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Equilibrium Binding of Magnesium(II) by *Escherichia coli* $tRNA^{fMet}$ †

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ABSTRACT: Equilibrium dialysis measurements show that $tRNA^{fMet}_1$ in 0.17 *M* Na^+ has one strong Mg^{2+} binding site, $K = 3 \times 10^4\ M^{-1}$, and approximately 26 weak binding sites with $K = 4 \times 10^2\ M^{-1}$, with RNA concentration measured in moles of tRNA per liter and $T = 4°C$. The data fit significantly less well to a model with two strong sites and a large class of weak sites. Binding is noncooperative. Our results differ from previous experiments showing cooperative binding because the binding equilibrium is not coupled to a cooperative conformational change of the macromolecule. Measurements at relatively high Na^+ concentrations and low temperature ensure that the tRNA is in the "native" region of the conformational phase diagram for all Mg^{2+} concentrations.

Even though the three-dimensional structure of yeast phenylalanine tRNA has been determined (Kim et al., 1973; Robertus et al., 1974) and good evidence exists that all tRNAs may have a similar structure (Kim et al., 1974; Klug et al., 1974), many details of tRNA function remain to be understood. Two obvious unanswered questions are the role of the tightly bound Mg^{2+} ions, and the possible importance of tRNA conformational changes when bound to the ribosome. In this and the following paper we explore these interrelated questions. For two reasons our attention will be restricted to the behavior of tRNA separated from other macromolecular components. First, thorough characterization of the binding and conformational properties of purified tRNA seems a necessary prelude to understanding more complex systems. Second, and more persuasively, we have to admit that we do not yet possess techniques for definitive characterization of tRNA conformational properties in more complex mixtures.

The purpose of this first paper will be to establish some simple facts about the equilibrium binding of Mg^{2+} by tRNA. It has been known for some time that divalent metal ions markedly stabilize the "native" conformation of tRNA (Fresco et al., 1966). Equilibrium studies of the binding of divalent (or trivalent) ions to tRNA have been reported from several laboratories (Cohn et al., 1969; Danchin and Gueron, 1970; Sander and Ts'o, 1971; Danchin, 1972; Rialdi et al., 1972; Schreier and Schimmel, 1974; Wolfson and Kearns, 1974; Kayne and Cohn, 1974; Jones and Kearns, 1974). In the investigations in which measurements extended to low ratios of cations/tRNA it has usually been found that ion binding is cooperative: the first ions added are not bound as tightly as are subsequent increments. An exception is the report by Jones and Kearns (1974) of noncooperative Eu^{3+} binding.

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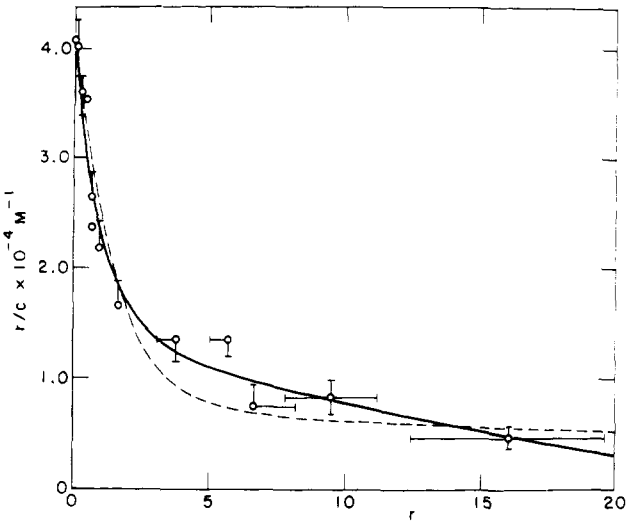

FIGURE 1: Scatchard plot of Mg^{2+} binding to $tRNA^{fMet}_1$ in 0.17 M Na^+, phosphate-cacodylate buffer (pH 7.0, 4°C). The solid curve is the best fit to eq 1 for $n_1 = 1$. The dashed curve is the best fit to eq 1 for $n_1 = 2$.

