experiments performed on DNA and RNA helices and hairpins have revealed that this linear relation holds up to about 0.1 M NaCl. At higher NaCl concentrations the T_m levels off and decreases above 1 M NaCl [22,35]. This is also observed for the melting of the secondary structure of the initiator tRNA although levelling off occurs at a somewhat higher Na^+ concentration ($\approx$ 1 M). The deviation from linearity at higher salt concentrations has been attributed to anion interaction and/or changes in solvent interaction with the nucleic acid [35]. Unexpectedly, this nonlinearity is not seen for the melting of $tRNA^{Phe}$ nor for the tertiary structure of the initiator tRNA of yeast for which the theory thus seems to be valid up to high NaCl concentrations (4 M NaCl).

4.3. Variation of the concentration of Na^+ in the presence of Mg^{2+} shows an antagonistic relation

At first glance, it is expected that Mg^{2+} would behave similarly to Na^+ in its interaction with

nucleic acids. However, it has been shown that the interaction of Mg^{2+}, in contrast to that of Na^+, follows a mass action law [12,26]. The apparent dissociation constant appears to be strongly dependent upon the ionic strength [12,13]. In a polyelectrolyte (like tRNA) ionic binding sites (as for Mg^{2+}) interact with each other in such a way that binding at any site affects the binding at all other sites because the work required to dissociate a small ion from a macro ion is not only a function of the ionic strength but also of the charge located on the macro ion. Therefore, the apparent dissociation constant is a function of the ionic strength as well as of the charge of the macro ion. For this situation the following expression has been derived for the apparent dissociation constant of the small ion [36]

$$pK_{app} = pK_{int} - 0.868wz_iZ \quad (1)$$

where K_{int} is the intrinsic dissociation constant, i.e., the dissociation constant for the situation in which the charge of the macro ion is zero; w represents the so-called electrostatic interaction factor, which is strongly dependent on the ionic strength and the size and shape of the macromolecule and therefore eq. 1 can only be applied provided the size and shape of the macromolecule do not alter as a result of binding; z_i denotes the charge of the small ion and Z is the total charge of the macro ion, e.g., when Mg^{2+} is bound to the tRNA, it is the charge of the tRNA without bound counterions plus twice the number of bound Mg^{2+}. The principal validity of eq. 1 for the binding of Mg^{2+} and Mn^{2+} to tRNA has been demonstrated [12]. The destabilizing effect that occurs when, in the presence of Mg^{2+}, the concentration of NaCl is raised can then be explained as follows. If we add limited amounts of Mg^{2+} to tRNA at relatively low ionic strength the binding of Mg^{2+} to helix and coil forms is different. Since the helix form binds more Mg^{2+} this form is stabilized and a higher melting point is observed [37]. The higher affinity for the helix form is not caused by a higher intrinsic binding constant, but is merely the result of a difference in the electrostatic interaction factor of the helix and coil forms [12]. When the ionic strength is increased while the concentration of Mg^{2+} is kept constant, the term $-0.868wz_iZ$ in eq. 1 becomes smaller and so the difference in the affinity of Mg^{2+} for helix and coil forms decreases. Therefore, the release of Mg^{2+} on melting is diminished. This effect is not completely compensated for by an increase in the net release of Na^+ and hence a lowering of T_m is observed. Elsewhere we will present detailed theoretical calculations of the melting temperature as a function of the concentrations of Na^+ and Mg^{2+} (Walters et al., unpublished data).

At high ionic strength the electrostatic interaction factor w in eq. 1 approaches zero so that electrostatic interactions are almost completely eliminated and binding of Mg^{2+} is only determined by the intrinsic dissociation constant. Previously, we have reported that the intrinsic dissociation constant for binding of Mg^{2+} to tRNA has a pK value of about 2.5 [12]. This means that binding of Mg^{2+} to tRNA still occurs at very high ionic strength. This prediction is confirmed by experimental observations. For instance, it has been found that the binding of the codon AUG to the initiator tRNA of yeast is affected by millimolar amounts of Mg^{2+} with 1 M NaCl in solution [38]. It has also been observed that the Y base fluorescence of $tRNA^{Phe}$ from barley embryos is affected by the addition of Mg^{2+} to a solution with an NaCl concentration of 2 M [39]. In the present report it is demonstrated that the T_m curves of the transition of $tRNA^{Phe}$ and the secondary structure of the initiator tRNA in the presence and absence of Mg^{2+} only coincide at about 2 M NaCl and for the tertiary structure of the latter tRNA even only at about 4 M NaCl, indicating that Mg^{2+} binding occurs up to this high Na^+ concentration. The experiments on the initiator tRNA show that its tertiary structure is most sensitive to Mg^{2+}.

The effect of a changing concentration of NaCl in the presence of a fixed level of Mg^{2+}, investigated here by differential melting techniques, has also been investigated by laser light and small-angle neutron scattering experiments on $tRNA^{Phe}$ [7,40,41] and by fluorescence of the Y base in $tRNA^{Phe}$ species [39,42]. The laser light scattering experiments show a decrease in the diffusion coefficient of this tRNA, in the presence of 1 mM

