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Thermodynamic Studies of Transfer Ribonucleic Acids. I. Magnesium Binding to Yeast Phenylalanine Transfer Ribonucleic Acid†

Giovanni Rialdi,‡ J. Levy,§ and R. Biltonen*

ABSTRACT: The thermodynamics of magnesium ion (Mg^{2+}) binding to yeast phenylalanine transfer ribonucleic acid ($tRNA^{Phe}$) have been determined calorimetrically. At low temperature, where $tRNA^{Phe}$ exists in its folded state, the enthalpy of Mg^{2+} binding was found to be 0 ± 100 cal/mole of ligand. This result is taken as evidence for the absence of a thermodynamically significant conformational change upon Mg^{2+} binding. Using the large heat of reaction between Mg^{2+} and EDTA, the extent of Mg^{2+} binding to $tRNA^{Phe}$ in both its folded state and unfolded state has been measured over a free Mg^{2+} concentration range of 0–2.5 mM. Mg^{2+} binding to folded $tRNA^{Phe}$ can best be represented in terms of two sets of independent binding sites characterized by occupancy numbers $N_{1A} = 4$ and $N_{2A} = 20$ with association constants $K_{1A} = 10^6\ M^{-1}$ and $K_{2A} = 1.1 \times 10^4\ M^{-1}$. Analysis of the combination of the present results and data previously obtained for mixed tRNA at higher Mg^{2+} concentration suggest the existence of a third set of independent binding sites. Mg^{2+} binding to unfolded $tRNA^{Phe}$ can be interpreted in terms of a single set of independent binding sites with $K_B = 7 \times 10^3\ M^{-1}$. These results show that Mg^{2+} binding to tRNA is thermodynamically characterized by a large, positive entropy change, presumably due to release of water from solvated Mg^{2+} upon binding. In addition it now appears that Mg^{2+} stabilizes the folded conformation of $tRNA^{Phe}$ simply because Mg^{2+} binds better to the folded form than to the unfolded form of the macromolecule.

The relationship between the structure and function of transfer ribonucleic acid has been extensively studied (Fresco *et al.*, 1966; Gantt *et al.*, 1969; Dudock *et al.*, 1970). An important result of such studies was the recognition that a unique three-dimensional conformation is necessary for the macromolecule to perform its biological function (Fresco *et al.*, 1966). The significance of the role of Mg^{2+} in the structure–function relationship has also been examined (Henley *et al.*, 1966; Lindahl *et al.*, 1966; Adams *et al.*, 1967; Reeves *et al.*, 1970; Ishida and Sueoka, 1968a,b; Ishida *et al.*, 1971; Robison and Zimmerman, 1971b). For example, Mg^{2+} has been found to influence the thermodynamic stability of the folded conformation of tRNA (Dudock *et al.*, 1970). Some investigators have also suggested that Mg^{2+} is an absolute requirement for the biologically active conformation (Lindahl *et al.*, 1966; Adams *et al.*, 1967; Reeves *et al.*, 1970). Since the interaction of Mg^{2+} with tRNA appears to be a rapid and reversible phenomenon (Ishida and Sueoka, 1968a), Mg^{2+} must exert thermodynamic, rather than kinetic, control on the structure of tRNA. For this reason a complete thermodynamic description of the interaction of Mg^{2+} with tRNA is necessary to understand this problem. Sander and Ts'o (1971) have studied the binding of Mg^{2+} to tRNA using a divalent cation-specific electrode, but were unable to obtain reliable data below free Mg^{2+} concentrations of about 0.1 mM. Others have investigated manganese binding to tRNA using proton magnetic relaxation (Cohn *et al.*, 1969) and electron spin resonance (Danchin and Gueron, 1970a) techniques. However, the characterization of the interaction has been limited because complete thermodynamic information has not been obtained.

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In this paper we wish to report a calorimetric study of Mg^{2+} binding to yeast phenylalanine tRNA ($tRNA^{Phe}$) which provides a complete thermodynamic description of the process over the range of 0–2.5 mM free Mg^{2+} concentration. Our results clearly show that the free energy change for the process is dominated by a favorable entropy change, that there exist at least two sets of independent binding sites on the macromolecule, and that Mg^{2+} does not induce a thermodynamically significant structural change in the macromolecule under the conditions of our experiments. Furthermore, an analysis of the combined results of our studies and those of Sander and Ts'o (1971) suggests that there are three sets of apparently independent Mg^{2+} binding sites on tRNA. There is no strong evidence of any cooperative interaction. Consequently, although Mg^{2+} greatly influences the thermodynamic stability of tRNA, it does not appear to be an absolute requirement for the existence of the folded form of the molecule.

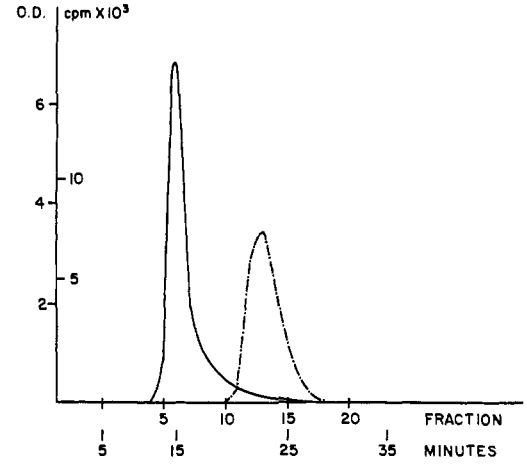

FIGURE 1: Elution profile of $tRNA^{Phe}$ dissolved in ^{32}P buffer solution and eluted with "cold" buffer. (——) A_{260}, (-·-·-) cpm.

