Thermodynamic Studies of Transfer Ribonucleic Acids. III. Thermodynamic Model for the Thermal Unfolding of Yeast Phenylalanine-Specific Transfer Ribonucleic Acid†

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ABSTRACT: A quantitative thermodynamic model for the thermal unfolding of yeast phenylalanine-specific transfer ribonucleic acid ($tRNA^{Phe}$) is developed. This model, based on calorimetric and equilibrium spectrophotometric studies, provides a relatively simple description of the influence of Mg^{2+} and temperature on the conformation and biological activity of tRNA. For example, it appears that Mg^{2+} does not directly induce any thermodynamically significant conformational change upon binding to tRNA. Mg^{2+} activation of tRNA is thus most likely to be due to the fact that the presence of Mg^{2+} insures that the folded (and presumably active) form of tRNA is thermodynamically more stable than the unfolded (and inactive) form. Consequently, it seems that Mg^{2+} is not an *integral* part of the active conformation of tRNA. This model is based on the following three premises which will be discussed in detail. The thermal unfolding of $tRNA^{Phe}$ can be well approximated as a two-state transition. Mg^{2+} binds better to folded, than unfolded, $tRNA^{Phe}$ and stabilizes the folded form by reducing the apparent $\Delta S°$ for unfolding and not by a change in the $\Delta H°$ of reaction. The variation in $\Delta H°$ with temperature is due to a heat capacity difference ($\Delta C_p°$) between the folded and unfolded forms of $tRNA^{Phe}$. A mathematical expression for $\Delta G°$ of unfolding as a function of temperature and Mg^{2+} concentration is derived. This expression is accurate to $\pm 1°$ with respect to the melting temperature and to ± 11 kcal/mole with respect to the $\Delta H°$ for unfolding.

There is conflicting evidence available concerning the influence of Mg^{2+} and temperature on the biological activity of tRNA. Several investigators have suggested that Mg^{2+} is an absolute requirement for the biological activity of certain species of tRNA (Lindahl *et al.*, 1966; Adams *et al.*, 1967; Reeves *et al.*, 1970) while others have observed activity in some tRNA species in the absence of Mg^{2+} (Ishida and Sueoka, 1968b; Ishida *et al.*, 1971). In those cases where Mg^{2+} was required, it has been suggested that Mg^{2+} induces a conformational change in the macromolecule, thereby rendering it active. Unfortunately no quantitative thermodynamic description of the tRNA–Mg^{2+} system has been available. However, recent calorimetric studies of Mg^{2+} binding to $tRNA^{Phe}$ [1] and equilibrium studies of its thermal unfolding characteristics have provided the necessary data from which a quantitative thermodynamic description can be constructed.

In the previous paper of this series (Levy *et al.*, 1972) a phenomenological characterization of the thermal unfolding of $tRNA^{Phe}$ as a function of Mg^{2+} concentration and temperature was reported. It was demonstrated that this reaction can be well approximated as a two-state transition. Consequently, the thermodynamic quantities for the thermal unfolding of $tRNA^{Phe}$ can be obtained from equilibrium data alone. The thermodynamic quantities, thus obtained, for $tRNA^{Phe}$ unfolding will be analyzed in this communication. This analysis will show that the stabilization of the folded form of $tRNA^{Phe}$ by Mg^{2+} is the result of a reduction in the apparent $\Delta S°$ for unfolding and not of a change in the $\Delta H°$ of reaction. It will also be shown that the variation in $\Delta H°$ with temperature is primarily due to a heat capacity difference between the folded and unfolded forms of $tRNA^{Phe}$. A simple and quantitatively consistent thermodynamic model for the thermal-unfolding behavior of $tRNA^{Phe}$ as a function of Mg^{2+} concentration and temperature to be developed in this paper is based on the results of the above analysis in conjunction with the results of an independent thermodynamic study of Mg^{2+} binding to $tRNA^{Phe}$ (Rialdi *et al.*, 1972). A mathematical expression for the $\Delta G°$ for unfolding as a function of temperature and Mg^{2+} concentration will be derived. This expression is accurate to $\pm 1°$ with respect to the melting temperature, T_m, and to ± 11 kcal/mole with respect to the $\Delta H°$ for unfolding.

The model to be developed here provides a basis for quantitative interpretation of the effect of Mg^{2+} on $tRNA^{Phe}$ conformation and biological activity. For example, there does not appear to be any thermodynamically significant conformational change on Mg^{2+} binding to $tRNA^{Phe}$. This suggests that Mg^{2+} activation of tRNA is probably due to the fact that the presence of Mg^{2+} insures that the folded (and presumably active) form of tRNA is thermodynamically more stable than the unfolded (and inactive form) under the assay conditions. Consequently, it appears that Mg^{2+} is not an *integral* part of the active conformation of tRNA.

Development and Testing of the Model

Premises of the Model. TWO-STATE APPROXIMATION. The reversible thermal-unfolding transition of $tRNA^{Phe}$ is well

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[1] Abbreviations used are: $tRNA^{Phe}$, yeast phenylalanine-specific transfer ribonucleic acid; $tRNA^{Leu}$, yeast leucine-specific transfer ribonucleic acid; $tRNA^{Ala}_{lab}$, one of the yeast alanine-specific transfer ribonucleic acids; $tRNA^{Trp}$, *Escherichia coli* tryptophan-specific transfer ribonucleic acid; $T_{max\ stab}$, temperature of maximum stability.

approximated as a two-state reaction under all conditions studied. The validity of this approximation is well supported by data obtained from spectrophotometric, calorimetric, and kinetic experiments (Levy *et al.*, 1972). Consequently, at any given Mg^{2+} concentration and ionic strength, the thermal-unfolding reaction can be phenomenologically represented as an equilibrium mixture of folded (A) and unfolded (B) molecules

$$A \rightleftharpoons B \tag{1}$$

An apparent equilibrium constant can be defined as

$$K_{app} = [B]/[A] \tag{2}$$

and the apparent free energy change given by

$$\Delta G^\circ_{app} = -RT \ln K_{app} \tag{3}$$

On the basis of the two-state approximation, essentially all $tRNA^{Phe}$ molecules exist in either the folded state (A) or the unfolded state (B) at equilibrium. It is the folded form which is thermodynamically stable at lower temperatures, and the unfolded form which is stable at higher temperatures. The labels A and B do not refer to single, well-defined molecular species, but rather, represent two distinct thermodynamic distributions. Hence, the thermodynamic differences between the two states reflect the differences between the average molecular species in each distribution. This thermodynamic description of the unfolding of $tRNA^{Phe}$ is analogous to that applied to the reversible thermal unfolding of globular proteins (Brandts, 1964; Lumry *et al.*, 1966).