Mg^{2+}, when the ionic strength is increased beyond 0.1 M. At first this was interpreted as a major conformational rearrangement of the molecule to a less compact structure [40], however modification experiments subsequently performed at the same ionic strength did not detect such major rearrangement [8], in complete agreement with neutron scattering and NMR measurements which showed no change in the overall structure under comparable conditions [7,15]. It is more likely that the altered diffusion behavior is caused by changes in the shape and size of the counterion shield and layer of hydration [7,8] or by changes in the dynamics of the structure [8]. The fluorescence measurements of the Y base in $tRNA^{Phe}$ [39,42], which probe the structure of the anticodon loop of this tRNA, revealed an antagonistic relation between Mg^{2+} and Na^+ with respect to their effect on this structural element, in a way very similar to that observed in the present study for the effect of these cations on the stability of tRNA. We have been able to confirm such an antagonistic effect on the structure of the anticodon loop of $tRNA^{Phe}$ from yeast by ^1H- and ^{31}P-NMR measurements (unpublished results). All these observations add to the view that the overall structure of $tRNA^{Phe}$ is rather insensitive to alterations in the ionic composition of the solution, but that it may induce (subtle) local changes in the molecule (e.g., the anticodon loop), in its direct solvent environment and in its stability or dynamics.

4.4. Influence of the concentrations of polyamines and Mg^{2+} in the presence of Na^+

Although polyamines are recognized as important candidates for the stabilization of tRNA in vivo [43], only two limited reports have appeared on the (optically detected) thermal unfolding of tRNA in the presence of these polycations [44,45]. Hence, the stabilizing effect of the polyamines spermine, spermidine and putrescine was studied for yeast $tRNA^{Phe}$ and compared with that of Mg^{2+}. At low concentrations of these cations the T_m vs. concentration curve was found to be steeper for spermine and spermidine than for Mg^{2+} which is likely to reflect the higher affinity of spermine and spermidine as compared with Mg^{2+} [29]. Nevertheless, at higher concentrations Mg^{2+} is more efficient in stabilization. In the few reports that have been published, the binding data were analysed without taking into account a gradual change in the apparent binding constant as a result of the change in the electrostatic interaction [28,44]. The fact that at low concentrations of cations the T_m vs. concentration curves were found to be steeper for spermidine and spermine than for Mg^{2+} suggests that at these low concentrations the apparent binding constant is significantly higher for spermidine than for Mg^{2+}. This finding does not mean that the intrinsic binding constant for spermidine and spermine should also be higher than for Mg^{2+}. According to eq. 1 the contribution of the electrostatic interaction is much more important for spermidine and spermine than for Mg^{2+}, since $z_i = 3$ for spermidine and $z_i = 4$ for spermine. The difference in stabilizing power between Mg^{2+} and putrescine suggests that the latter ion has a lower intrinsic binding constant. For putrescine $z_i = 2$, the same as for Mg^{2+}; nevertheless, the T_m vs. concentration curve indicates that putrescine binds much less strongly.

At increasing concentrations of Mg^{2+}, spermidine and spermine the T_m curves of the melting transitions of the tRNAs studied reach distinct plateaus. Such a plateau region can be expected if there is no net release of bound small molecules on melting. Therefore, the simplest explanation for this observation is that helix and coil forms have the same number of binding sites which are nearly all occupied at high enough concentrations of the small molecule ligands. Thus, on melting there is no net release of bound ions. In this situation melting is also not accompanied by electrostatic free energy changes, since the charge of the tRNA molecule approaches zero.

The denaturing experiments performed with the initiator tRNA in the presence of Mg^{2+} and polyamines provide some clue as to the structural elements best stabilized by either cation. Binding of polyamines results in an increase of the T_m of both transitions observed in the differential melting curve of this tRNA but, when compared with Mg^{2+}, spermine and spermidine appear to be slightly better in stabilizing the secondary structure, while Mg^{2+} is clearly much better in stabiliz-

ing the tertiary structure. It has been pointed out by Teeter et al. [11] that the potential cation binding sites in the structure of tRNA can be divided by shape into electronegative pockets and clefts. The clefts are mainly formed by the secondary structure as helical grooves and the pockets by the tertiary structure at sites where the polynucleotide chain makes a sharp turn. A possible mechanistic explanation for our observations then is that elongated cations like polyamines are better suited to bind to clefts than to pockets in contrast to spherical Mg^{2+} which will bind to both with a fairly equal affinity. This picture of Mg^{2+} and polyamine interaction is compatible with the results from experiments on $tRNA^{Phe}$ dissolved in a buffer with mixtures of Mg^{2+} and spermidine ions. Over a broad range of spermidine concentrations, addition of Mg^{2+} always results in a higher T_m, while an increase in T_m by spermidine is only observed when less than about 5 mM Mg^{2+} is present in solution (at an Na^+ concentration of 125 mM). With these results in mind, it is no surprise to see that the polyamines in the polymix buffer (which contains 5 mM Mg^{2+}) – constructed by Jelenc and Kurland [5] to minimize error frequencies in polypeptide synthesis – have hardly any effect on the overall stability of $tRNA^{Phe}$. When Mg^{2+} is present at concentrations of about 1 mM, a concentration preferred by Loftfield and co-workers in their studies on error frequencies of aminoacylation [46], low concentrations of spermidine do have a pronounced effect on the stability of tRNA.

The specificity of Mg^{2+} and polyamine stabilization of tRNA, as described in this paper, probably also applies to other RNAs such as those in ribosomes. It has been demonstrated by competition experiments, in which Mg^{2+} was replaced by polyamines, that the structural and functional integrity of 50 S and 30 S subunits could only be maintained when the amount of Mg^{2+} was kept above a critical level of about 0.1 Mg^{2+} per rRNA phosphate [47,48]. This suggests that a particular conformation of a structural element of these RNAs, similar to the tertiary structure of tRNAs, can only be stabilized by Mg^{2+}.

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