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ON THE COORDINATION PROPERTIES OF Eu^{3+} BOUND TO tRNA

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The luminescence properties of Eu^{3+} have been used to investigate the binding and coordination properties of the ion with tRNA, as an attempt to resolve the discussion of whether metal ions bind to tRNA in solution only by Debye-Hückel screening, or whether direct coordination to specific sites may occur. Binding studies with *Escherichia coli* $tRNA_f^{met}$ (taking advantage of 4-thiouracil-sensitized Eu^{3+} emission) distinguish three classes of binding affinities. Two of these are single sites with affinities approx. 10^4 and approx. 10^3 tighter than the nonspecific affinity of Eu^{3+} for native DNA. Mg^{2+} competes for binding at both these sites. Measurement of the lifetime and excitation spectrum of Eu^{3+} bound to the highest affinity site shows that the ion has two to five non-phosphate ligands in its inner coordination sphere. The existence of this coordinated site demonstrates that electrostatic screening is not the only mechanism for metal ion interaction with tRNA. The coordination properties of the high-affinity Eu^{3+} site do not agree with the properties of any of the metal ion sites found in the two $tRNA^{phe}$ crystal forms. Possible reasons for this discrepancy are discussed; it may be that ions bind differently to isolated molecules in solution than to molecules packed in a crystal lattice.

1. Introduction

DNA and RNA molecules, by virtue of their high negative charges, are always found associated with a dense cloud of counterions. These counterions have an important influence on the conformations and interactions of a polynucleotide [1,2]. With DNA, two modes of ion interaction have been distinguished: purely electrostatic, Debye-Hückel screening accounts for the association of alkali metal ions such as Na^+ [1], while most transition metals strongly coordinate to phosphate oxygen or to bases [3]. Some metal ions such as Mg^{2+} bind in a predominantly electrostatic mode but may spend a fraction of their time coordinated to phosphate oxygen [4].

A particularly intriguing case of ion-nucleic acid interactions is presented by transfer RNA molecules. X-ray crystallographic studies of $tRNA^{phe}$ have shown four or five sites occupied by metal ions (e.g., Mg^{2+}, Sm^{3+}) coordinating to different parts of the molecule [5–7]. It has been suggested that these ligated ions are important in stabilizing the tertiary structure of the tRNA [8,9]. In support of this numerous thermodynamic studies have observed a class of four to six divalent ions binding tightly and cooperatively to tRNA, stabilizing the native structure of the molecule (summarized in ref. 9). These and other data suggest that metal ions may play two roles in RNA structure: besides the expected partial neutralization of phosphate charges by Debye-Hückel screening, ions may also preferentially stabilize particular structures by direct coordination to specific sets of ligands.

However, there have been some reasons to question the importance of specific site ion coordination for tRNA structure. For instance, the thermodynamically observed class of tight, cooperatively bound ions is adequately explained by the increased number of Debye-Hückel screening ions

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expected to surround the more compact form of tRNA after tertiary structure formation [10]; special high-affinity sites need not be postulated. In support of this, ions incapable of direct coordination, such as $Co(NH_3)_6^{3+}$, have been observed to bind cooperatively to tRNA and stabilize the tertiary structure [11]. ^{31}P-NMR experiments suggest that all tRNA phosphates are perturbed at low Mn^{2+}/tRNA ratios, with no preferential interactions at specific sites [12]. These data have been used to argue that tRNA in solution never chelates metal ions in the same 'site-specific' way observed in the $tRNA^{phe}$ crystal [12]. To clarify the significance of the tRNA-metal ion coordination complexes seen in crystal studies it is essential to find out whether the same complexes are formed with tRNA in solution, and to determine the affinities of the individual sites for metal ions.

To investigate ion coordination to tRNA in solution fairly specific information about the environment of a bound ion in solution is needed. Recently, it has been demonstrated that europium ion luminescence contains considerable information about the number and types of ligands coordinating to the ion [13]. Specifically, the luminescence lifetime is related to the number of water molecules remaining in the ion inner coordination sphere, and the position of one Eu^{3+} excitation peak is a rough measure of the number of negatively charged ligands coordinated. This kind of information might be able to provide a connection between crystallographic and solution observations. The aim of this paper is to investigate the suitability of Eu^{3+} luminescence as a probe for ion-tRNA interactions.