The thermal unfolding of tRNA is accompanied by dramatic changes in viscosity, sedimentation constant, optical activity, and ultraviolet absorption (Millar and Steiner, 1966; Fresco, 1963; Fresco *et al.*, 1966; Henley *et al.*, 1966; Levy *et al.*, 1972). From such changes in physical properties, it has been concluded that the structural variation promoted by a temperature increase is characterized by the rupture of hydrogen bonds between base pairs, resulting in the formation of extended single-stranded molecules. Thus the folded state, A, consists of a distribution of molecules in which the predominant hydrogen-bonding interactions are intramolecular and in which base-stacking interactions are maximized. The unfolded state, B, consists of a distribution of molecules in which the hydrogen bonding is predominantly between the nucleic acid bases and the water solvent and in which the degree of base stacking may vary from 0 to 100% depending upon the temperature. To the extent that the two-state approximation is valid, all molecular species exist in one of these two extreme states, and states of intermediate folding are negligibly populated.

The characteristics of the thermal unfolding of $tRNA^{Phe}$ are strongly influenced by the free Mg^{2+} concentration. Increased Mg^{2+} concentrations stabilize the folded form of $tRNA^{Phe}$ as evidenced by increased T_m values and, hence, increased ΔG° values at any given temperature. Consequently, although Mg^{2+} binds to both forms of $tRNA^{Phe}$, it must bind better to the folded form. This fact has been independently verified by calorimetric studies of Mg^{2+} binding to folded and unfolded $tRNA^{Phe}$ (Rialdi *et al.*, 1972).

The effect of Mg^{2+} on the thermal-unfolding transition of $tRNA^{Phe}$ can thus be described by the following reaction scheme (eq 4). A and B refer to the folded and unfolded forms

$$\underset{\text{I}}{A \cdot Mg_{r_A}} \rightleftharpoons r_A Mg^{2+} + \underset{\text{II}}{A \underset{+Mg^{2+}}{\overset{-Mg^{2+}}{\rightleftharpoons}} B} + r_B Mg^{2+} \rightleftharpoons \underset{\text{III}}{B \cdot Mg_{r_B}} \tag{4}$$

of $tRNA^{Phe}$, respectively. Reactions I and III represent the binding reactions of Mg^{2+} to $tRNA^{Phe}$ in states A and B, respectively, where r_A and r_B are the average number of Mg^{2+} bound to A and B at any given free Mg^{2+} concentration. (These two reactions actually consist of multiple binding steps characterized by individual microscopic binding constants and the number of binding sites.) Reaction II refers to the two-state thermal-unfolding transition in the absence of Mg^{2+}. The thermal-unfolding transition, as a function of Mg^{2+} and temperature, is thus a composite reaction involving all three steps as provided by the scheme given in eq 4.

BINDING OF Mg^{2+} TO FOLDED AND UNFOLDED $tRNA^{Phe}$. Rialdi *et al.* (1972) have established that the binding of Mg^{2+} to both forms of $tRNA^{Phe}$ can be described in terms of sets of independent but equivalent binding sites. In particular they found no evidence of cooperative interaction between binding sites in either form of the macromolecule over the range of 0–2.5 mM free Mg^{2+} concentration. It thus follows that Mg^{2+} binding to $tRNA^{Phe}$ can be described simply in terms of the site binding constants, K_i and K_j, which represent the association constants for Mg^{2+} binding to site i of $tRNA^{Phe}$ in state A and site j of $tRNA^{Phe}$ in state B, respectively. If we now define $[A_0]$ and $[B_0]$ as the concentrations of $tRNA^{Phe}$ molecules in states A and B with no Mg^{2+} bound, it follows that the total concentration of all molecules in states A and B is

$$[A] = [A_0] \prod_{i=1}^{N_A} (1 + K_i[Mg^{2+}]) \tag{5a}$$

and

$$[B] = [B_0] \prod_{j=1}^{N_B} (1 + K_j[Mg^{2+}]) \tag{5b}$$

where N_A and N_B represent the total number of binding sites in states A and B, respectively.

Rialdi *et al.* (1972) further found that Mg^{2+} binding to $tRNA^{Phe}$ can be described in terms of only two sets of independent but equivalent sites in state A. In addition, they established that the total number of binding sites in the two states were the same within experimental error. Consequently, eq 5a and b can be further simplified to

$$[A] = [A_0](1 + K_{1A}[Mg^{2+}])^{N_1}(1 + K_{2A}[Mg^{2+}])^{N_2} \tag{6a}$$

$$[B] = [B_0](1 + K_{1B}[Mg^{2+}])^{N_1}(1 + K_{2B}[Mg^{2+}])^{N_2} \tag{6b}$$

where N_1 and N_2 are the number of stronger and weaker binding sites in state A, respectively, K_{1A} and K_{2A} are the association constants of the stronger and weaker sites in state A, and K_{1B} and K_{2B} are the association constants for these same sites in state B. It is to be noted that K_{1B} may equal K_{2B}.

