The conformation of the tRNA^{Phe} anticodon loop monitored by fluorescence

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ABSTRACT

The intrinsic fluorescence of the Wye base was used to study the conformational change of the anticodon loop of yeast tRNA^{Phe} brought about by the addition of magnesium. The fluorescence emission and excitation spectra show dramatic changes as magnesium is added to the solution. The rotational relaxation time changes from 6 nsec without added magnesium to 33 nsec with 10 mM magnesium at an ionic strength of 0.1 M. Stern-Volmer quenching by iodide or iodoethanol shows greater access of the base to the quencher with no added magnesium. A plausible interpretation of this data is that the base stack of the anticodon loop is altered by tilting or twisting the Wye base with respect to the adjacent bases and the base becomes parallel to its neighbors upon the addition of magnesium.

INTRODUCTION

The role of magnesium in tRNA structure has been extensively explored (1). Such varied techniques as laser light scattering (2), X-ray diffraction (3,4,5), NMR (6), fluorescence (7), electron spin resonance (8), viscosity, absorbance and sedimentation velocity (9) have all been directed at an attempt to assess the role of this required cation. Recently, it has been suggested that there is no specific role for magnesium and that the putative specific effects are in actuality only the neutralization of the anionic phosphate backbone (10). Reality is probably somewhere between extremes: the binding is specific in that the size of the hydration sphere of magnesium allows it to fit in precise locales from which larger cations are excluded whereas smaller ones fit too loosely and of course this binding is driven by the necessity of neutralizing charge.

This study shall focus on the intrinsic fluorescence of yeast tRNA^{Phe} as modulated by the addition of magnesium. The intrinsic fluorophore, Wybutine (Wye) is located in the anticodon loop at position 37. The X-ray diffraction studies of Quigley et al. (3) and Holbrook et al. (4) locate one magnesium in the anticodon loop possibly coordinated to phosphate 37. The

fluorescence of Wye is dramatically sensitive to magnesium; the shape of the emission spectrum changes, the polarization, lifetime and quenching properties are all sensitive to magnesium. The spectral changes have been interpreted as representing different tRNA conformations but the question arises as to what is the low magnesium conformation.

The conformation of tRNA in the presence of magnesium is, by all solution techniques, consistent with the crystal structure. As tRNA will not crystallize without magnesium, (a definite clue to the importance of this ion in structural maintenance) it is left to spectral studies to deduce the low (or no) magnesium conformation. Two of the suggested structures (interpreting the conformation at different levels) are a hinged tRNA molecule which opens and closes in response to magnesium (11) or a base stack change in the anticodon loop (12) to a 5' stack. Neither of these structures is probably totally correct but facets of each are incorporated in the true structure. Recognition of the dynamic nature of nucleic acid structures is now part of all structural descriptions of these molecules. A stack change of some sort is the most obvious interpretation of the fluorescence. However, it remains to be determined if, in fact, this is a change between 3' stack and 5' stack. The quenching data yields a quenching constant (this study) for the no magnesium situation which is too small to be consistent with the base being fully exposed to solvent. Another type of base stack change seems to fit the data.

In addition to the anticodon loop magnesium obviously binds to other regions of tRNA resulting in conformational changes of the entire molecule, e.g. hydrodynamic properties such as the diffusion coefficient (2,11) and the rotational relaxation time (this study) change in response to the amount of magnesium present. The diffusion coefficient and fluorescence changes promoted by magnesium do not seem to be mere polyelectrolyte effects as maintaining a constant ionic strength with and without magnesium does not give the same diffusion coefficient nor the same fluorescence. There is a wealth of data to utilize in the attempt to describe the nature of the stack change. The biological relevance of different tRNA conformations is unknown but observations on phenomena such as codon recognition (13) imply that the conformational dynamics of tRNA may be of importance.

MATERIALS AND METHODS

Yeast tRNA^{Phe} was purchased from Boehringer Mannheim. The tRNA was dissolved in a buffer composed of 10 mM Tris, pH 7.4, and 100 mM KCl, dialyzed overnight, and used with no further purification. The tRNA stock

solutions were maintained in this buffer to avoid aggregation (14). Magnesium was added to the buffer from a 1 M stock solution of MgCl_2 . As no attempt was made to remove the magnesium included in the sample of tRNA, the sample referred to as no magnesium is actually no added magnesium. Concentration of tRNA was determined by absorbance at 260 nm, $\epsilon = 5.02 \times 10^5$ (15). In order to obtain good fluorescence data the concentration of tRNA was varied between 1×10^{-6} M and 8×10^{-6} M (i.e., below 1×10^{-6} M the fluorescence signal is too small and above 8×10^{-6} M the inner filter effect will reduce the intensity).