2. Materials and methods

All solutions were made up with deionized water distilled from $KMnO_4$, and filtered through sterile 0.2 μm membranes (Gelman). $EuCl_3$ was purchased from Aldrich, tRNAs from Boehringer-Mannheim, and calf thymus DNA from Sigma. RNA and DNA samples were dissolved in 0.2 M 4-morpholineethanesulfonic acid (Calbiochem) adjusted to pH 6.0 with NaOH, and dialyzed exhaustively against the same buffer at 2 mM concentration. $EuCl_3$ solutions were made up fresh weekly and standardized by fluorescence titration with dipicolinic acid.

A Perkin-Elmer MPF 44B fluorimeter was used for fluorescence titrations. All titrations were carried out in a volume of 200–300 μl in a 4×4 mm cuvette thermostatted at 20 or 5°C by a circulating water bath. The excitation slit was kept at 2 nm to minimize photochemical damage.

Lifetime measurements were made on an apparatus constructed by Dr. T. Herrmann at the University of Maryland Medical School which will be described in detail elsewhere (T. Herrmann and A. Shamoo, unpublished data). The excitation source was a Molectronics nitrogen-pumped dye laser with a bandwidth of about 0.01 nm and an energy per pulse of approx. 0.4 mJ. Emission was detected at 612 nm with a Photochemical Research Associates photon counting lifetime apparatus equipped with a red-sensitive photomultiplier. The same apparatus was also used for scanning through the Eu^{3+} excitation peak. About 10 μM of Eu^{3+} in H_2O could be detected. Measurements were made at ambient (22°C) temperatures.

All anions tested showed some interaction with Eu^{3+}, detected either by a red shift in the $^7F_0 \rightarrow {}^5D_0$ transition or, in the case of Cl^-, by quenching of the luminescence intensity. (No effect of Cl^- (1 mM to 1 M) was observed on the $^5D_0$ luminescence intensity when the $^7F_0 \rightarrow {}^5L_6$ transition was excited.) Because of this quenching NO_3^- was generally used as the anion in the laser experiments, so that a reading of the Eu^{3+} intensity could be taken before adding tRNA. Experiments with Cl^- gave the same lifetimes, and the 4-thiouracil-sensitized emission experiments gave identical results with Cl^- or NO_3^-.

All experiments reported here were done at pH 6.0. Eu^{3+} undergoes significant irreversible reaction with hydroxide anion above pH 7 and reproducible results were not obtained above this pH.

3. Results

3.1. Electrostatic effects dominate Eu^{3+}-DNA binding

The binding of Eu^{3+} to helical DNA was examined first, both to make sure that Eu^{3+} does