EXISTENCE OF A ΔC_p°. The apparent enthalpy change for unfolding of $tRNA^{Phe}$ as described by eq 4 can be expressed in the following way.

$$\Delta H^\circ = \Delta H_I + \Delta H_{II} + \Delta H_{III} \tag{7}$$

ΔH_{II} is the enthalpy of unfolding in the absence of Mg^{2+},

$\Delta H°_0$. ΔH_I and ΔH_{III} are the enthalpy changes related to dissociation and association of Mg^{2+} from $tRNA^{Phe}$ in states A and B under the experimental conditions and can be expressed as

$$\Delta H_I = -r_A \Delta H_A \quad (8a)$$

$$\Delta H_{III} = r_B \Delta H_B \quad (8b)$$

where ΔH_A and ΔH_B are the average enthalpy change per site for Mg^{2+} binding in the two forms of the macromolecule. Thus

$$\Delta H° = \Delta H°_0 - r_A \Delta H_A + r_B \Delta H_B \quad (9)$$

The $\Delta H°$ for unfolding is a strong function of the melting temperature (Levy *et al.*, 1972) as seen in Figure 1. It appears that this temperature dependence of the $\Delta H°$ is most likely due to a heat capacity difference between forms and not to an effect of magnesium ion. This statement is supported by calorimetric experiments which have shown that $\Delta H_A = 0$ (Rialdi *et al.*, 1972) and by a comparison of the magnitude of the Mg^{2+} binding constants to state A and B which suggests that ΔH_B is also probably close to zero (Rialdi *et al.*, 1972). Although this latter point has not been experimentally established, it can be shown that the observed variation in $\Delta H°$ with T_m is not very likely to be the result of Mg^{2+} binding to state B.

The results presented in Figure 1 are well represented as a linear function of temperature (Levy *et al.*, 1972). This includes both the data represented as open circles, which were obtained at the observed T_m at various concentrations of Mg^{2+}, and the data represented as closed circles, which are estimates of $\Delta H°$ at several temperatures, as obtained from a single transition curve in the absence of Mg^{2+}. The fact that both sets of results are well represented by the same empirical relationship strongly indicates that $\Delta H°$ is *only* a function of temperature. A further demonstration that the temperature dependence of $\Delta H°$ is not the result of Mg^{2+} binding is provided by the broken curve in Figure 1 which was calculated from eq 9 using the fact that $\Delta H_A = 0$ kcal/mole (Rialdi *et al.*, 1972), experimental values of the parameters for Mg^{2+} binding to the B form of $tRNA^{Phe}$ (Rialdi *et al.*, 1972), and assuming $\Delta H_B = 3$ kcal/mole and $(d\Delta H°_0/dt) = 0$. Although a monotonic increase of $\Delta H°$ with T_m (and hence Mg^{2+} concentration) can be qualitatively rationalized in this manner, this basis provides an unsatisfactory explanation for a linear increase of $\Delta H°$ with T_m. We thus conclude that the T_m dependence of $\Delta H°$ cannot be the result of Mg^{2+} binding alone and must be primarily the result of a heat capacity increase upon unfolding of the macromolecule.

Derivation of the Expression for $\Delta G°$. At any given Mg^{2+} concentration, the equilibrium constant for the thermal unfolding of $tRNA^{Phe}$ can be written in terms of eq 5a and 5b.

$$K_{app} = [B]/[A] = [B_0]\left\{\prod_{j=1}^{N_B}[1 + K_j(Mg^{2+})]\right\} \Big/ [A_0]\left\{\prod_{i=1}^{N_A}[1 + K_i(Mg^{2+})]\right\} \quad (10)$$

Thus

$$\Delta G° = -RT \ln K_0 - RT \sum_{j=1}^{N_B} \ln (1 + K_j[Mg^{2+}]) + RT \sum_{i=1}^{N_A} \ln (1 + K_i[Mg^{2+}]) \quad (11)$$

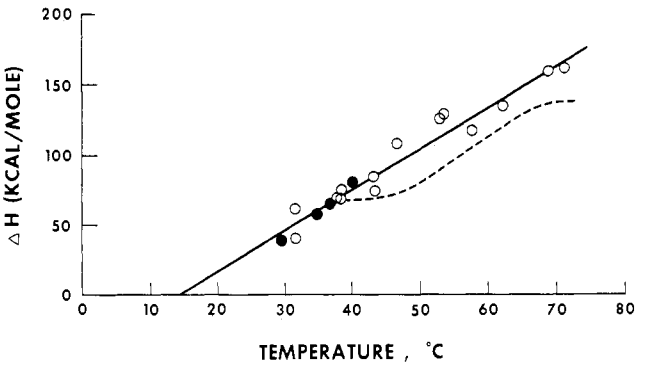

FIGURE 1: Van't Hoff heat as a function of temperature for the unfolding of $tRNA^{Phe}$. The solution conditions are identical with those described in the previous report (Levy *et al.*, 1972). The open circles represent data obtained from several experiments at different Mg^{2+} concentration; the filled circles represent data obtained from a single melting experiment in the absence of Mg^{2+}. The solid line was calculated assuming a constant $\Delta C_p° = 3$ kcal/mole-deg. The broken line was calculated assuming $\Delta C_p° = 0$ and $\Delta H_B = 3$ kcal/mole; see text for details.

where $K_0 = [B_0]/[A_0]$, T is the absolute temperature, and R is the universal gas constant. This general expression can be further simplified in terms of the results of Rialdi *et al.* (1972) (see eq 6a and b)

$$\Delta G° = \Delta G°_0 - N_1 RT \ln \frac{(1 + K_{1B}[Mg^{2+}])}{(1 + K_{1A}[Mg^{2+}])} - N_2 RT \ln \frac{(1 + K_{2B}[Mg^{2+}])}{(1 + K_{2A}[Mg^{2+}])} \quad (12)$$

where $\Delta G°_0 = RT \ln K_0$ is the standard free energy difference between states B and A in the absence of Mg^{2+}.

Since $\Delta H°$ appears to be Mg^{2+} concentration independent, its observed temperature dependence must be due to an approximately constant heat capacity difference between forms. Thus the standard free energy change for reaction II in eq 4, the thermal unfolding of $tRNA^{Phe}$ in the absence of Mg^{2+}, is

$$\Delta G°_0 = \Delta H°_0 - T\Delta S°_0 = \Delta H°_0' + \Delta C_p°(T - T_0) - T\left(\Delta S°_0' + \int_{T_0}^{T} \Delta C_p° d \ln T\right) \quad (13)$$

where $\Delta H°_0'$ and $\Delta S°_0'$ are the standard enthalpy and entropy change for unfolding in the absence of Mg^{2+} at that melting temperature, T_0, and $\Delta C_p°$ is the constant heat capacity change upon unfolding. Since the enthalpy of Mg^{2+} binding to both forms of $tRNA^{Phe}$ is zero, the thermodynamic influence of Mg^{2+} on the unfolding reaction appears as an entropic contribution to the overall free energy change. This contribution, as given in eq 12, is