Fluorescence spectra were measured using the SLM 8000 spectrofluorimeter equipped with glan-thompson polarizers in a T configuration. The cell compartment temperature was controlled by a Lauda RC3 water bath. The data for the Perrin plots was obtained by adding spectroscopic grade glycerol to tRNA solutions (percentages determined by weight), then varying both percentage of glycerol and temperature to change viscosity. The polarization was measured ratiometrically measuring $(I_{\parallel}/I_{\perp})_V$ and $(I_{\parallel}/I_{\perp})_H$ where $I_{\parallel}/I_{\perp}$ is the ratio of emission intensity at emission polarizer settings parallel and perpendicular to the excitation and V or H refer to the excitation polarizer orientation. The polarization is then calculated from the relation

$$P = \frac{R_N - 1}{R_N + 1}$$

where

$$R_N = \frac{(I_{\parallel}/I_{\perp})_V}{(I_{\parallel}/I_{\perp})_H}$$

Due to the weak signal produced by Wye, a background subtraction was done for all polarization measurements (16).

Stern Volmer quenching experiments were analyzed according to the relation:

$$F_0/F - 1 = K[Q]$$

where $K = k\tau_0$, and F_0 and F are the fluorescence emission intensities in the absence and presence of the quencher, $[Q]$, τ_0 is the lifetime of the fluorophore with no quencher present and k is the rate constant for quenching.

The lifetime at the wavelengths used for the Perrin and quenching experiments, namely excitation at 320 nm and emission at 430 nm was measured by phase resolved lifetime measurements on the SLM 4800 fluorimeter. The lifetime was evaluated using both a scattering reference and a sample reference according to the relations

$$\tau = (1/2\pi f) \tan\phi$$

$$\tau = (1/2\pi f)(1/M^2 - 1)^{1/2}$$

where τ is the lifetime, f is the modulation frequency, ϕ is the phase of the emitted light and M is the modulation of the emitted light.

RESULTS

The effect of magnesium on the fluorescence emission spectrum of Wye in yeast tRNA^{Phe} is rather dramatic as is shown in Figure 1. There are two emission peaks which vary in intensity relative to one another in response to magnesium. The excitation spectra (Figures 2 and 3) show that each emission peak has a different excitation maximum. The intensity is quite different for the two excitation profiles as indicated in the figure legend. The spectra in figure 3 are so much more intense than those in figure 2, that the photomultiplier voltage had to be reduced in order to record the excitation spectra for the 455 nm emission. These spectra clearly show that more than one electronic transition is being measured in the fluorescence of Wye base in tRNA^{Phe}. Elucidation of the complicated nature of the overlapping transitions will require further experiments. As would be expected from such complicated emission spectra, there is a heterogeneity in the lifetimes also. One indication of a heterogeneous sample is that the lifetimes as calculated from phase and modulation are different (17). Such was the case detected for

Figure 1. Emission spectra of yeast tRNA^{Phe} with no added magnesium (1) and with 15 mM magnesium added (2). The excitation wavelength was 315 nm.

Figure 2. Excitation spectra of yeast tRNA^{Phe} with no added magnesium (1) and with 15 mM magnesium added (2). The emission wavelength was 405 nm. The photomultiplier voltage was 450 V.

Wye in either buffer system. At the excitation and emission wavelengths of 320 and 435 nm the apparent lifetime is the same for both phase and modulation with values of 6.1 nsec when magnesium is present and 4.5 nsec when no magnesium is added at frequencies of modulation of 18 and 30 MHz. These lifetime values were used for evaluating the quenching and Perrin data.

The Perrin plot (Figure 4) was constructed using glycerol to change the viscosity of the solution. The points plotted are averages of at least two

Figure 3. Excitation spectra of yeast tRNA^{Phe} with the emission wavelength equal to 455 nm. The numbering is the same as Figure 2. The photomultiplier voltage was 250 V.

Figure 4. Perrin plot of polarization. Exciting wavelength is 335 nm and the emission is monitored at 405 nm. Panel A, plus 15 mM magnesium and panel B, no added magnesium

different measurements. The data were accumulated by adding a weighed aliquot of glycerol, then measuring at temperatures between 10°C and 30°C, and repeating with more glycerol. No temperature variation in the fluorescence emission was found within this temperature range, likewise no fluorescence variations were detected up to 50% (w/w) glycerol. Table 1 summarizes the fluorescence parameters. The rotational relaxation times from the Perrin plot show considerable variation with and without added magnesium.