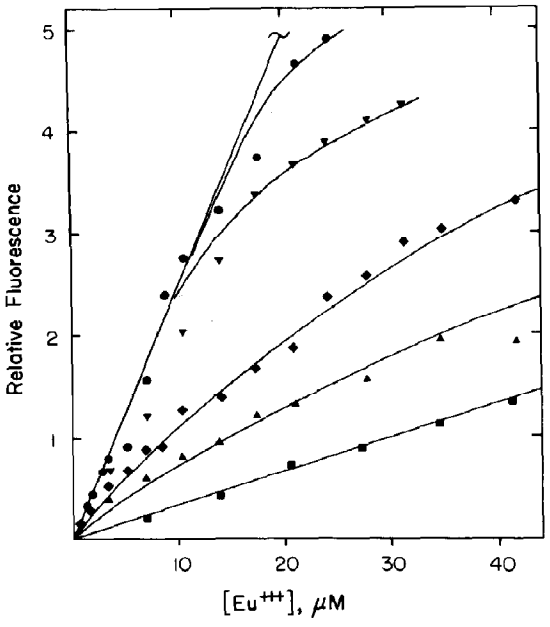

Fig. 1. Titration of DNA with $EuCl_3$. Calf thymus DNA (62.2 μM in phosphates) was titrated with $EuCl_3$ at the indicated NaCl concentrations. Excitation was at 280 nm. Curves drawn are overlap isotherms [15] with site size = 3 phosphates. (●) 27 mM NaCl, $K = 10^7$ M^{-1}; (▼) 133 mM, $K = 1.6 \times 10^5$ M^{-1}; (◆) 200 mM, $K = 1.2 \times 10^4$ M^{-1}; (▲) 400 mM, $K = 3.5 \times 10^3$ M^{-1}; (■) 27 mM, no DNA added.

not have any unexpected effects on nucleic acids and to have a 'baseline' of nonspecific binding against which any unusual tRNA sites may be compared. Titrations of DNA with $EuCl_3$ at various monovalent salt concentrations are shown in fig. 1. At the lowest salt concentration Eu^{3+} bound stoichiometrically to the DNA until nearly one charge equivalent of Eu^{3+} had been added per DNA phosphate (i.e., one-third Eu^{3+} per phosphate). At this point the DNA generally started to aggregate, as indicated by scattering of the excitation beam. This phenomenon is presumably the well known collapse of DNA induced by trivalent ions [14]. At higher salt concentrations the titration curves are fit by 'overlap' binding isotherms which take into account the inherent anticooperativity of ligands covering more than one base or base-pair on DNA [15]. Least-squares analysis of the data, allowing both the Eu^{3+} binding affinity and the site size to vary, gives the expected site size of approx. 3 phosphates [1,2].

The dependence of the Eu^{3+} affinity for DNA on Na^+ concentration is fairly precipitous, with $d(\log K)/d(\log[Na^+])$ equal to 3.1, approximately as expected (about three Na^+ should be displaced from the DNA counterion atmosphere by the binding of one trivalent ion [1,2]). Using the Manning theory of ion binding to DNA, the affinity of a trivalent ion for DNA in the presence of 0.2 M competing monovalent ion is calculated to be 1.0×10^3 M^{-1} [1]. The actual binding affinity of Eu^{3+}, 1.2×10^4 M^{-1} at 0.2 M Na^+, is about one order of magnitude larger than predicted. This extra binding affinity may be the result of direct Eu^{3+} coordination to DNA phosphates (see below). Altogether, these data show that Eu^{3+} behaves as predicted for a trivalent ion associating with DNA in a predominantly electrostatic manner.

The Eu^{3+} $^5D_0$ excited-state lifetime can be interpreted in terms of the inner sphere hydration of the ion. Water coordinated to Eu^{3+} vibrationally relaxes the excited state and the decay rate is directly proportional to the number of water molecules in the inner coordination sphere. Measurements on a number of crystalline europium compounds of known hydration have calibrated the proportionality [16]. The decay rate of the DNA-bound Eu^{3+} is 7.0 ± 0.1 ms^{-1}, which corresponds to 7.0 hydrating water molecules. This observed hydration places an upper limit on the number of DNA ligands which can be coordinating to the bound ions. Eu^{3+} usually contains 8 to 9 ligands in its inner coordination sphere [16,17], though coordinations as low as 6 may have been observed [17]. Thus, Eu^{3+} bound to DNA coordinates between 0 and 2 DNA ligands.

Further information about the Eu^{3+} ligand environment can be gained from an examination of the $^7F_0 \rightarrow {}^5D_0$ transition ($\approx$ 579 nm). This peak is nondegenerate and never split by a ligand field, and the transition is red-shifted by negative charges in the ion inner coordination sphere. There is a rough correlation of the magnitude of the red shift with the number of coordinated charges; the proportionality has been calibrated with complexes of known coordination [13,18]. Fig. 2 shows that the