Experimental Section

Phenylalanine tRNA ($tRNA^{Phe}$), isolated from Brewer's yeast and purified according to the method of Wimmer *et al.* (1968), was purchased from Boehringer-Mannheim (lot 7470106) and used without further purification. The reported activity was approximately 1000 pmoles of phenylalanine accepted/A_{260} unit and represented 96–99% of the total biological activity. The EDTA used was Fisher ACS certified grade. All other reagents were of the highest purity available.

$tRNA^{Phe}$ solutions were prepared by dissolving 5 mg of material in 10 ml of a stock solution of buffer containing 5 mM sodium phosphate (pH 7.2) and 5 mM NaCl. This solution was exhaustively dialyzed overnight at 4° against a solution of 3 mM EDTA in the same buffer to remove all divalent cations. Further dialysis against the stock buffer solution removed the EDTA. The $tRNA^{Phe}$ sample was then diluted with buffer to a concentration of approximately 10^{-5} M and dialyzed against a stock buffer solution containing a known amount of $MgCl_2$. Stock solutions of $MgCl_2$ were titrated with EDTA, using Eriochrome Black T as the indicator, to determine the Mg^{2+} concentration. The concentration of all $tRNA^{Phe}$ samples was determined spectrophotometically at room temperature using an extinction coefficient of 550,000 l./mole at 257 nm (Levy, 1971).

Equilibration of unfolded $tRNA^{Phe}$ with a buffer solution containing a known concentration of Mg^{2+} was achieved by rapid passage of a $tRNA^{Phe}$ solution through a G-25 Sephadex column at high temperature where the macromolecule was unfolded. These columns were made and equilibrated with buffer containing a known concentration of $MgCl_2$ at high temperature (75–82°). Since these columns were extremely fragile, new ones were prepared for each experiment. The $tRNA^{Phe}$ solutions were maintained on the column for approximately 15 min. This length of time was sufficient for equilibration as demonstrated by the elution profile, shown in Figure 1, which was obtained for a sample of $tRNA^{Phe}$ dissolved in ^{32}P radioactively labeled phosphate buffer and then eluted with nonradioactive phosphate buffer at 82°. Essentially complete separation of the ^{32}P label from the $tRNA^{Phe}$ shows that equilibration was accomplished. The $tRNA^{Phe}$ samples, thus equilibrated with Mg^{2+} at high temperature, were then eluted from the column and used in the calorimetric experiments to determine Mg^{2+} binding to unfolded $tRNA^{Phe}$.

All calorimetric measurements were performed with an LKB batch microcalorimeter, the details of which have previously been described (Monk and Wadso, 1968). Approximately 4 ml of a $tRNA^{Phe}$ solution and 2 ml of 3 mM EDTA buffer solution (or $MgCl_2$ solution), both at the same pH, were mixed in the calorimeter, and the heat absorbed was measured. The exact amounts of components added to the calorimeter cell were determined by weighing the filling syringes before and after filling. The reference cell contained equivalent amounts of buffer solution. The pH of the final $tRNA^{Phe}$–EDTA mixture was measured and found to vary by less than 0.01 pH unit from that of the original solutions. The heats of dilution of all components were measured separately and subtracted from the observed heats to obtain the heat of reaction. The calibration of the calorimeter was performed electrically and checked by measuring the heat of dilution of sucrose.

Results

Experimental Rationale. When $tRNA^{Phe}$, in the absence of Mg^{2+},[1] is mixed with a known amount of $MgCl_2$ the total heat of mixing is

$$Q = Q_t + Q_m + Q_x \quad (1)$$

where Q_t and Q_m are the heats of dilution of $tRNA^{Phe}$ and $MgCl_2$ and Q_x is the heat of reaction between Mg^{2+} and the macromolecule. The respective heats of dilution were obtained in separate experiments and subtracted from the measured heat to obtain the heat of reaction. In a series of experiments at 25° in which the final *total* concentration of Mg^{2+} was either 0.33 or 1.0 mM, the heat of reaction was found to be 0 ± 2 kcal/mole of $tRNA^{Phe}$ at both concentrations. In all experiments the $[Mg^{2+}]/[tRNA^{Phe}]$ ratio was greater than 60:1. At these concentrations of Mg^{2+}, approximately 20 moles of Mg^{2+} is known to be bound per mole of tRNA (see following results) (Sander and Ts'o, 1971). On the basis of these results it can be concluded that the heat of Mg^{2+} binding to $tRNA^{Phe}$ is 0 ± 100 cal/mole of ligand.

Despite the lack of any significant heat change associated

[1] The absence of Mg^{2+} was verified by the fact that the heat of mixing of this solution with excess EDTA produced no measurable heat except for that of dilution of the various components. This means that less than 0.3 mole of Mg^{2+}/mole of $tRNA^{Phe}$ were present in our solutions.

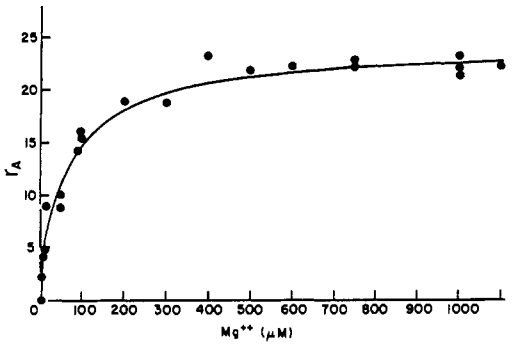

FIGURE 2: Binding of Mg^{2+} to RNA^{Phe} as a function of Mg^{2+} concentration at 25°, pH 7.2, mM Na-phosphate buffer, 5 mM NaCl. Solid line calculated assuming $N_{1A} = 4$, $N_{2A} = 20$, $K_{1A} = 10^6\ M^{-1}$, and $K_{2A} = 1.1 \times 10^4\ M^{-1}$. See text for details.