$$T\Delta S_{Mg} = N_1 RT \ln \frac{(1 + K_{1B}[Mg^{2+}])}{(1 + K_{1A}[Mg^{2+}])} + N_2 RT \ln \frac{(1 + K_{2B}[Mg^{2+}])}{(1 + K_{2A}[Mg^{2+}])} \quad (14)$$

and the standard free energy for unfolding, as a function of temperature and Mg^{2+} concentration can be simply written as

$$\Delta G° = \Delta G°_0 - T\Delta S_{Mg^{2+}} \quad (15)$$

Validity of the Model. The quantitative validity and usefulness of the proposed thermodynamic model can be verified by a quantitative comparison between the experimental results and various parameters calculated using eq 13, 14, and 15. The thermodynamic parameters selected for this comparison are ΔH°, T_m, and ΔS_{Mg}. The experimental ΔH° was obtained by van't Hoff analysis of the spectrophotometric data (Levy *et al.*, 1972). The T_m, defined as that temperature at which the fraction of molecules unfolded was 0.5, was determined experimentally. The "experimental" ΔS_{Mg} was calculated at various Mg^{2+} concentrations from

$$\Delta S_{Mg} = \Delta S^\circ - \Delta S^\circ_0 = (\Delta H^\circ/T_m) - (\Delta S^\circ_0{}' + \Delta C_p^\circ \ln T_m/T_0) \quad (16)$$

ΔS° is the observed entropy change for unfolding at a given Mg^{2+} concentration and is obtained from the experimental ΔH° at the T_m for that Mg^{2+} concentration and ΔS°_0 is the entropy change for unfolding in the absence of Mg^{2+} at the T_m for unfolding in the presence of the given Mg^{2+} concentration.

It has already been argued that ΔH° is a linear function of the temperature, independent of Mg^{2+} concentration. This has been shown in Figure 1 where the points represent experimental data, and the solid line was calculated assuming $\Delta H^\circ_0{}' = 70$ kcal/mole at 38.1° and $\Delta C_p^\circ = 3$ kcal/mole-deg. The average deviation between the calculated and experimental values is ± 11 kcal/mole.

The Mg^{2+}-concentration-dependent entropy term, ΔS_{Mg}, is shown in Figure 2 as a function of log [Mg^{2+}] with the "experimental" values represented as the filled circles. The involvement of both high and low affinity Mg^{2+} binding sites is indicated by the apparent biphasic nature of the data. This result is qualitatively consistent with the Mg^{2+} binding results of Rialdi *et al.* (1972). A quantitative consistency of these two sets of results can also be demonstrated by computer simulation of various ΔS_{Mg} curves using eq 14 and systematically varying N_1, N_2, K_{1A}, K_{2A}, K_{1B}, and K_{2B}.

The most restrictive model is one in which it is assumed that form A possesses a single set of independent binding sites which do not exist in form B. This is equivalent to assuming $K_{1B} = 0$ and $K_{2A} = K_{2B}$. This model does provide a qualitative representation of the present results as shown by broken curve A, which was calculated for $N_{1A} = 3$ and $K_{1A} = 2 \times 10^5$ M^{-1}. However, a systematic deviation between the calculated curves and the values of ΔS_{Mg} suggests that this model is incorrect. In addition, the implied assumptions in this model that form A possess more sites than form B and that all other Mg^{2+} binding sites are equivalent in both forms are in contradiction with the results of Rialdi *et al.* (1972). For these reasons this model was tentatively rejected, and a search for a more appropriate model initiated.

In all subsequent calculations N_1 was restricted to the values 4 ± 1 and $(N_1 + N_2)$ was assumed to equal 24, in accordance with the Mg^{2+} binding results. Assuming that both forms of $tRNA^{Phe}$ each possessed only one set of independent and equivalent binding sites (*i.e.*, $K_{1A} = K_{2A}$ and $K_{1B} = K_{2B}$) a series of curves was generated which showed serious systematic deviation at either low or high Mg^{2+} concentration. These results clearly indicated that any model which assumed an identical number of binding sites in both forms of $tRNA^{Phe}$ must also assume the involvement of both a stronger and a weaker set of binding sites.

In a subsequent series of calculations it was assumed that 3×10^5 $M^{-1} \leq K_{1A} \leq 2 \times 10^6$ M^{-1}, $K_{2A} = 1.1 \times 10^4 \pm 2 \times 10^3$ M^{-1} and $K_{1B} = K_{2B} = 7 \times 10^3 \pm 2 \times 10^3$ M^{-1}. These restrictions are set by the estimates of the various binding constants, with respective errors, obtained from independent Mg^{2+} binding studies (Rialdi *et al.*, 1972). The simulated curve of this series which best fits the experimental values is curve B in Figure 2. The average deviation between this curve, calculated assuming $N_1 = 3$, $K_{1A} = 3 \times 10^5$ M^{-1}, $K_{2A} = 10^4$ M^{-1}, and $K_{1B} = K_{2B} = 7 \times 10^3$ M^{-1}, and the experimental estimates is ± 3 cal/mole-deg. However, the observed deviation is largely systematic, particularly in the Mg^{2+} concentration range of 50–500 μM. This systematic deviation can be partially reduced, but not eliminated, by assuming an error in our estimate in T_0, the melting temperature of $tRNA^{Phe}$ in the absence of Mg^{2+}. Furthermore, the assumed value of K_{1A} is not in good agreement with the Mg^{2+} binding results of Rialdi *et al.* (1972). This suggested that a model of two sets of sites in the folded form and one set in the unfolded form was inadequate at low Mg^{2+} concentration.

The best agreement between experimental and calculated values of ΔS_{Mg} was obtained when it was assumed that the unfolded form of $tRNA^{Phe}$ possessed a set of strong binding sites in addition to the weak set. This assumption is not incompatible with the Mg^{2+} binding studies (Rialdi *et al.*, 1972). Therefore, an additional series of calculations was performed, maintaining the same restrictions as in the previous series except that K_{1B} was allowed to vary independently of K_{2B}. The simulated curve of this series, which best fits the experimental data within an average deviation of ± 1 cal/mole-deg, is the solid curve in Figure 2 calculated assuming $N_{1A} = N_{1B} = 4$, $K_{1A} = 10^6$ M^{-1}, $K_{2A} = 10^4$ M^{-1}, $K_{1B} = 5 \times 10^5$ M^{-1}, and $K_{2B} = 5 \times 10^3$ M^{-1}. In this case, the systematic deviation is eliminated[2] and the value assumed for K_{1A} is now in good agreement with that obtained from the Mg^{2+} binding data (Rialdi *et al.*, 1972).