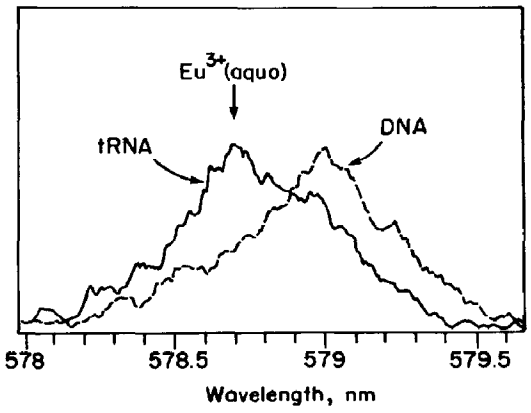

Fig. 2. Eu^{3+} excitation spectra. The luminescence emission intensity (612 nm) of Eu^{3+} bound to calf thymus DNA (0.28 mM DNA phosphates, 73 μM $EuCl_3$, 0.10 M $NaNO_3$), or to $tRNA_f^{met}$ (6.8 μM $EuCl_3$, 0.10 M $NaNO_3$) is shown as a function of excitation wavelength. Essentially all of the Eu^{3+} added is bound to DNA or tRNA under these conditions.

excitation maximum of Eu^{3+} bound to DNA has been red-shifted by about 0.4 nm from the Eu^{3+} (aquo) position; this corresponds to 1.3 negative charges coordinating to each Eu^{3+}, with an uncertainty of about ± 0.5 in this value [13]. With the hydration data discussed above, this result requires that DNA bound Eu^{3+} be coordinated to one or two DNA ligands, with one or both of the ligands phosphate oxygen. The most likely interpretation of the Eu^{3+} thermodynamic and luminescence data is that all Eu^{3+} bound to DNA are coordinated directly to one phosphate oxygen. Note that all the Eu^{3+} are coordinating to the same number of phosphates; if there were two populations of bound ions (with respect to phosphate coordination) two excitation peaks would have been observed.

3.2. *There are several classes of Eu^{3+}-tRNA affinities*

To follow the binding of Eu^{3+} to tRNA it is convenient to use the well known ability of the