with Mg^{2+} binding to $tRNA^{Phe}$, the binding reaction can still be monitored calorimetrically by making use of the large heat of reaction between EDTA and Mg^{2+}. The experiments were carried out in the following manner. A buffered solution of $tRNA^{Phe}$ was extensively dialyzed at 4° against a large excess volume of buffer solution containing a known concentration of $MgCl_2$. Thus, except for a small Donnan membrane correction (Tanford, 1961), the concentration of free Mg^{2+} is identical in both solutions. The total concentration of Mg^{2+} in the $tRNA^{Phe}$ solution is

$$M_t = (1 + \delta)M + r_A C_t \qquad (2)$$

where the total concentration of Mg^{2+} in the dialysate is M, C_t is the concentration of $tRNA^{Phe}$, r_A is the average number of moles of Mg^{2+} bound per mole of $tRNA^{Phe}$ in its folded state at 25° and δ is the Donnan correction factor which is small and can be approximately calculated knowing the salt concentration. Equal volumes of the $tRNA^{Phe}$ solution and the dialysate were then separately mixed with buffer solution containing an excess of EDTA in the calorimeter. The two respective heats of mixing are

$$Q(\text{tRNA}) = Q_{dil} + (1 + \delta)Q_E + r_A V C_t(\Delta H_E - \Delta H_A) \qquad (3)$$

and

$$Q(\text{buffer}) = Q_{dil}' + Q_E \qquad (4)$$

Q_{dil} and Q_{dil}' are the sum of the heats of dilution of the components in each solution and were measured separately. V is the volume of the $tRNA^{Phe}$ solution and dialysate used. ΔH_E is the molar heat of reaction between free Mg^{2+} and EDTA, and ΔH_A is the molar heat of binding of Mg^{2+} to $tRNA^{Phe}$. $Q_E = MV\Delta H_E$. It thus follows that

$$\Delta Q = Q(\text{tRNA}) - Q_{dil} - (Q(\text{buffer}) - Q_{dil}') \qquad (5)$$

$$= \delta Q_E + r_A V C_t(\Delta H_E - \Delta H_A)$$

Since $\Delta H_A = 0 \pm 100$ cal/mole

$$r_A = \frac{\Delta Q - \delta Q_E}{V C_t \Delta H_E} \qquad (6)$$

Thus a knowledge of ΔQ, V, C_t, ΔH_E, and δ allows a determination of r_A as a function of Mg^{2+} concentration. V and C_t were determined as indicated in the Experimental Section. δ was calculated in the usual manner at the various $MgCl_2$ concentrations and was found to always be less than $1.7(C_t/M)$, this high value being obtained at 2.5 mM $MgCl_2$ concentration. The value of ΔH_E, determined in a separate series of experiments by mixing known amounts of $MgCl_2$ with excess EDTA, was found to be 7.6 ± 0.1 kcal/mole under the ionic conditions of all experiments.

The derivation of eq 6 is based on two assumptions. (1) EDTA does not interact with $tRNA^{Phe}$ to produce any measurable heat and (2) the concentration of EDTA used in all experiments is sufficient to remove all Mg^{2+} bound to the $tRNA^{Phe}$. The first assumption is supported by the fact that mixing $tRNA^{Phe}$, in the absence of Mg^{2+}, with excess EDTA results in zero heat production. The second assumption merely influences the meaning of r_A. If the concentration of EDTA used failed to remove some bound Mg^{2+} because the binding constant of Mg^{2+} to $tRNA^{Phe}$ was extremely large then these experiments would fail to "measure" the number of such bound Mg^{2+}, and our estimates of r_A would be systematically lower than the true values. However, our final EDTA concentration was approximately 10^2 times greater than the final $tRNA^{Phe}$ concentration in all experiments. Therefore, the $tRNA^{Phe}$ could effectively compete with the EDTA for the Mg^{2+}, only if the Mg^{2+} binding constant for $tRNA^{Phe}$ were on the order of 10^2 times greater than the Mg^{2+} binding constant for EDTA, or about $10^{10}\ M^{-1}$.

Binding of Mg^{2+} to Folded $tRNA^{Phe}$. The values of r_A obtained as a function of free Mg^{2+} concentration at 25° are tabulated in Table I, and graphically represented in Figure 2. The details of the binding curve are not apparent in Figure 2, but a representation of the data in terms of a Scatchard plot (Scatchard, 1949) (r_A/M *vs.* r_A), shown in Figure 3, clearly exhibit the saliant features. It appears that over a range of 0–2.5 mM Mg^{2+}, Mg^{2+} binding to $tRNA^{Phe}$ can be described in terms of approximately 25 binding loci per macromolecule. Graphical extrapolation, using several representations of the data (Klotz and Hunston, 1971), indicate that the number of binding loci, N_A, is 24 ± 1. Sander and Ts'o (1971) have previously observed that a Scatchard representation of similar data for mixed yeast tRNA was linear over a concentration range of about 0.1–2 mM Mg^{2+}. Our data over the equivalent range ($r_A > 10$) can also be represented in terms of a single set of binding sites as shown by the broken line labeled A in Figure 3. The apparent binding constant obtained from the slope of that line is $1.6 \times 10^4\ M^{-1}$ which is in excellent agreement with the "intrinsic" binding constant of $1.7 \times 10^4\ M^{-1}$ reported by Sander and Ts'o (1971). However, the extreme nonlinearity of our Scatchard representation clearly shows that all sites can not be both independent and equivalent. Since the data can not be accounted for in terms of a single set of independent binding sites, the next simplest analytical model is one using three parameters—a single set of binding sites with interaction between sites. Assuming a one-dimensional finite lattice with a regular distribution of sites, possible binding curves were simulated to "fit" the data by systematically varying values for the intrinsic binding constant and the interaction parameter. No representation could be found which would "fit" the data without serious systematic deviation at either the low or high Mg^{2+} concentration portion of the curve. Consequently a model of the next highest degree of complexity, two sets of independent and identical binding sites, was assumed. It will be shown that this model represents the data satisfactorily throughout the entire range of Mg^{2+} concentrations studied.