The biphasic nature of the influence of Mg^{2+} on the thermal stability of $tRNA^{Phe}$ is most clearly shown by the two broken curves labeled C and D in Figure 2, which were calculated for the stronger and weaker sets of sites, respectively, in the latter model. The agreement between the "experimental" values of ΔS_{Mg} and those calculated by a binding model, upon which were imposed restrictions set by independent Mg^{2+} binding studies, clearly support our interpretation of the thermal unfolding behavior of $tRNA^{Phe}$ as a function of temperature and Mg^{2+}.[3]

[2] In this last model a rather large deviation between experimental and calculated values at 1 mM Mg^{2+} is observed. Although this indicates an inadequacy in the model, a simple explanation is available. A combination of the Mg^{2+} binding results of Rialdi *et al.* (1972) with those of Sander and Ts'o (1971) suggested the existence of a third set of Mg^{2+} binding sites, with an apparent binding constant of about 10^2 M^{-1}. Mg^{2+} binding to these sites would begin to influence the thermal stability of $tRNA^{Phe}$ at about 1 mM Mg^{2+}. Since we have not incorporated a third set of sites into our model, deviation between the calculated and experimental thermodynamic quantities would be expected at high Mg^{2+} concentration.

[3] In addition to the above binding models which appear, in varying degrees, to quantitatively represent the "experimental" results for ΔS_{Mg}, two other models could certainly be as adequate. One model is such that form A of $tRNA^{Phe}$ possesses several Mg^{2+} binding sites not present in state B but each of which has different intrinsic affinities for Mg^{2+}. This possibility appears to be unlikely, however, since the Mg^{2+} binding studies of Rialdi *et al.* (1972) indicate a similarity in the number of binding sites in both forms of $tRNA^{Phe}$. The other possible model is one in which both forms possess the same number of sites with a cooperative interaction between sites. This model cannot be eliminated by the present results, but would appear to be incorrect since Rialdi *et al.* (1972) did not find any evidence for the existence of any cooperative interaction between Mg^{2+} binding sites in state A.

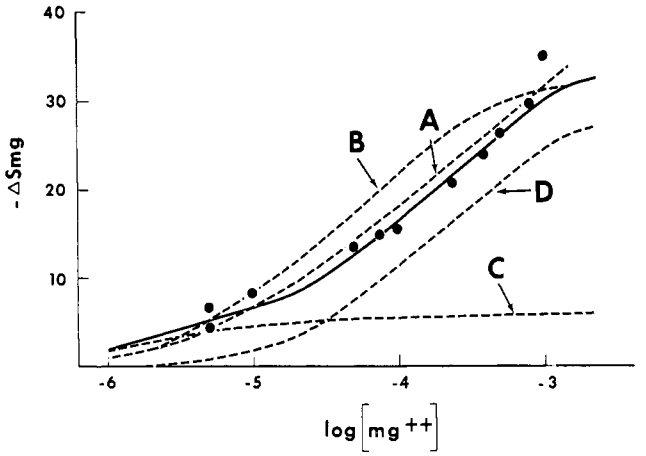

FIGURE 2: The Mg^{2+} entropy contribution to ΔG° as a function of the log [Mg^{2+}]. The points were calculated according to eq 16. The curves A and B were calculated assuming binding models as described in the text. The solid curve was calculated according to eq 14, assuming the binding parameters given in Table I. The curves C and D represent the contributions of the strong and weak sets of sites, respectively, as derived from a decomposition of the solid curve.

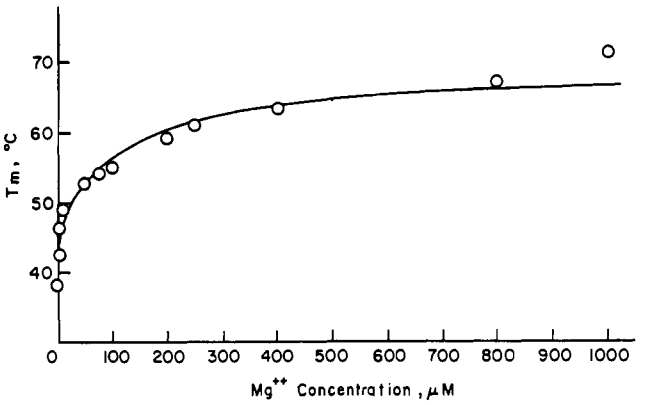

FIGURE 3: The melting temperature, T_m, of $tRNA^{Phe}$ as a function of Mg^{2+} concentration. The solid curve was calculated by finding the root to eq 15, assuming the thermodynamic parameters given in Table I.

An additional demonstration of the quantitative agreement between our model and the experimental results is shown in Figure 3 where the melting temperature is presented as a function of Mg^{2+} concentration. The solid curve was calculated by finding the root of eq 15 assuming the values of $\Delta H^\circ{}_0'$, $\Delta S^\circ{}_0'$, ΔC_p° given above and the same Mg^{2+} binding parameters used to calculate the solid curve shown in Figure 2. The average deviation between the curve and the experimental values of T_m is $\pm 1^\circ$.

The good agreement obtained between the calculated and experimental ΔH°, ΔS_{Mg}, and T_m values indicates that the premises of our thermodynamic model are valid in view of the data presently available and shows that such a model can provide a quantitative representation of the thermal-unfolding characteristics of $tRNA^{Phe}$ as a function of temperature and Mg^{2+} concentration. Thus, the most plausible explanation for the stabilization of $tRNA^{Phe}$ by Mg^{2+} is that Mg^{2+} binds better to the folded form of $tRNA^{Phe}$ than to the unfolded form and thus shifts the equilibrium between these forms, thereby reducing the apparent equilibrium constant and increasing the apparent free energy change for the unfolding reaction. The free energy increase is due to a decrease in the apparent ΔS°, and not to an increased ΔH°. The reduction in ΔS° for thermal unfolding results from the positive ΔS for binding of Mg^{2+} to $tRNA^{Phe}$, which is due to the release of water molecules formerly complexed by Mg^{2+} ions, being greater in the folded form than in the unfolded form of $tRNA^{Phe}$. Since the ΔH° for the transition is not Mg^{2+} dependent, its apparent temperature dependence must really be a true temperature dependence and must be the result of a heat capacity difference between folded and unfolded forms of $tRNA^{Phe}$.