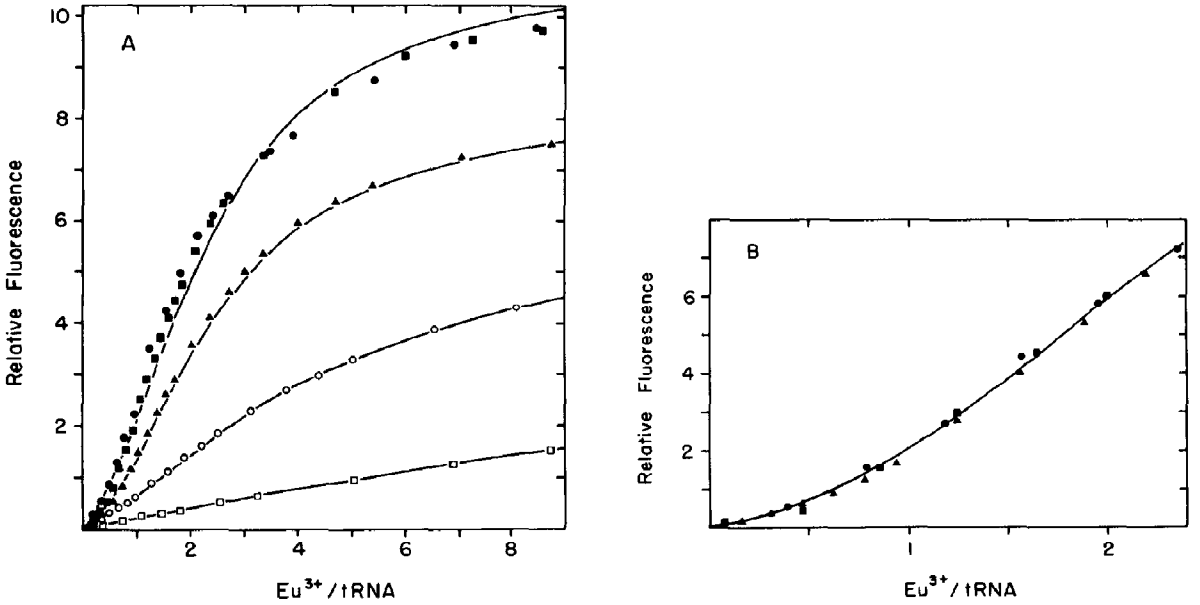

Fig. 3. Titrations of $tRNA_f^{met}$ with $EuCl_3$. (A) Salt dependence. Excitation wavelength, 344 nm; tRNA concentration, 2.5–3.0 μM. (□) 2.0 M NaCl, $K = 1.22\times10^4$ M^{-1}; (○) 1.2 M NaCl, $K_1 = 7.5\times10^5$ M^{-1}, $K_2 = 7.2\times10^4$ M^{-1}; (▲) 0.6 M NaCl, $K_1 = 6\times10^6$ M^{-1}, $K_2 = 8\times10^5$ M^{-1}, K_3 (25 sites) $= 3\times10^4$ M^{-1}; (■) 0.2 M NaCl, $K_1 = 1.5\times10^8$, $K_2 = 2.5\times10^7$, K_3 (25 sites) $= 10^6$; (●) 0.1 M NaCl. Calculation of the curves assumed that only K_2 contributes to the observed luminescence. (B) tRNA concentration dependence. NaCl concentration, 0.2 M. (●) 0.96 μM, (▲) 2.4 μM, (□) 9.6 μM $tRNA_f^{met}$.

single 4-thiouracil in $tRNA_f^{met}$ to sensitize Eu^{3+} emission by energy transfer [19,20]. Titrations of $tRNA_f^{met}$ with Eu^{3+} at various Na^+ concentrations are shown in fig. 3. The shapes of the titration curves and their variation with salt imply the existence of at least three different affinities of $tRNA_f^{met}$ for Eu^{3+}, all of which are considerably stronger than the DNA affinity already described.

Consider first the titration at 2 M NaCl. At this high salt, binding is sufficiently weak that the concentration of bound Eu^{3+} is insignificant compared to the total concentration added, and a single binding affinity of $K = 1.2 \times 10^4$ M^{-1} is calculated from a double-reciprocal plot of the data. This must reflect interactions at or near the 4-thiouracil, and is surprisingly tight. At 2 M Na^+, the concentration of monovalent ions in bulk solution must be about as high as the concentration bound near the tRNA phosphates, so that no increase in entropy is gained by a multivalent ion displacing bound Na^+ into solution. (With DNA, the bound layer of monovalent ions is predicted to be 1.2 M [1], and the affinity of Eu^{3+} for DNA should only be 10 M^{-1} at 1–2 M Na^+.) Eu^{3+} must therefore derive considerable free energy from an interaction with tRNA that is not available with DNA.

At 1.2 M NaCl the curve is still fitted by a single-site isotherm of $K = 7.2 \times 10^4$ M^{-1}, above about 5 Eu^{3+} added per tRNA molecule. The increase in K over the 2 M curve is roughly as expected from the salt dependence of DNA binding. In the first part of the curve there is a slight but reproducible inflection. This sigmoid character becomes more distinct at lower salt concentrations as the number of ions bound becomes comparable to the total number added. It can be taken into account by assuming that one Eu^{3+} interacts with an affinity of 7.5×10^5 M^{-1} in some region of the tRNA not sensitizing Eu^{3+} emission, i.e., that there is sequential binding to sites of decreasing affinity. It should be emphasized that the inflections in these curves are not due to cooperative uptake of ions at a critical free ion concentration, as observed with Mg^{2+} binding to tRNA at low monovalent salt concentrations [9]. Titrations over a wide range of tRNA concentrations are virtually superimposable when plotted as a function of the ratio Eu^{3+}/tRNA (fig. 3B), while the lag in cooperative binding curves should occur roughly near the same absolute concentration of Eu^{3+} independent of tRNA concentration (see the discussion of $tRNA^{Phe}$ below).