TABLE I: Extent of Mg^{2+} Binding to $tRNA^{Phe}$ as a Function of Mg^{2+} Concentration, pH 7.2, 5 mM Na-Phosphate, 5 mM NaCl.

$[Mg^{2+}]$ (μM/l.)	r_A[a] (exp)	r_A[b] (calcd)	Δr[c]
0	0	0	0
1	2.6	2.2	−0.4
	2.8		−0.6
3	4.7	3.6	−1.1
	4.9		−1.3
5	4.2	4.4	+0.2
	4.6		−0.2
10	4.8	5.6	+0.8
	5.0		+0.6
15	9.0	6.6	−2.4
50	8.8	11.0	+2.2
	8.9		+2.1
	10.0		+1.0
90	14.3	13.8	−0.5
100	15.6	14.4	−1.2
	15.8		−1.4
200	18.8	17.7	−1.1
300	18.8	19.3	+0.5
400	23.3	20.3	−3.0
500	21.8	20.9	−0.9
600	22.2	21.4	−0.8
750	22.2	21.8	−0.4
	22.8		−1.0
1000	22.0 ± 0.7[d]	22.3	+0.3
1100	22.0	22.5	+0.5
2500	23.3	23.3	0

[a] Experimental values obtained as discussed in text. [b] Calculated values assuming $N_{1A} = 4$, $N_{2A} = 20$, $K_{1A} = 1 \times 10^6$ M^{-1}, and $K_{2A} = 1.1 \times 10^4$ M^{-1}. [c] $\Delta r = r_A$ (calcd) − r_A (exp). [d] Average of six experiments.

Recently, Klotz and Hunston (1971) derived, for the general model of m sets of independent and equivalent sites, the appropriate mathematical relationships for the intercepts and slopes obtained by the usual methods of graphical analysis of binding data. They derived expressions for four apparent binding constants which are defined by

$$K_\gamma = \sum_{i=1}^{m} N_i K_i^\gamma \qquad (7)$$

where $\gamma = -1$, 0, 1, and 2, and N_i is the number of independent and equivalent sites of type i with association constant K_i. Using these mathematical relationships, unique solutions for all relevant parameters, N_i and K_i, can be obtained only in cases with one or two independent sets of loci. Assuming two sets of sites initial estimates of $N_{1A} \cong 4$, $K_{1A} \cong 8 \times 10^5$ M^{-1}, $N_{2A} \cong 20$, and $K_{2A} \cong 10^4$ M^{-1} were obtained. Various binding curves were then computer simulated by systematically varying these initial estimates, assuming $N_A = N_{1A} + N_{2A} = 24$. The set of parameters which best fit our binding data, with an average deviation of ±0.7 mole of Mg^{2+} bound per mole of $tRNA^{Phe}$, was found to be $N_{1A} = 4$, $N_{2A} = 20$, $K_{1A} = 1 \times 10^6$ M^{-1}, and $K_{2A} = 1.1 \times 10^4$ M^{-1}. The errors in N_{1A} and N_{2A} and K_{2A} were estimated to be ±1 and ±2 × 10^5 M^{-1}, respectively. The reliability of the estimate of K_{1A} is best given by the range of values which can reasonably fit the data. If K_{1A} were less than 3×10^5 M^{-1} or greater than 2×10^6 M^{-1}, serious systematic deviation between the calculated and experimental values of r_A at low binding would be observed. This is demonstrated in Figure 3 where the broken lines labeled B and C were calculated for both of these extreme values for K_{1A} assuming the values of the other parameters to be those given above. Although the error in our estimate of K_{1A} is large, we can still safely conclude that the simplest model which can satisfactorily fit our data is one containing two sets of independent and equivalent binding sites characterized by the values given above. The adequacy of this representation is demonstrated in Table I, where calculated values of r_A are compared with the experimental values, and in Figures 2 and 3, where the solid lines were calculated using this model. The complete thermodynamic quantities, obtained from the following equations assuming a standard sate of 1 mole/l., are summarized in Table II.

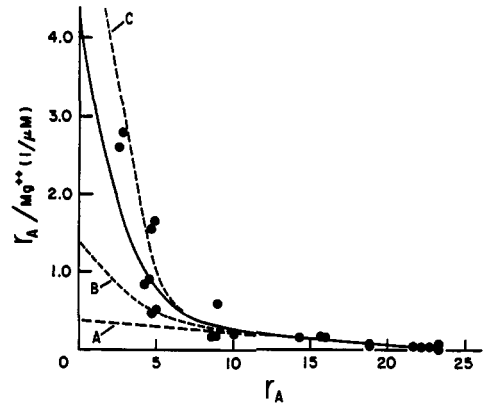

FIGURE 3: $r_A/[Mg^{2+}]$ *vs.* r_A using the data in Figure 2. The solid line was calculated using the same binding parameters as for Figure 2. Line A was calculated assuming a single set of binding sites with $N = 24$ and $K = 1.6 \times 10^4$ M^{-1}. Lines B and C were calculated assuming N_{1A}, N_{2A}, and K_{2A} were the same as for the solid line but with $K_{1A} = 3 \times 10^5$ M^{-1} and 2×10^6 M^{-1}, respectively. See text for details.

$$\Delta G^\circ = -RT \ln K$$
$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \qquad (8)$$

Binding of Mg^{2+} to Unfolded $tRNA^{Phe}$. Although the Mg^{2+} binding characteristics of folded $tRNA^{Phe}$ are important, they do not, in themselves, directly provide an explanation of the stabilization of $tRNA^{Phe}$ by Mg^{2+}. For such an explanation it is minimally necessary to know the *difference* between Mg^{2+} binding to folded and unfolded $tRNA^{Phe}$.