Earlier work from our laboratory (Cole et al., 1972) points out a simple reason for cooperative Mg^{2+} uptake. Most studies of divalent ion binding have begun with tRNA solutions in low (<10 mM) Na^+ concentrations, without Mg^{2+}. Under these conditions the phase diagram of all tRNAs so far examined (Cole et al., 1972) shows an "extended form" conformation that is different from native tRNA. Addition of Mg^{2+} converts the molecule to the native structure. Binding is expected to be cooperative because it is coupled to a macromolecular conformational change.

At the same time, the phase diagram, and further conformational studies (Yang and Crothers, 1972; Stein and Crothers, 1976), suggests conditions for avoiding this coupling and hence observing the binding equilibrium by itself. When the Na^+ concentration is reasonably high (>50 mM) no appreciable slow conformational change can be observed on adding Mg^{2+} to $tRNA^{fMet}_1$ or $tRNA^{Tyr}_1$ (both *Escherichia coli*). (The same statement does *not* apply to "denaturable" tRNAs like yeast $tRNA^{Leu}_3$ or *E. coli* $tRNA^{Glu}_2$.) Hence for $tRNA^{fMet}_1$ we expect no coupling of binding to a conformational change when the experiment begins at high Na^+ concentration.

The experiments reported here show that binding of Mg^{2+} to the high salt (native) form of $tRNA^{fMet}_1$ is noncooperative. There is one strong Mg^{2+} binding site, and a larger number of much weaker sites. We should caution that these results may depend on the tRNA, since experiments on wheat germ $tRNA^{Gly}_1$ (A. Stein, unpublished results) show more than one strong binding site, although again the binding is noncooperative.

In the following paper we explore the influence of Mg^{2+} on the thermodynamic and kinetic properties of $tRNA^{fMet}_1$. One important conclusion of that work related to Mg^{2+} binding is that the strong binding site disappears when the tertiary structure unfolds. This puts some limits on where the site could be located in the three-dimensional structure.

Materials and Methods

(*a*) *Buffer.* tRNA samples for equilibrium dialysis were dissolved in a buffer containing 1 mM phosphate, 10 mM sodium cacodylate, and 154 mM sodium perchlorate (pH 7) (total Na^+ concentration 0.166 M).

(*b*) *Transfer RNA.* Purified uncross-linked $tRNA^{fMet}_1$ was obtained from Oak Ridge National Laboratory. We are grateful to Dr. Z. Egan, Dr. A. D. Kelmers, and Dr. P. E. Cole for their cooperation in providing this sample.

The methionine acceptor activity was 2000 ± 50 pmol/A_{260} unit. Samples were prepared for the optical measurements by dialysis against 0.05 M EDTA-containing buffer (pH 7) to remove divalent ions and then by extensive dialysis against buffer containing the stated Mg^{2+} concentration.

(*c*) *Equilibrium Dialysis Measurements.* The plexiglas dialysis cell consisted of two compartments separated by a membrane fashioned from Fisher 1.3-in. dialysis tubing. tRNA (1.0 ml) in buffer at a concentration of 20.5 μM was dialyzed against 150 ml of buffer at the desired Mg^{2+} concentration for 48 hr or more in a cold room thermostated at 4°C. The larger compartment was stirred using a Teflon-coated magnetic stirrer. Mg^{2+} concentrations of both compartments were measured using a Perkin-Elmer Model 305 B graphite furnace atomic absorption spectrophotometer, and the concentration of tRNA was measured by its absorbance at 260 nm after a 50-fold dilution with 5×10^{-4} M Mg^{2+} containing buffer.