The curves at 0.2 and 0.1 M NaCl are nearly identical, despite the 8-fold difference in Eu^{3+} affinity expected for a 2-fold change in Na^+ concentration. The Eu^{3+} binding observed here must therefore be essentially stoichiometric at this tRNA concentration (every Eu^{3+} added to the solution is bound to tRNA), as indeed an extrapolation of the binding constants obtained at 1.2 and 2 M NaCl would imply. However, the curves approach saturation of the 4-thiouracil-sensitized emission quite gradually. To reconcile this curvature with stoichiometric binding requires the assumption of a third, weaker class of binding sites for Eu^{3+}, not showing energy transfer from 4-thiouracil.

The curves drawn in fig. 3 have been calculated making the following assumptions about the three classes of binding affinites. (i) The salt dependence of the different affinity sites is $d(\log K)/d(\log [Na^+]) \simeq -3$; (ii) the intermediate (4-thiouracil-sensitized) site affinity is extrapolated from the data at 1.2 and 2 M NaCl; (iii) 25 independent weak sites are assumed (one-third of the RNA phosphates, or the maximum plausible saturation of tRNA with a trivalent ion). The data are fitted reasonably well using these assumptions, and the affinities can vary over only a limited range and still give a good fit. The stoichiometries of the stronger sites are unambiguously determined; the data cannot be fit well with either two strong or two intermediate affinity sites, even if the restriction that $d(\log K)/d(\log[Na^+]) = -3$ is abandoned. The fit is much less sensitive to the number of weak sites; 15 sites with 50% greater affinity fits the data nearly as well. Sites intermediate in affinity between the 4-thiouracil and weak sites can also be accommodated by the data. The important conclusion from these titrations is that there are two strong sites with affinities of approx. 10^7 and approx. 10^8 M^{-1} at 0.2 M Na^+.

3.3. *One Eu^{3+} is highly coordinated to tRNA*

The excitation spectrum of one equivalent of Eu^{3+} bound to $tRNA_f^{met}$ at 0.1 M NaCl is shown