In principle, Mg^{2+} binding to unfolded $tRNA^{Phe}$ can be studied in a manner analogous to that just described for folded $tRNA^{Phe}$. However, in this case, the equilibrium dialysis would have to be performed at temperatures sufficiently high (greater than 70°) to maintain the $tRNA^{Phe}$ in its unfolded state at all Mg^{2+} concentrations (Levy, 1971). Unfortunately unfolded $tRNA^{Phe}$ rapidly aggregates (Levy, 1971), and because a long time is required for equilibration by dialysis, the $tRNA^{Phe}$ would irreversibly aggregate during the preparative procedure. Therefore, in order to prepare unfolded $tRNA^{Phe}$ equilibrated at all Mg^{2+} concentrations,

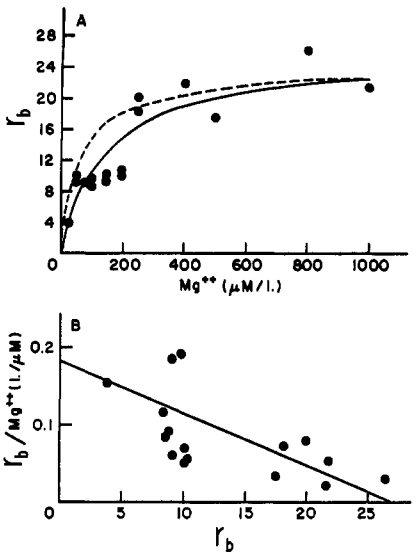

FIGURE 4. (A) Binding of Mg^{2+} to unfolded $tRNA^{Phe}$ at approximately 80°. Solid line was calculated for $N_B = 27$, $K_B = 7 \times 10^3$ M^{-1}. Dotted line is the calculated binding curve for folded $tRNA^{Phe}$ at 25°. (B) $r_B/[Mg^{2+}]$ *vs.* r_B using the data in Figure 4A. The solid line was calculated using the same binding parameters as for Figure 4A.

TABLE II: Thermodynamic Quantities for Mg^{2+} Binding to $tRNA^{Phe}$ at 25°, pH 7.2, 5 mM Sodium Phosphate, 5 mM NaCl.

Folded $tRNA^{Phe}$	Unfolded $tRNA^{Phe}$
$N_{1A} = 4 \pm 1$	
$N_{2A} = 20 \pm 1$	$N_B = 27 \pm 3$
$K_{1A} = 1 \times 10^6$ M^{-1} [a]	
$K_{2A} = 1.1 \times 10^4 \pm 2 \times 10^3$ M^{-1}	$K_B = 7 \times 10^3 \pm 2 \times 10^3$ M^{-1}
$\Delta G_{1A} = -8.2 \pm 0.5$ kcal/mole	
$\Delta G_{2A} = -5.5 \pm 0.1$ kcal/mole	$\Delta G_B = -5.3 \pm 0.2$ kcal/mole
$\Delta H_A = 0 \pm 100$ cal/mole [b]	$\Delta H_B = 0$ [c]
$\Delta S_{1A} = 28 \pm 2$ cal/(mole deg)	
$\Delta S_{2A} = 18.4 \pm 0.6$ cal/(mole deg)	$\Delta S_B = 17.8 \pm 0.7$ cal/(mole deg)

[a] See text for estimate of error. [b] Average value for all binding sites. [c] Assumed value; see text for discussion.

it was necessary to devise a method whereby equilibration could be rapidly achieved at high temperature. The technique used consisted of rapidly (~15 min) passing the $tRNA^{Phe}$ solution through a Sephadex column previously equilibrated with a known concentration of Mg^{2+} as described in the Experimental Section. The total concentration of Mg^{2+} in the eluent $tRNA^{Phe}$ solution is

$$M_t = M + r_B C_t \quad (9)$$

where M is the concentration of free Mg^{2+} and r_B the average number of moles of Mg^{2+} bound per mole of unfolded $tRNA^{Phe}$. After elution, the solution was rapidly cooled to room temperature where the $tRNA^{Phe}$ refolded, and the concentration of both free and bound Mg^{2+} changed because the binding characteristics of folded and unfolded $tRNA^{Phe}$ are presumably different. Hence, at low temperature: $M_t = M - \Delta r(C_t) + (r_B + \Delta r)(C_t)$, where Δr is the average number of Mg^{2+} which are bound to or released from $tRNA^{Phe}$ as it refolds at low temperature. This change in the concentration of free and bound Mg^{2+} does not prevent the determination of r_B as can be seen in the following equations. The heat of mixing measured at 25° (where the ΔH of binding of Mg^{2+} to the folded form is essentially zero) is given by

$$Q(\text{tRNA}) = Q_{dil} + Q_E - \Delta r V C_t \Delta H_E + (r_B + \Delta r) V C_t (\Delta H_E - \Delta H_A) \quad (10)$$

Since $\Delta H_A = 0$

$$Q(\text{tRNA}) = Q_{dil} + Q_E + r_B V C_t \Delta H_E \quad (11)$$

Thus r_B can be directly determined. The results which were obtained by this method are summarized in Figure 4. These results have a large statistical scatter (for reasons to be discussed later) and cannot be used to obtain a highly reliable estimate of the binding parameters. However, least-squares fitting of the data, assuming a single set of independent and equivalent binding sites, did provide an estimate of $N_B = 27 \pm 3$, $K_B = 7000 \pm 2000$ M^{-1}. Thus, although we cannot unequivocally conclude the existence of only one set of sites for unfolded $tRNA^{Phe}$, we can state that, over the range of Mg^{2+} concentration studied, folded and unfolded $tRNA^{Phe}$ phenomenologically possess approximately the same number of sites and that all sites are of a stronger affinity in the folded than in the unfolded form. It is also to be noted that K_B, the estimated association constant for the unfolded form, is very similar to estimates of the Mg^{2+} binding constant (K ~6000 M^{-1}) for single-strand polyribonucleotides (Sander and Ts'o, 1971). The thermodynamic quantities calculated for Mg^{2+} binding to unfolded $tRNA^{Phe}$ are also summarized in Table II.