There are at least two possible effects being reflected in the Perrin plot; polyelectrolyte behavior and a specific magnesium effect. The fluorescence emission spectra are sensitive to ionic strength but the Wye spectral pattern in magnesium containing buffers (see Figure 1 curve 2) cannot be elicited without magnesium; i.e., the fluorescence intensity can be

TABLE I. FLUORESCENCE PARAMETERS

Solution	Ionic strength	Quantum Yield ^a	P_0^b	τ^c	ρ^b
no Mg^{++} added	.11 M	.04	.308	4.5 nsec	37 nsec
15 mM Mg^{++}	.155 M	.09	.308	6.1 nsec	36 nsec

^a Quantum yield relative to Quinine sulfate (18).

^b Calculated from the Perrin Plot shown in Figure 4.

^c Lifetimes measured by phase modulation fluorimetry excited at 320 nm and emission monitored at 430 nm.

increased by adding more salt but there is no change in the shape of the no magnesium emission spectrum and the value of the polarization does not increase as it does when magnesium is added. For these reasons it was assumed that there were two effects. To pursue this thesis Perrin plots were performed for a no added magnesium sample and a 10 mM magnesium sample at ionic strengths of 0.05, 0.1, 0.15 and 0.2 M. These results are summarized in Table II. These conditions were chosen in order to allow comparisons with the laser light scattering data (11).

The stack change from a 3' anticodon loop stack to a 5' stack would remove base 37 (Wye) from a stack thus exposing this base to solvent which should make it much more readily quenched. This should be experimentally accessible by variations in Stern-Volmer quenching, these experiments were

TABLE II. IONIC STRENGTH VARIATION PERRIN DATA^a

A. 10 mM Mg^{++}	ionic strength	P_0	ρ
	.05	.24	25 nsec
	.10	.24	33 nsec
	.15	.24	41 nsec
	.20	.24	41 nsec
B. no added Mg	.05	.12	4.0 nsec
	.10	.12	6.0 nsec
	.15	.12	4.0 nsec
	.20	.12	0.6 nsec

^a The lifetimes at 4.5 and 6.1 nsec were used to calculate the data and the excitation and emission wavelengths were 320 and 430 nm.

Figure 5. Stern-Volmer quenching for yeast tRNA^{Phe}. The solid circles show KI as a quencher and the open circles show iodoethanol as a quencher. Panel A represents tRNA with no magnesium added whereas Panel B shows tRNA plus 15 mM MgCl₂.

performed. The traditional choice for a quencher has been iodide salts (usually potassium iodide). As polynucleotides are polyanions this does not seem to be the most appropriate type of quencher (see Lehrer, 19 for the effect of charged polypeptides on iodide quenching). Acrylamide was used as a quencher, to no avail; it does not quench the Wye base fluorescence. (Data not shown.) Finally, iodoethanol was tried as a nonionic, small, potential quencher. This is probably a better quencher for Wye base and for most polynucleotides. The quenching data is better behaved for iodoethanol.

TABLE III. QUENCHING DATA

<u>Solvent</u>	<u>Quencher</u>	<u>K</u>	<u>k</u>
no Mg ⁺⁺	KI	3.5	7.8×10^8
+ Mg ⁺⁺	KI	3.1	4.8×10^8
no Mg ⁺⁺	Iodoethanol	5.4	1.2×10^9
+ Mg ⁺⁺	Iodoethanol	4.2	6.9×10^8

Figure 5 shows quenching for tRNA with no added magnesium (A) and shows quenching with magnesium added (B). The KI quenching is less linear (solid circles in both figures) than iodoethanol and the intensity measurement for KI quenching required waiting for the signal to stabilize. The iodoethanol signal was stable with no time fluctuations after addition of subsequent aliquots. Table III reports the values extracted from the slope of the lines in Figure 5. The variability in the quenching constant K , or quenching rate constant k , supports the conclusion that KI is not a good quencher for Wye base in tRNA. Both sets of data are consistent with the contention that Wye base is more exposed in the very low magnesium situation. It is interesting to compare the low quenching rate constant of 7×10^8 to that found for ethidium bromide intercalated into DNA of 1×10^8 (20), as presumably in both case base stacking is protecting the fluorophore from collision with the quencher.