Based on this model, the following expression for the ΔG° for unfolding as a function of temperature and free Mg^{2+} concentration was developed.

$$\Delta G^\circ = \Delta H^\circ{}_0' + \Delta C_p^\circ(T - T_0) - T(\Delta S^\circ{}_0' + \Delta C_p^\circ \ln T/T_0) - N_1RT \ln (1 + K_{1B}[Mg^{2+}]) - N_2RT \ln (1 + K_{2B}[Mg^{2+}]) + N_1RT \ln (1 + K_{1A}[Mg^{2+}]) + N_2RT \ln (1 + K_{2A}[Mg^{2+}]) \quad (17)$$

The constants in this expression which provide the best fit between the experimental and calculated thermal-unfolding behavior of $tRNA^{Phe}$ are summarized in Table I for the best of the binding models and compared with the experimental results of Rialdi *et al.* (1972).

Discussion

Interpretation and Significance of the Heat Capacity Change. It has been shown that there is a heat capacity difference (ΔC_p°) between the folded and unfolded forms of $tRNA^{Phe}$. This apparent ΔC_p° is, in fact, a true ΔC_p° between forms and is not due to a Mg^{2+} effect. This conclusion is based on the observation that no enthalpy change accompanies Mg^{2+} binding to $tRNA^{Phe}$ in state A and upon arguments that the heat of Mg^{2+} binding to unfolded $tRNA^{Phe}$ is also zero. From a least-squares analysis of all the data points of ΔH° *vs.* temperature, the value of ΔC_p° was found to be 3.0 kcal/mole-deg. Additionally, the values of the slopes of ΔS° *vs.* $\ln T/T_0$ curves, which are equal to ΔC_p°, were found to be identical at all Mg^{2+} concentrations and also to be equal to the ΔC_p° obtained from the variation of ΔH° with temperature.

TABLE I: Thermodynamic Parameters[a] for the Unfolding of $tRNA^{Phe}$.

N_{1A}	4	4 ± 1[b]
K_{1A}	1×10^6 M^{-1}	1×10^6 M^{-1}[b]
N_{2A}	20	20 ± 1[b]
K_{2A}	1×10^4 M^{-1}	$1.1 \pm 0.2 \times 10^4$ M^{-1}[b]
N_{1B}	4	
K_{1B}	5×10^5 M^{-1}	
N_{2B}	20	
K_{2B}	5×10^3 M^{-1}	$7 \pm 2 \times 10^3$ M^{-1}[b,c]
$N_{1B} + N_{2B}$	24	27 ± 3[b,c]

[a] $T_0 = 38.1^\circ$, $\Delta H^\circ{}_0' = 70$ kcal/mole, $\Delta S^\circ{}_0' = 225$ cal/mole-deg, $\Delta C_p^\circ = 3$ kcal/mole-deg. [b] Rialdi *et al.* (1972). [c] Because of experimental limitations Rialdi *et al.* (1972) were unable to distinguish the existence of both a strong and weak set of binding sites in state B. Thus their estimate for K_{2B} is a weighted average value.

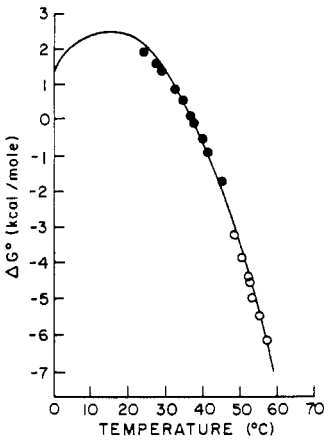

FIGURE 4: The standard free energy, ΔG°, as a function of temperature in the absence of Mg^{2+}. The solid curve was calculated according to eq 17, assuming the parameters in Table I. The filled points are experimental values obtained for $tRNA^{Phe}$ in the absence of Mg^{2+}. The open circles were derived from experimental values obtained in the presence of 50 μM Mg^{2+} and corrected for the $T\Delta S_{Mg}$ contribution according to eq 14.

ΔC_p° values of this magnitude have previously been observed for many protein unfolding reactions (Brandts, 1964, 1965, 1969; Tanford, 1968; Biltonen and Lumry, 1969, 1971) and several polynucleotide and nucleic acid reactions (Bunville *et al.*, 1965; Rawitscher *et al.*, 1963). In proteins the source of the ΔC_p° is attributed to the disruption of hydrophobic interactions with the resultant transfer of hydrophobic amino acids from the nonaqueous protein interior to an aqueous environment. In nucleic acids and polynucleotides, ΔC_p° results from the variation in the amount of residual base stacking in the unfolded state with temperature and may also result from disruption of hydrophobic interactions with the concomitant transfer of bases from the interior nonaqueous environment to the external aqueous one. In the case of unfolding of globular proteins, it appears that the magnitude of ΔC_p° is related to the size of the cooperative unfolding unit (Brandts, 1969; Biltonen and Lumry, 1969). Further study is required to determine whether or not a similar situation exists for the unfolding of tRNA.

The large ΔC_p° for the thermal unfolding of $tRNA^{Phe}$ suggests that the macromolecule can be denatured by either heating or cooling. This can be seen by the following argument. An extrapolation of the values of ΔS° at a given Mg^{2+} concentration to low temperature using the calculated value of ΔC_p° indicates that the entropy change for the unfolding reaction at that Mg^{2+} concentration becomes zero at some temperature. For example, in the absence of Mg^{2+}, ΔS° changes sign at approximately 16°. Since $(\partial \Delta G^\circ/\partial T)_p = -\Delta S^\circ$, the slope of ΔG° *vs.* temperature changes from positive to negative at that temperature. Thus ΔG° attains a maximum value at approximately 16°. This is demonstrated in Figure 4 where ΔG° is plotted as a function of the temperature. At the temperature where ΔG° attains its maximum value, $T_{max\ stab}$, the equilibrium between the folded and unfolded forms can be shifted toward the unfolded form by either raising or lowering the temperature. Temperatures of maximum stability resulting from large heat capacity differences between forms have previously been observed experimentally for the thermal unfolding of some globular proteins (Brandts, 1965, 1969; Biltonen and Lumry, 1969, 1971). The existence of a $T_{max\ stab}$ provides an explanation of why proteins can be denatured by both high and low temperatures and possesses a temperature of optimal activity under a given set of conditions (Brandts, 1967). Similarly, it is expected that tRNA may lose activity at both high and low temperature since the molecule can be unfolded at temperatures both above and below the temperature of maximum stability.