Discussion

Sources of Error. Before beginning any discussion of the significance of these results, it is useful to clearly delineate the possible sources of error in the data. The useful sensitivity of the calorimetric instrument used in these experiments is about 10–50 μcal (including corrections for the differential heat of mixing), whereas the total amount of $tRNA^{Phe}$ used in each experiment was approximately 3–5 $\times 10^{-8}$ mole. Thus the absolute error in any measurement is on the order of 2 kcal/mole of $tRNA^{Phe}$. It is this error which is reported for ΔH_A, the heat of Mg^{2+} binding to $tRNA^{Phe}$. This represents an error in N of approximately ±0.3. An additional systematic error results from any error in the extinction coefficient (Levy, 1971) used to calculate the concentration of $tRNA^{Phe}$. This error is estimated to be less than 5% and, therefore, should not seriously affect either the accuracy of ΔH_A (which is limited only by instrument sensitivity), or the shape of the Mg^{2+} binding curves (*i.e.*, the values of the obtained binding constants). However, N_A, the extrapolated value for the total number of binding sites per $tRNA^{Phe}$ molecule, could be incorrect by 5% or approximately ±1. This error is on the

order of the statistical error of the extrapolation, and it is this error which is reported.

The errors outlined above are minor when compared to the other errors which exist. To calculate r_A, the average number of moles of Mg^{2+} bound per mole of $tRNA^{Phe}$, the measured heat had to be corrected for several other sources of heat production (*cf.* eq 4). The heats of dilution and the error in Mg^{2+} concentration due to the Donnan membrane effect were generally small, but Q_E, the heat of reaction of free Mg^{2+} with EDTA, was very large, particularly at high concentrations of free $MgCl_2$. At 1 mM free Mg^{2+}, for example, M was ten times greater than $r_A C_t$. Thus the heat in which we were interested amounted to about 10% of the actual heat measured. In our experiments Q could be determined with a precision of about $\pm 0.3\%$. At 1 mM Mg^{2+} the random error in r was therefore about ± 0.7. This is reflected in the standard error of ± 0.7 obtained for a series of 6 experiments at 1 mM $MgCl_2$ (*cf.* Table I). The significance of this error, of course, varied linearly with Mg^{2+} concentration. This, in fact, is a limitation of the calorimetric technique in measuring ligand binding when the heat of a secondary reaction is used to monitor the binding reaction. If ΔH_A had been sufficiently large, then the extent of binding could have been directly measured, as has been done for other systems (Bolen *et al.*, 1971; Bjurulf *et al.*, 1970).

While the sensitivity and precision of the calorimeter place certain limitations on the accuracy of our data, it is the reproducibility of the preparation of the solutions used in the experiments which most severely limits the accuracy of these measurements. In another series of experiments, where the thermal stability of $tRNA^{Phe}$ was measured spectroscopically as a function of free Mg^{2+}, difficulty in reproducing the data at very low Mg^{2+} concentration was observed. This apparently was the result either of not effectively achieving complete equilibrium during dialysis or of divalent cation contamination by glassware. Our laboratory, as well as others (Reeves *et al.*, 1970), has found that equilibration of the EDTA concentration across dialysis membranes is very slow. Dialysis was therefore performed for long times in the manner described in the Experimental Section. Nevertheless, the possibility of an error due to this effect exists, and such an error would produce large relative errors in the apparent free Mg^{2+} concentration, particularly at low concentrations of $MgCl_2$. For this reason Q_E was never calculated, but instead, was directly measured in the calorimeter. Even with this precaution, however, our results at very low Mg^{2+} concentration could have significant systematic error. This error is estimated to be maximally about $\pm 10^{-6}$ M in terms of the actual free Mg^{2+} concentration.

The errors resulting from equilibration of $tRNA^{Phe}$ with $MgCl_2$ on Sephadex columns were particularly large as evidenced by the scatter of the data shown in Figure 4. This was certainly not due to any limitations in the calorimetric technique, but most likely due to preparation of the $tRNA^{Phe}$ solutions. The equilibration columns were used at high temperature, where they are extremely fragile, and new columns had to be made for each experiment. The length of time during which the $tRNA^{Phe}$ was maintained at high temperature was somewhat variable ($\pm 10\%$), and it is known that some aggregation occurs in the unfolded state. Consequently, the scatter of the data for Mg^{2+} binding to unfolded $tRNA^{Phe}$ is probably the result of variation in the degree of aggregation of our samples.

Significance of ΔH_A. The finding that ΔH_A, the enthalpy of Mg^{2+} binding to $tRNA^{Phe}$ in the folded state, is equal to zero very strongly suggests that Mg^{2+} does not produce any thermodynamically significant alteration in the conformation of $tRNA^{Phe}$. If Mg^{2+} binding did produce a conformation change, then the apparent heat of binding would be given by $\Delta H_A = \Delta H_C + r_A \Delta H_{bind}$, where ΔH_{bind} is the true binding enthalpy change, and ΔH_C is the conformational enthalpy change. Consequently, the only way to observe $\Delta H_A = 0$ and simultaneously have a significant conformational change in $tRNA^{Phe}$ structure is if a fortuitous cancellation of the conformational enthalpy and the term $r_A \Delta H_{bind}$ occurred. Such a cancellation is extremely unlikely. Additional support for the lack of a conformational change comes from the fact that ΔH_A was found to be zero at two different free Mg^{2+} concentrations, where the degree of binding (r_A) varied. Since ΔH_A is independent of r_A, ΔH_{bind} must be zero, and consequently ΔH_C must also be zero. In addition no change in the absorption spectrum of $tRNA^{Phe}$ as a function of Mg^{2+} concentration was observed at 25° (Levy, 1971). We thus conclude that Mg^{2+} does not directly alter the structure of $tRNA^{Phe}$.