Samples from each compartment were prepared for analysis against standards which were concurrently prepared in the same way. Samples (20-μl) from each compartment were added to 20 μl of 0.1 M EDTA (pH 10) and 20 μl of water, and then diluted to read approximately 1 OD (at the primary Mg line 285 nm), about 1 μM for the 20-μl injection used. The compartments were refilled after sampling. Magnesium acetate standard solutions (20-μl) were prepared in the same manner except that 20 μl of buffer to which no Mg^{2+} had been added replaced the 20 μl of water. This buffer was determined to be 0.28 μM in Mg^{2+} by the method of additions (Slavin, 1968). It was found that addition of EDTA (pH 10) eliminated chemical interference from the buffer and tRNA and produced calibration curves nearly linear to an OD of 2. Polyethylene containers were exclusively used, and all containers, pipets, and sampling syringes (nonmetal) were extensively rinsed with doubly deionized water (Mg^{2+} concentration $<10^{-8}$ M) and dried before use.

It was demonstrated that equilibrium was attained in the dialysis experiment. Mg^{2+} was dialyzed in for about half of the points and out for the rest. No concentration difference existed between compartments in the absence of tRNA. Also, tRNA absorbance measurements indicated that the tRNA compartment did not leak. With the high Na^+ concentration used the Donnan effect is negligible. The integrity of the tRNA throughout the dialysis run was demonstrated by the reproducibility of points on the binding isotherm and by assay and relaxation measurements upon completion of the run.

Results and Discussion

Figure 1 shows the binding data represented as a Scatchard plot. The error bars represent the error propagated into the quantities r (Mg^{2+} bound per tRNA) and r/c by an error of 3% in measuring concentrations, where c is the free Mg^{2+} concentration. The curve through the points is given by

$$\frac{r}{c} = \frac{K_1 n_1}{1 + K_1 c} + \frac{K_2 n_2}{1 + K_2 c} \qquad (1)$$

for two classes of noninteracting sites where $K_1 = 2.94 \times 10^4$ M^{-1}, $n_1 = 1$, $K_2 = 4.17 \times 10^2$ M^{-1}, $n_2 = 26$ (obtained

by a simple iterative procedure). The dotted curve corresponds to the best fit to two classes of independent sites with $n_1 = 2$. The other parameters are: $K_1 = 1.77 \times 10^4\ M^{-1}$, $K_2 \rightarrow 0$, $n_2 \rightarrow \infty$ such that $K_2 n_2 = 0.5 \times 10^4\ M^{-1}$. This model fits the data significantly less well. Due to the high Na^+ concentration the weak sites could not be accurately characterized. However, a value of 27 for the total number of sites is in close agreement with the values reported by others, most of which were at lower Na^+ (Cohn et al., 1969; Danchin and Gueron, 1970; Sander and Ts'o, 1971; Danchin, 1972; Rialdi et al., 1972; Schreier and Schimmel, 1974). Since competition with Na^+ is expected to decrease the apparent Mg^{2+} binding constant, but probably not greatly affect the number of sites, the parameters corresponding to the solid fitted curve in the figure are more likely to be correct. For a class of weak sites similar to that observed by others, considering the competition with Na^+, the steep decrease in r/c at small r values is not consistent with $n_1 > 1$. A value of $n_1 = 2$ can be accommodated, although with significantly worse fit to the data, if there exists a very large class of extremely weak sites. A model with several classes of sites could clearly be adjusted to fit the data accurately.