Biological Implications of the Model. We would now like to raise a few questions related to the effect of Mg^{2+} on the conformation and biological activity of tRNA, and discuss the answers in terms of the proposed model.

1. Does Mg^{2+} binding change the $tRNA^{Phe}$ conformation? Several workers have observed changes in the fluorescence of the Y base in yeast $tRNA^{Phe}$ (Römer *et al.*, 1970; Eisinger *et al.*, 1970) and beef liver $tRNA^{Phe}$ (Zimmerman *et al.*, 1970) as a function of Mg^{2+} concentration. These observations suggest that $tRNA^{Phe}$ undergoes some type of structural change on Mg^{2+} binding. The results presented here for yeast $tRNA^{Phe}$ suggest that there is not a *major* conformational change in the usual sense of the term, *i.e.*, there is no gross change in the molecular size or shape or in the number or arrangement of hydrogen bonds and other noncovalent interactions. This follows from the conclusion that the enthalpy changes for the unfolding transition appear to be independent of Mg^{2+} concentration within experimental error. These results are totally consistent with the previous observation that the enthalpy change for Mg^{2+} binding to folded $tRNA^{Phe}$ was calorimetrically determined to be 0 ± 100 cal/mole of Mg^{2+} (Rialdi *et al.*, 1972) and indicate that no thermodynamically significant conformational change occurs on Mg^{2+} binding. In addition titration of $tRNA^{Phe}$ at 20° with $MgCl_2$ produced no change in ultraviolet absorption over that resulting from dilution of the $tRNA^{Phe}$ by addition of titrant (Levy, 1971).

It is entirely possible, however, that Mg^{2+} binding may produce a subtle-type conformational change such as a tightening of the tRNA phosphate backbone. This tightening could produce the observed fluorescence changes by reducing the amount of solvent quenching (Eisinger and Lamola, 1971) and would probably decrease the positive entropy change expected for Mg^{2+} binding to tRNA without producing any enthalpy change. In view of the results reported (Rialdi *et al.*, 1972; Levy *et al.*, 1972), it is unlikely that Mg^{2+} binding produces any *major* conformational change in $tRNA^{Phe}$ and any conformational change which does occur on Mg^{2+} binding is probably very local in nature.

2. Is Mg^{2+} required for the biologically active conformation? Fresco and coworkers (Lindahl *et al.*, 1966; Adams *et al.*, 1967) have concluded from studies of the activity of yeast leucine-specific tRNA that Mg^{2+} was an obligate part of the biologically active structure of the molecule and that without Mg^{2+} another inactive form of tRNA existed. This inactive form (isolated under conditions where denaturing reagents were used) could be "renatured" to a form with normal activity by heating in the presence of Mg^{2+}. Similarly, Reeves *et al.* (1970) have found that denatured yeast $tRNA^{Ala}_{lab}$ regains 80% of its activity lost at 0° by heating at 37° with Mg^{2+}. Reeves *et al.* (1970) suggested that this tRNA possessed two low-temperature conformations—one existing in the absence of Mg^{2+} and one in the presence of Mg^{2+}. Only the form existing in the presence of Mg^{2+} was active.

The model presented here suggests a different interpretation for these observations. It will be assumed in this interpretation that our model for $tRNA^{Phe}$ unfolding as a function of Mg^{2+} and temperature is qualitatively accurate for describing the thermal-unfolding transitions of yeast $tRNA^{Leu}$

and yeast $tRNA^{Ala}_{lab}$. This model includes the two-state approximation for the thermal-unfolding transitions, the stabilization of the folded forms of these molecules by Mg^{2+}, and a heat capacity increase on unfolding such that a temperature of maximum stability exists. The possible existence of a temperature of maximum stability for tRNA thermal unfolding is supported by the observation that when *Escherichia coli* $tRNA^{Trp}$ is heated the absorbance achieves a minimum value about 30° below the apparent T_m (Ishida and Sueoka, 1967). This may be interpreted as a $T_{max\ stab}$ from which either increasing or decreasing the temperature produces an increase in the degree of unfolding and in the absorbance.

The only other assumption that is necessary is that the unfolded form is kinetically stable at low temperature. Thus even if the folded form is thermodynamically more favorable at low temperature under the assay conditions, the unfolded form in which the tRNA was isolated cannot readily cross the activation barrier between forms at low temperature. Raising the temperature increases the rate constant for the folding reaction so that the unfolded form can convert to the folded form, which is presumably the active form. Because Mg^{2+} stabilizes the folded form, its presence simply assures that the folded form will be thermodynamically favored at the final temperature. Support for this assumption that the kinetics of tRNA folding are highly temperature dependent comes from the observation that the rate of conversion of inactive to active *E. coli* $tRNA^{Trp}$ is very slow at low temperature and increases about 30-fold when the temperature is increased from 25 and 35° (Ishida and Sueoka, 1968a).

Thus the observation of Fresco and coworkers (Lindahl *et al.*, 1966; Adams *et al.*, 1967) that heating in the presence of Mg^{2+} produces $tRNA^{Leu}$ which is biologically active can be explained in the following way. The elevated temperature produces favorable kinetics for the folding reaction so that any $tRNA^{Leu}$ molecules, which had been "trapped" in the unfolded form under the denaturing isolation conditions, can readily cross the activation barrier to the folded active form, which is the thermodynamically stable form in the presence of Mg^{2+}. Thus the activity of the sample increases after heating in the presence of Mg^{2+} since now essentially all molecules are in the folded active form. Support for this mechanism of activation comes from observations of Fresco and his coworkers (Lindahl *et al.*, 1966; Adams *et al.*, 1967). It was found that on "renaturing" or "activating," $tRNA^{Leu}$ undergoes a decrease in absorbance, becomes more compact, and less accessible to ribonuclease attack. All of these changes would be expected when tRNA refolds. The fact that only small changes in these observables occur does not mean that a major conformational change such as folding is not taking place. It simply means that not all of the molecules are refolding because not all were originally unfolded. This is supported by the fact that the activity of an "inactive" sample was actually 60% of that of the fully active or "renatured" $tRNA^{Leu}$.