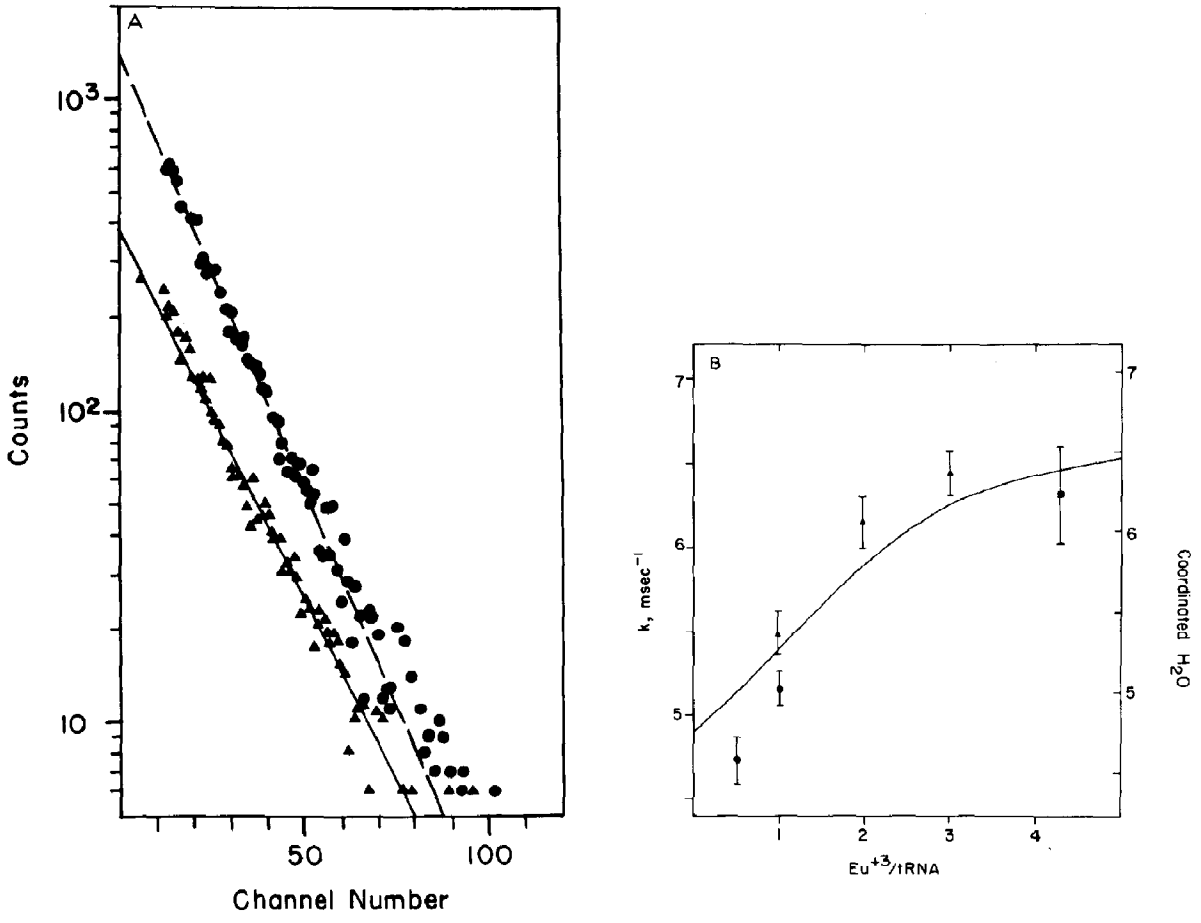

Fig. 4. (A) Luminescence decay of Eu^{3+} bound to $tRNA_f^{met}$. Conditions: 13.6 μM tRNA, 5 mM Mes (pH 6.0), 0.1 M $NaNO_3$, and 13.6 (▲) or 40.8 (●) μM $EuCl_3$. Excitation at 578.8 nm and emission at 612 nm. Decays from 4000 laser pulses were collected at 10 μs per channel and an interval of 100 ms. (B) Luminescent decay rate constants of tRNA-bound Eu^{3+} (in 0.2 M NaCl). The solid curve is calculated assuming the binding affinities of fig. 3 and site 1 hydration = 4 waters, sites 2 and 3 = 7 waters. (■) 7.2 μM tRNA, (▲) 13.6 μM, (●) 20.5 μM. Eu^{3+} binding under these conditions is essentially stoichiometric. The number of coordinated water molecules is calculated from the difference between the decay rate constants measured in H_2O (shown here) and ^2H_2O (see text).

in fig. 2. The peak is not shifted from the Eu^{3+}(aquo) wavelength (578.7 nm), suggesting that no phosphate is coordinating to the ion (see section 4). At higher saturations (up to 4 Eu^{3+}/tRNA) a broad maximum between 578.7 and 579.0 nm is still observed.

Lifetime decay measurements of tRNA-bound Eu^{3+} always show a single exponential (fig. 4A), though the lifetimes vary considerably with the degree of saturation (fig. 4B). This is interpreted as rapid exchange (on the time scale of the $^5D_0$ excited-state lifetime) between environments of different hydration, i.e., the average lifetime of all bound Eu^{3+} is being measured [13]. The curve drawn in fig. 4 was calculated assuming that the most tightly bound Eu^{3+} has a hydration of four waters and all other bound Eu^{3+} have seven waters. The binding affinities determined by the titration shown in fig. 3 were used to calculate the average occupancy of each class of sites. The data are