Neither the relaxation measurements reported in the following paper (Stein and Crothers, 1976) nor our previous considerations of the phase diagram show evidence for any new cooperative interaction regions when Mg^{2+} is added to $tRNA^{fMet}_1$ in 0.17 M Na^+. Hence it is not surprising that Mg^{2+} binding is not cooperative since it is not coupled to a conformational change of the tRNA. In contrast, Danchin (1972) reported that Mn^{2+} binding to *E. coli* $tRNA^{fMet}$, several other purified tRNAs, and unfractionated tRNA exhibited cooperativity within the strong class of binding sites (approximately 6). Identical Scatchard plots were reported for yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Val}_1$, and *E. coli* $tRNA^{fMet}$. Unfractionated *E. coli* tRNA and *E. coli* $tRNA^{Tyr}$ were qualitatively similar. Schreirer and Schimmel (1974) have also reported similar results for yeast $tRNA^{Phe}$. The measurements of Danchin were performed in sodium-free 0.1 M triethanolamine hydrochloride buffer which probably corresponds to the low salt region of the tRNA phase diagram. It is possible that the same is true of the Schreier and Schimmel measurements. For *E. coli* $tRNA^{fMet}_1$, $tRNA^{Val}_1$, and $tRNA^{Tyr}_1$, at least, a large cooperative conformational rearrangement occurs as Mg^{2+} is added to the low salt buffer due primarily to electrostatic effects (Cole et al., 1972). This cooperative rearrangement would be expected to cause cooperative binding of divalent ions since the compact "native" tRNA is expected to bind divalent ions more strongly than the electrostatically "extended" form of tRNA.

The result that $tRNA^{fMet}_1$ has but a single strong binding site is somewhat surprising. Some evidence exists for a strong metal ion binding site in the region of the D helix in some tRNAs. Jones and Kearns (1974) using nuclear magnetic resonance (NMR) found that several Eu^{3+} ions are tightly bound to yeast $tRNA^{Phe}$, and that the binding is sequential rather than cooperative in high Na^+ salt. The resonance corresponding to GC(11) in the D arm exhibits a pronounced downfield shift with Eu^{3+}/tRNA ratios less than 1.0. The base pair AU(6) resonance in the acceptor stem is also gradually shifted by a large amount with Eu^{3+}.

Wolfson and Kearns (1974) studied Eu^{3+} binding to *E. coli* $tRNA^{fMet}$ (probably a mixture of 1 and 3) by fluorescence decay techniques. The Eu^{3+} fluorescence arises predominantly by 4-thiouridine sensitized energy transfer indicating that the binding sites observed are located very close to the 4-thiouridine residue at position 8. A 10^{-4} M solution of Eu^{3+} in 0.15 M Na^+ buffer was titrated with tRNA. Two lifetimes are resolved. The integrated intensity corresponding to the short-lived emission sharply peaks at a ratio of 3 Eu^{3+} ions/tRNA, and the long-lived component plateaus at 1 Eu^{3+}/tRNA. They conclude that there are at least two different types of strong Eu^{3+} sites by the observation of more than one emission lifetime. It is also concluded that at least two of the approximately three strong sites are located very close to the 4-thiouridine, and that Eu^{3+} binding is much stronger than the binding of Mg^{2+}.

Wolfson and Kearns' data, however, show that at a ratio of 1 Eu^{3+}/tRNA the long-lived component of the fluorescence decay has reached its maximum intensity whereas the short-lived component is near zero intensity. This result implies that there exists a single binding site very close to the 4-thiouridine residue which has a greater affinity for Eu^{3+} than the other class of sites in this region which become occupied at higher Eu^{3+}/tRNA ratios. This agrees with our finding of a single strong Mg^{2+} binding site.

Of particular interest is the location of the single strong Mg^{2+} binding site in $tRNA^{fMet}_1$. Data in the accompanying paper (Stein and Crothers, 1976) indicate that the strong site is lost when the tertiary structure unfolds. Hence the site must be located in the D arm–tertiary structure region. Furthermore, since Na^+ ions do not compete effectively for binding, the site must have a special chelation geometry for phosphate binding, or it may possibly allow ligation of Mg^{2+} to electronegative atomic centers. Two alternatives which meet these criteria are suggested by the structural work on $tRNA^{Phe}$, either of which could probably be adapted to $tRNA^{fMet}_1$. Robertus et al. (1974) single out an interesting site with an ion chelated between the oxygens of phosphates 23 and 24 and the amino nitrogens of G(25) and G(45). Kim et al. (1974) suggest that the three electronegative atoms N(7) of G(9) and O(6) and N(7) of G(12) form an arrangement which could easily bind a metal cation. $tRNA^{fMet}_1$ has a G(12)·C(24) base pair and a G in position 9, thereby possibly forming the same arrangement of electronegative atoms.