It is to be noted that both fluorescence changes (Eisinger *et al.*, 1970; Robison and Zimmerman, 1971a) and variation in the apparent sedimentation coefficient of $tRNA^{Phe}$ (Romer *et al.*, 1970) have been observed as a function of the total Mg^{2+} concentration. The "Y" base of $tRNA^{Phe}$ shows an enhancement of fluorescence intensity upon binding Mg^{2+} (Eisinger *et al.*, 1970; Eisinger and Lamola, 1971; Robison and Zimmerman, 1971a). This could well be due to a reduction of solvent quenching upon "tightening" of the $tRNA^{Phe}$ structure in the region where Mg^{2+} binds (Eisinger and Lamola, 1971). Romer and coworkers (1970) have observed an increase in $s_{20,w}$ of $tRNA^{Phe}$ upon addition of Mg^{2+}. This could be the result of a change in the partial specific volume of the macromolecule, which would be expected upon ion binding, but which was not taken into account. Neither of these events need be of general thermodynamic significance as reflected in the enthalpy of Mg^{2+} binding. Consequently our results do not appear to contradict any previous work.

Thermodynamics of Mg^{2+} *Binding to Folded* $tRNA^{Phe}$. The calorimetric data show that, at Mg^{2+} concentrations less than 2.5 mM, the binding of the divalent cation to $tRNA^{Phe}$ can be described in terms of two distinct independent and equivalent sets of sites. These data are quantitatively consistent with those of Sander and Ts'o (1971) over the concentration range of 2 mM Mg^{2+} to ~100 μM Mg^{2+}, as will be demonstrated. Because of the limited sensitivity of the divalent cation-specific electrode, they were unable to demonstrate the existence of the set of very strong binding sites observed in these studies. However, the existence of a strong set of divalent cation binding sites on tRNA has previously been demonstrated by proton magnetic relaxation studies (Cohn *et al.*, 1969) of Mn^{2+} binding to *Escherichia coli* bulk tRNA and purified $tRNA^{Phe}$. The results of Cohn *et al.* (1969) are qualitatively consistent with our results although they interpret their data in terms of negative cooperative interactions between strong sites in addition to the existence of a set of weaker sites. It is not clear, however, that their data require a mathematically more complicated model than the one presently proposed. Recently electron spin resonance studies of Mn^{2+} binding to *E. coli* bulk tRNA (Danchin and Gueron, 1970a,b) have indicated an apparent positive cooperative interaction between the first 6–10 Mn^{2+} bound. Such a cooperative effect has not previously been observed, and is also inconsistent with the results presented here for Mg^{2+} binding to yeast $tRNA^{Phe}$. We feel this difference is not due to intrinsic differences in the binding of Mn^{2+} and Mg^{2+}, but is probably either the result of a systematic error in one of the two studies, or more likely due

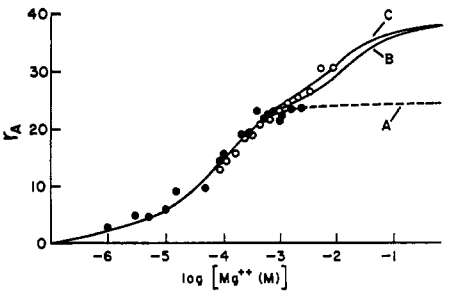

FIGURE 5: r_A *vs.* log [Mg^{2+}]. (●) represents the present data and (○) calculated (Sander, 1970) for mixed tRNA assuming 78 phosphate groups/tRNA. The latter data were obtained at a concentration of 10 mM tRNA phosphate in 1.3 mM sodium phosphate buffer. Line A was calculated assuming the binding parameters given in Figure 2. Lines B and C were calculated assuming an additional third set of independent and equivalent sites with $N_{3A} = 14$ and $K_{3A} = 70$ M^{-1} and 100 M^{-1}, respectively.

to the possibility that some tRNA species are partially unfolded at room temperature in the absence of Mg^{2+} or Mn^{2+}. This latter possibility is suggested by recent studies (Levy, 1971) of the thermodynamics of the unfolding of $tRNA^{Phe}$, which indicate that the folded form is only marginally stable at room temperature in the absence of Mg^{2+}. If several of the different species in the mixed *E. coli* tRNA used in the Mn^{2+} studies were less stable than yeast $tRNA^{Phe}$ under similar conditions, and hence partially unfolded, initial binding of Mn^{2+} would cause refolding to occur, and hence an apparent positive cooperative binding effect would be observed. Obviously the validity of this conjecture awaits further study. In the sense that both Mn^{2+} and Mg^{2+} possess at least two sets of binding sites at ion concentrations less than ~3 mM, it thus appears that the binding of the two divalent cations to tRNA is similar.

The fact that $\Delta H_A = 0$ shows that a large positive entropy change is the main thermodynamic driving force for the binding of Mg^{2+} to folded $tRNA^{Phe}$. This fact is consistent with similar results obtained for the binding of Mg^{2+} to various mononucleotides (Belaich and Sari, 1969; Zimmer *et al.*[2]) in aqueous solution. Krakauer (1971) has also concluded that Mg^{2+} binding to polyribonucleotides is thermodynamically dominated by a positive entropy change. The source of this favorable entropy change is probably due to the release of water molecules which are strongly coordinated to fully solvated Mg^{2+} (Belaich and Sari, 1969; Krakauer, 1971).

The existence of two sets of Mg^{2+} binding sites on $tRNA^{Phe}$ could be due to the conformational characteristics[3] of $tRNA^{Phe}$. A likely possibility would be that the strong sites are such that the number of coordinated water molecules in the Mg^{2+}–$tRNA^{Phe}$ complex is minimal, whereas in the weaker sites more water molecules are able to coordinate with Mg^{2+} in the complex (Danchin and Gueron, 1970b). This difference could be the result of variations in the number and geometrical arrangement of potential interacting groups of the "site."