The observations of Reeves *et al.* (1970) are also consistent with this interpretation of the activation of tRNA by Mg^{2+}. They found that Mg^{2+} stabilizes $tRNA^{Ala}_{lab}$—the T_m and sharpness of the transitions increase with Mg^{2+} concentration and $tRNA^{Ala}_{lab}$ can be refolded by titration with Mg^{2+} at 25°. That refolding can occur at 25° means that this tRNA is probably partially unfolded at 25° in the absence of Mg^{2+}, but folded in the presence of Mg^{2+}. (Thus $tRNA^{Ala}_{lab}$ is thermodynamically less stable than $tRNA^{Phe}$ in the absence of Mg^{2+} at the same temperature.) $tRNA^{Ala}_{lab}$ is less active at 0° than at 25° in the absence of Mg^{2+} indicating that without Mg^{2+} $tRNA^{Ala}_{lab}$ may be less folded at 0° than at 25°. Therefore, a temperature of maximum stability probably exists between 0 and 25° such that above and below the latter temperature the degree of unfolding increases. In the presence of Mg^{2+}, the activity is essentially identical at 0 and 25° because $\Delta G°$ for unfolding is probably so large at both temperatures that essentially all molecules are in the folded state.

Reeves *et al.* (1970) found that although addition of Mg^{2+} to the $tRNA^{Ala}_{lab}$ at 0° was not sufficient to increase the activity, addition of Mg^{2+} at 0° followed by heating to 37° did increase activity. Also if Mg^{2+} were added to the $tRNA^{Ala}_{lab}$ at 25° and the temperature then lowered to 0°, $tRNA^{Ala}_{lab}$ regained essentially total activity. These observations can be explained by our model, in the following way. At 25° $tRNA^{Ala}_{lab}$ is most likely partially unfolded in the absence of Mg^{2+}, but folded in the presence 1 mM Mg^{2+}. Therefore, it is expected that at 25° $tRNA^{Ala}_{lab}$ will display less activity in the absence of Mg^{2+} than in its presence. In 1 mM Mg^{2+} $tRNA^{Ala}_{lab}$ will probably still be folded at 0° because $\Delta G° \gg 0$, and therefore, will have the same activity at 0 and 25°. However, since $tRNA^{Ala}_{lab}$ in the absence of Mg^{2+} may have a temperature of maximum stability between 0 and 25° it will be less folded at 0°, and therefore, will display less activity than at 25°. Since the activation energy barrier for folding is probably high at low temperature, addition of Mg^{2+} at 0° would not induce rapid refolding even though the folded form may be thermodynamically more stable than the unfolded form at 0° in the presence of Mg^{2+}. Addition of Mg^{2+} at 0° followed by heating to 37° can now induce folding because the rate constant for folding is most likely increased at 37° and because the folded form is probably still thermodynamically more stable than the unfolded form at 37° in the presence of high [Mg^{2+}]. In this way $tRNA^{Ala}_{lab}$ regains 80% of its activity lost at 0° in the absence of Mg^{2+} by addition of Mg^{2+} at 0°, followed by heating to 37°.

A corollary of this hypothesis for explaining Mg^{2+} activation of tRNA is that Mg^{2+} is *not required* as an *integral part* of the active conformation of tRNA. This corollary is supported by evidence presented here suggesting that no *major* conformational change occurs on Mg^{2+} binding to tRNA. Additionally, Ishida and Sueoka (1968b) have found that *E. coli* $tRNA^{Trp}$ can be "activated" by heating in the presence of EDTA and high concentrations of Na^+ and concluded that divalent cations are not absolutely necessary for the active conformation of $tRNA^{Trp}$. Thus those tRNA molecules which appear to require Mg^{2+} for activity are most likely those whose folded (active) forms are thermodynamically unfavorable at the assay temperature in the absence of Mg^{2+}. Those which do not require Mg^{2+} for activity most likely exist in the folded form in the absence of Mg^{2+} under assay conditions.

Further Testing of the Proposed Model. The general validity of the proposed model for the thermally induced unfolding of transfer ribonucleic acids can, and should be, tested in several ways. For instance, the general phenomenological characteristics of unfolding of several additional species of tRNA as a function of temperature and Mg^{2+} concentration should be determined. In addition specific details of the thermal transition of $tRNA^{Phe}$ should be quantitatively verified. For example, the validity of the two-state approximation can be best established by differential scanning calorimetry, as has been done for a variety of protein systems. In this respect it should be pointed out, however, that both nucleic acid chemical purity and reversibility of the the thermal transition must be strictly established before the results of such

experiments are to have significant bearing on this question.

Details of the presently proposed model can also be tested. For example, direct heat capacity measurements with folded and unfolded tRNA can be made to either verify or refute the existence of the large ΔC_p° accompanying unfolding. Such experiments performed with proteins (Biltonen *et al.*, 1971) have established that a large increase in heat capacity occurs upon thermal unfolding of the macromolecule. Also experimental determination of the existence, or absence, of the high Mg^{2+} affinity sites to unfolded $tRNA^{Phe}$ is potentially possible.

Of more biological significance is the possible existence of "cold denaturation" of tRNA. Such a phenomenon is consistent both with our model and limited experimental evidence as has been discussed. The actual existence of a temperature of maximum stability of tRNA can probably be best verified by kinetic experiments. Two experimental approaches to answer this question would be direct determination of proton exchange or nuclear magnetic resonance studies of solvent exchangable protons of tRNA as a function of temperature. Thus the proposed model of the unfolding of tRNA as a function of temperature and Mg^{2+} concentration, which appears to be of some biological significance, can be further tested by appropriate experimentation. Since this thermodynamic model provides a quantitative framework which may help in the interpretation of a variety of experimental data relating to tRNA, such testing is of the utmost importance.

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