Finally, we would like to warn against generalizing our finding of one tightly bound Mg^{2+} to other tRNAs. Work on wheat germ $tRNA^{Gly}_1$ (A. Stein, unpublished results) indicates more than one strong site. Since the functional role of the tightly bound Mg^{2+} is not known, there is no reason to assume that Mg^{2+} binding cannot differ from one tRNA class to another. Specifically, Mg^{2+} binding may be different for initiator tRNAs. A number of purified tRNAs will have to be examined before this point can be settled.

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Conformational Changes of Transfer RNA. The Role of Magnesium(II)†

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ABSTRACT: Magnesium ions added to $tRNA^{fMET}_1$ selectively stabilize the dihydrouridine helix–tertiary structural region. Low Mg^{2+} levels have little direct effect on the remaining three cloverleaf helices, but these are prevented from melting independently when their intrinsic T_m is surpassed by the T_m of the tertiary structure. At high Mg^{2+} concentration the thermal unfolding of $tRNA^{fMet}_1$ is approximately a two-state, concerted transition from the globular native structure to the random coil, in contrast to the sequential unfolding observed without Mg^{2+}. We interpret the kinetics of refolding to mean that the D helix serves as a required nucleus for the rate-limiting step of tertiary structure formation. We found that unfolding of the tertiary structure leads to loss of the tightly bound Mg^{2+} ions, and showed with a Mn^{2+}-sensitive fluorescent indicator that the rate of Mn^{2+} release is the same as the rate of unfolding the tertiary structure. Hence the tightly bound divalent ion must be located in a site formed by the tertiary structure–D helix region of the molecule.

Many biological macromolecules, both proteins and nucleic acids, undergo conformational changes in the course of their function. Hemoglobin when binding oxygen, and DNA while undergoing replication are well-known examples. Conformational changes must occur during the complex reactions of ribosome-mediated protein synthesis, but the mechanical events in this fundamental biological process remain obscure. One of the key components in protein synthesis is tRNA. Its biochemical role as carrier of amino acid and anticodon are well known. More hypothetical is the possibility that tRNA performs an important mechanical function in protein synthesis by undergoing major conformational changes when bound to the ribosome. There is some evidence suggesting that tRNA structure may unfold when bound to the 30S subunit (Schwarz et al., 1974; Erdman et al., 1973). Furthermore, one can see distinct mechanistic advantages in unfolding enough of the tRNA tertiary structure to allow independent motion of amino acid acceptor and anticodon portions of the molecule (Crothers, 1975).

Our current series of experiments has focused on conformational changes of purified $tRNA^{fMet}$ in solution. Some, but certainly not all, of these changes may be related to tRNA function; others need to be characterized in order to understand how newly synthesized tRNA (or its precursor) can fold up into the native structure. The molecular mechanism of thermal unfolding of $tRNA^{fMet}$ in the absence of Mg^{2+} has previously been investigated in some detail by a combination of relaxation kinetics and nuclear magnetic resonance (NMR) spectroscopy (Crothers et al., 1974). Four distinct relaxations were found and characterized, corresponding to effectively independent sequential melting of the tertiary structure–D helix region and the three remaining helical arms.

In this paper we present the effect of increasing concentrations of Mg^{2+} on these known relaxations. The influence of Mg^{2+} on the thermal stability and dynamic properties of particular regions of the molecule is elucidated, as is the molecular pathway of thermal unfolding in the presence of Mg^{2+}. We also present a mechanism for the rate-determining step in refolding the native structure, involving formation of tertiary bonds to the D helix, which serves as a re-

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