DISCUSSION

The sensitivity of Wye base fluorescence to magnesium is well documented (7,12,13 and many more). There is general agreement that the fluorescence in a buffer containing magnesium and a relatively large amount of salt (typically 100 mM) results from a tRNA structure that is close to the crystal structure. The question has become how to characterize the low magnesium form. The dramatic increase in quantum yield and lifetime are presumed to result from favorable stacking interactions in the anticodon loop (21,22). The most prevalent interpretation for the low magnesium form is a change of anticodon loop structure to a 5' stacked form (7,12,23). The amount of information available from Wye fluorescence is both varied and intrinsically interesting. Erlich et al. (7) just refer to stacked and unstacked forms of the anticodon loop, whereas Rigler et al. (24) and Labuda and Pörschke (25) prefer the interpretation of multiple conformations of the anticodon loop with magnesium selecting a specific conformation. The overlapping transitions shown in Figures 1, 2, and 3 could be interpreted as either multiple transitions for the Wye base or populations of different environments for Wye with magnesium changing the proportions. At this point these interpretations do not appear to be separable with current data; more lifetime and other data are required. Recognizing this uncertainty, the assumption of multiple conformations will be presumed.

The data in Table I give essentially the same quantum yield and P_0 as that observed by Eisinger et al. (22) but the lifetimes and rotational relaxation times show interesting variations. In a previous study measuring

the anisotropic decay of tRNA^{Phe} (21), lifetimes of 6.2 nsec with 0.0026 M Mg⁺⁺ and 4.4 nsec with no added magnesium were found which are in excellent agreement with the lifetimes reported here (Table I) which were measured using phase modulation. This indicates that as little as 0.0026 M Mg⁺⁺ can promote the spectral shift to the magnesium form. This is not meant to imply that the lifetime data are all in agreement; the discrepancies seem to be a result of where within the multiple transitions the lifetime is being measured. The phase modulation measurements show the existence of multiple transitions with multiple lifetimes; excitation at 320 with emission at 430 had lifetimes of 6.1 and 4.5 nsec (this study and 21) but Blumberg et al. (23) exciting at 336 nm and monitoring emission above 400 nm found a lifetime of 4.6 nsec with 10 mM magnesium and Ehrlich et al. (7) exciting at 313 nm with the emission monitored at 435 found lifetimes ranging from 2 nsec with no magnesium to 4.5 nsec with 10 mM magnesium added. Thusly the two studies using the same spectral excitation and emission (this study and 21) show agreement in the lifetime but varying either the excitation or emission wavelengths result in different lifetimes (7 and 23). The spectra reported in Figures 1, 2 and 3 are a summation of overlapping transitions for which phase resolution fluorescence measurements gave a single, lifetime only at 320 nm and 430 nm. Hopefully, future experiments using the technique of phase suppression should resolve the transitions (17).

The other effect, namely the rotational relaxation time is also complicated, Beardsley et al. (21) got 9.6 nsec and 9.2 nsec at ionic strength of 0.028 M with and without magnesium. The experimental conditions closest to theirs is the 0.05 M ionic strength where the rotational relaxation times are 25 nsec and 4 nsec (see Table II). There is thusly a large difference in these values. It is possible that the discrepancy of 9.6 nsec versus 25 nsec represents an ionic strength effect or not enough magnesium. Whereas a rotational relaxation time of 20 nsec was found at an ionic strength of .035 M in 10 mM magnesium (23) which compared favorably to 25 nsec at .05 M also with 10 mM Mg⁺⁺.

The existence of tRNA conformers with different magnesium affinities was suggested by Rigler et al. (24) measuring fluorescence anisotropic decay of a tRNA^{Phe} with ethidium bromide covalently substituted for Wye. The anisotropic decay was best fit with 2 rotational relaxation times, 14 and 29 nsec (one for each lifetime). The data in Table II taken from comparable solution conditions shows 6 and 33 nsec. The agreement between these two types of measurements is probably as good as is possible as one is measuring

a steady state population (Perrin plots) and the other the polarized decay after a nanosecond excitation pulse.