The results which have been reported in this paper have been limited to the concentration range of 0–2.5 mM free Mg^{2+}. However, our results for $tRNA^{Phe}$ can be combined with those of Sander and Ts'o (1971) for mixed yeast tRNA to provide a representation of Mg^{2+} binding to tRNA over the range of 0–0.01 M Mg^{2+} which is shown in Figure 5. Good agreement between the two sets of experiments is observed. Our results obtained over the range of 0–2.5 mM Mg^{2+} can be described in terms of two sets of independent sites with a total of 24 loci. The dotted line, labeled A, was calculated for such a model. However, the results of Sander and Ts'o (1971) indicate additional binding beyond 24 sites/molecule at higher Mg^{2+} concentrations. Analysis of the combined results indicate that this further binding is probably best described in terms of a third set of independent sites which number approximately 14 and have an association constant, $K_{3A} \simeq 100$ M^{-1}. This conclusion is based on the observation that $dr_A/[d \ln (Mg^{2+})]$ is increasing in the vicinity 10^{-3} M Mg^{2+}. The lines labeled B and C in Figure 5 were calculated assuming a third set of independent binding sites with $N_{3A} = 14$ and $K_{3A} = 70$ and 100 M^{-1}, respectively. While reasonable agreement between the calculated and experimental curves is found, it must be understood that other models, including those with interaction between sites, could possibly be developed which would satisfactorily represent the combined results. More detailed analysis of the combined results is not warranted, however, since systematic errors between the two sets of data most certainly exist.

Thermodynamics of Mg^{2+} Binding to Unfolded $tRNA^{Phe}$. The enthalpy change of Mg^{2+} binding to $tRNA^{Phe}$ in the unfolded state, ΔH_B, has not been determined, but it is quite likely that it is also about zero. There are several reasons for this conclusion. First, if $\Delta H_B < 0$ in unfolded $tRNA^{Phe}$, one would expect stronger binding of Mg^{2+} to unfolded than to folded $tRNA^{Phe}$. This is inconsistent with these results and with the fact that Mg^{2+} thermodynamically stabilizes the folded form. Second, if ΔH_B was significantly greater than zero, one would expect much weaker binding to the unfolded form compared to the folded form than is actually observed. The difference in ΔG, the standard free energy of Mg^{2+} binding, between the weak sites of the folded form ($K \sim 1.1 \times 10^4$ M^{-1}) and the unfolded form ($K \sim 7 \times 10^3$ M^{-1}) is approximately 200 cal. If the entropy increase upon binding was identical for both forms, then ΔH_B for the unfolded form would be only 200 cal per site. Third, the thermodynamics of $tRNA^{Phe}$ unfolding as a function of Mg^{2+} concentration recently determined by Levy (1971) can not be explained in a simple fashion if ΔH_B for unfolded $tRNA^{Phe}$ is significantly greater than zero. It thus follows that the primary driving force for Mg^{2+} binding to unfolded $tRNA^{Phe}$ is a positive entropy change. Consequently, the thermodynamic characteristics of Mg^{2+} binding to both folded and unfolded $tRNA^{Phe}$ appear to be qualitatively similar.

In summary, our data indicate the existence of at least two sets of Mg^{2+} binding sites in folded $tRNA^{Phe}$, both of which are stronger than the apparent single set found for the unfolded form. In all cases the binding reaction is driven by a large positive entropy change, most likely resulting from the release of water molecules. There is no evidence of Mg^{2+} promoting a thermodynamically significant conformational change in the molecule. Thus the simplest explanation for the stabilizing effect of Mg^{2+} on $tRNA^{Phe}$ is that Mg^{2+} binds with a stronger affinity to the folded than unfolded form. This conclusion is consistent with recent thermodynamic studies on

[2] S. Zimmer, G. Rialdi, and R. Biltonen, manuscript in preparation.

[3] The possibility exists that the strong binding sites of $tRNA^{Phe}$ are due to the "unusual" base composition. This is based upon analysis of the Mg^{2+}-dependence of the thermal-unfolding transition of $tRNA^{Phe}$ which indicates that both folded and unfolded forms may have a set of strong binding sites ($K \geq 5 \times 10^5$) (J. T. Levy and R. Biltonen, to be published). While the present data neither support nor refute this possibility, it is a point worthy of further study, and will also be discussed in a following communication.

the unfolding of $tRNA^{Phe}$ as a function of Mg^{2+} concentration (Levy, 1971; Levy and Biltonen[4]).

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[4] J. Levy and R. Biltonen, manuscript in preparation.

Specificity and Spectral Resolution of an L-Glutamate Dehydrogenase–Monocarboxylic Amino Acid Complex†

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ABSTRACT: We recently reported differential spectroscopic evidence demonstrating the existence of an L-glutamate dehydrogenase–L-leucine complex. Extension of these spectroscopic studies shows that a variety of monocarboxylic amino acids can combine with the enzyme to form such a complex and that two groups of these complexes can be distinguished by their difference spectra. The difference spectra consist of two components: (1) a blue-shifted tryptophan perturbation spectrum which occurs in complexes of all of the amino acids and (2) a red-shifted tyrosine perturbation spectrum which appears only in complexes formed by amino acids possessing long aliphatic side chains. The ability of amino acids of one class to displace the amino acids of the other class in these complexes indicates a common binding site for all of the amino acids. The ligand requirements for complex formation and for formation of a 279-nm peak allow a simple estimation of the maximum distance between the two enzyme chromophores involved in the formation of this enzyme–ligand complex.

Recently we established the presence of a glutamate dehydrogenase–L-leucine complex with ultraviolet differential spectroscopic methods and suggested that this complex may be related to the leucine activation of the glutamate dehydrogenase reaction (Prough *et al.*, 1972). It was shown that the dissociation constant of this complex was approximately 270 μM and that the dissociation constant was independent of

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