Another technique which has focused on the separation of ionic effects and magnesium effects is laser light scattering (11). The data in Table II were obtained at solution conditions similar to those used in the laser light scattering study. The Wye base fluorescence rotational relaxation time shows distinct differences with and without magnesium as does the translational diffusion study but that is about the only similarity. Laser light scattering shows large changes in $D_{20,w}^0$ between .1 and .2 ionic strength with 10 mM Mg^{++} the fluorescence data does not show large changes. The data presented in Table II does show very striking differences between the no added magnesium and the 10 mM magnesium conditions regardless of the ionic strength. Also the data in Tables I and II are not consistent. The lifetimes measured for excitation at 335 nm and emission at 405 nm which were the spectral parameters used for the Perrin Plot shown in Figure 4 were not homogeneous; i.e. the lifetimes quoted were those calculated from the phase data and were different from those calculated from modulation. Also the P_0 values are not the same in Table I and II which is consistent with the change in excitation wavelength. The situation with magnesium present shows quite similar relaxation times at the different excitation wavelength which is consistent with an interpretation of isotropic rotation for the fluorophore. Without magnesium the situation is entirely different. The P_0 is not the same as that found for the magnesium sample at the same excitation and emission wavelengths. The most reasonable interpretation is that there is segmental motion in the tRNA under these conditions and that Wye base is not particularly constrained. The P_0 changes also support the idea of multiple transitions. Excitation at 320 versus 335 is dominated by different transitions (lifetime and Perrin data). The unknown nature of the precise electronic transitions makes detailed interpretation impossible, particularly an interpretation of the rotational relaxation times of 37 nsec versus less than 10 nsec for the different excitation wavelengths with no magnesium added, different types of motion of the fluorophore must be dominating at the different wavelengths. The strong variation of $D_{20,w}^0$ with ionic strength shows that comparisons of Mg^{+2} effects must consider the ionic strength (11). These two different spectroscopic techniques show overall changes in the size and/or shape of tRNA elicited by different solvent conditions.

The quantum yield and lifetime increases for Wye fluorescence are consistent with increased stacking. The quantum yield of Wye increases as the

size of the oligonucleotide containing it is increased (i.e. from free base to hexanucleotide to dodecanucleotide) and concomitantly the excitation and emission maxima blue shift (26). A change in the accessibility of the anticodon loop nucleotides is also indicated by the quenching data. The trend is the same with either KI or iodoethanol as quenchers. The base is more accessible to quencher in the no added magnesium situation but retarded by ionic effects when comparing KI to iodoethanol. The rate constant for quenching is twice as large with no added magnesium implying that the quencher has greater access to the base. The protection of a fluorophore from quenching by a base stack has been noted previously for ethidium bromide intercalated into DNA (20). Thus there is less stacking protection for the low magnesium situation.

What is the low magnesium form for tRNA^{Phe}? The Wye base is more exposed to the solvent. A smaller rotational relaxation time at high ionic strength is apparent so the base is less rigid with respect to the unit rotating. A change in base stacking seems evident. The fluorescence quantum yield and the quenching constants are consistent with the low magnesium form being less protected from solvent, i.e. less covered in a stack of bases. A change of stack to a 5' stack would have the base experiencing no protection by adjacent bases. While the fluorescence data could be interpreted as a 5' stack, the proton NMR for the Wye methyl groups does not seem consistent with Wye being unprotected (27,28). Shifting the bases from the 3' stack shown in the crystal structure to a 5' stack throws bases 37 and 38 out of a stacked array. There are 4 methyl groups on Wye base; two in ester links on the side chain, a C-CH₃ at C₁₁ and N-CH₃ at N₃. The side chain methyls are in essentially the same environment in the free base or in tRNA with and without magnesium, i.e. chemical shifts of about 3.67 ppm versus 3.64 (27,28). But the C-CH₃ and N-CH₃ do show chemical shifts with respect to the position of the resonances in the free nucleotide (28). The C-CH₃ is at 2.18 ppm in the mononucleotide and shifts to 2.0 ppm in tRNA with no magnesium and 2.08 ppm with added magnesium (data from Figure 5 in Kan et al., 1977). This indicates that this methyl group is even more shielded without magnesium. The N-CH₃ shows the same trend giving a resonance at 4.0 ppm in the mononucleotide, 3.8 ppm with no magnesium and 3.9 ppm with magnesium added to the tRNA.

A possible interpretation of the NMR methyl chemical shifts in the no magnesium situation is that the Wye base plane is tilted with respect to A38 and A36 which could result from changing the angle of the N-glycosidic bond,

x, or changing the sugar pucker. A tilt or propeller twist in the base could shield the side of Wye with the C-CH₃ and N-CH₃ and expose the other side of the base. This would increase the distance between the adjacent bases on one side so that quencher would have better access than in fully parallel bases. This would also change the overlap of the pi orbitals of the aromatic bases resulting in changes in electronic spectra. The scenario for magnesium binding would then be the following: the bases in the anticodon loop (at least the 3' side) are tilted or twisted with respect to each other with no magnesium and the Wye base is unconstrained, giving rotational relaxation times of less than 10 nsec. Magnesium binds to phosphate 37 tethering the base and holding it rigidly into the tRNA. The backbone flexes and the bases now line up parallel in the familiar crystal structure form. The entire loop is now rigid with respect to the whole molecule and the bases are constrained (ρ of 37 nsec for Wye). This type of model is a simplification which needs more data, such as anisotropic decay and description of the motional aspects but is a feasible model incorporating the fluorescence and NMR data for Wye base.

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