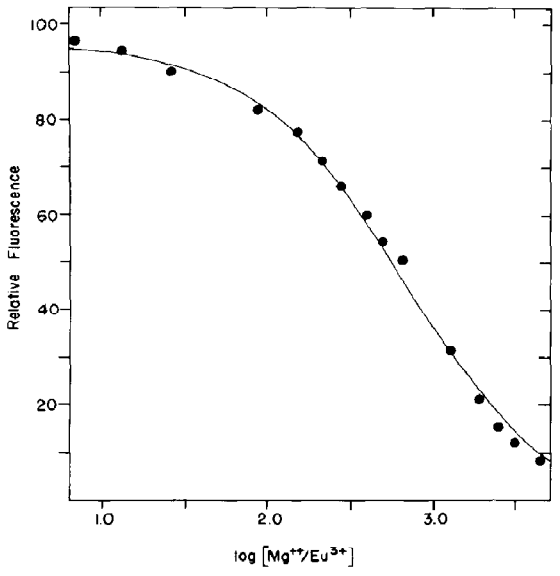

Fig. 5. Competition between Eu^{3+} and Mg^{2+} for tRNA binding: 2.7 μM $tRNA_f^{met}$ in 0.2 M NaCl was titrated with 3 equiv. $EuCl_3$ and then with $MgSO_4$. The decrease in 4-thiouracil-sensitized Eu^{3+} emission with added $MgSO_4$ is shown. The curve is calculated for Mg^{2+} affinities of 10^4 and 10^3 M^{-1}. See text for further explanation.

fitted reasonably well by this curve and it is clear that the strongest Eu^{3+}-binding site is highly dehydrated.

^2H_2O does not relax the Eu^{3+} $^5D_0$ state as H_2O does, and lifetimes measured in 100% ^2H_2O are nearly independent of Eu^{3+} hydration and environment in a variety of samples [16]. Measurement of tRNA-bound Eu^{3+} lifetime in ^2H_2O gives a typical decay rate of 0.404 ± 0.008 ms^{-1}. Previously, Wolfson and Kearns [19] observed the lifetime of Eu^{3+} bound to $tRNA_f^{met}$ in ^2H_2O by 4-thiouracil-sensitized emission. Their results differ from those shown here by resolving two exponential decays, both faster than 0.4 ms^{-1}. The reason for these unusual lifetimes and apparent slow exchange is uncertain. The variation in steady-state 4-thiouracil-sensitized emission with Eu^{3+}/tRNA ratio that they report does agree at least qualitatively with that shown here (fig. 3).

3.4. Mg^{2+} and Eu^{3+} compete for binding at strong sites

Stein and Crothers [21] have studied Mg^{2+} binding to $tRNA_f^{met}$ under conditions similar to those used here. The simplest interpretation of their data requires two classes of sites: one strong site ($K = 2.9 \times 10^4$ M^{-1}) and a number of weak sites ($n = 26$, $K = 417$ M^{-1}). Three or more classes of sites can be used to fit the data as well. For instance, an isotherm with one site of $K = 3 \times 10^4$ M^{-1}, one site of 4×10^3 M^{-1}, and 38 sites of 200 M^{-1} gives a Scatchard plot almost indistinguishable from the 'two classes' isotherm over the range of the data. It is tempting to identify the strong Mg^{2+}-binding site(s) with the strong Eu^{3+} sites. To see if this is a plausible interpretation, competition between Mg^{2+} and Eu^{3+} for $tRNA_f^{met}$ binding was observed using 4-thiouracil-sensitized Eu^{3+} emission. A competition curve is shown in fig. 5. At very high Mg^{2+} concentrations essentially all of the Eu^{3+} can be driven off the tRNA, as expected; the question is whether the Eu^{3+} is displaced by weak or tight binding Mg^{2+}.

To calculate competition curves all three classes of Eu^{3+} sites must be taken into account, since displacement of Eu^{3+} from one site will increase the concentration of free Eu^{3+} and influence the saturation level at all other sites. In the following calculations the set of Eu^{3+} binding affinities used to fit the 0.2 M NaCl curve of fig. 3 have been used. To simplify the problem somewhat Mg^{2+} binding to the third, weak class of sites has been neglected. The degree of Eu^{3+} saturation at these sites is relatively low (5.7% under the initial conditions of fig. 5) and probably not significantly affected until the Mg^{2+} concentration is above approx. 10 mM. The data were fitted by fixing the Mg^{2+} affinity for the intermediate affinity (4-thiouracil-sensitized) Eu^{3+} site at various values and varying the Mg^{2+} affinity for the strong Eu^{3+} site to obtain the optimum fit. Based on the Mg^{2+} binding data of Stein and Crothers [21], the range of Mg^{2+} affinities considered for the intermediate site was limited to 10^2–10^4 M^{-1}. Mg^{2+} affinities between 800 and 10^3 M^{-1} gave very good fits of the data when used with strong site affinities from 10^3 to 10^4, respectively.

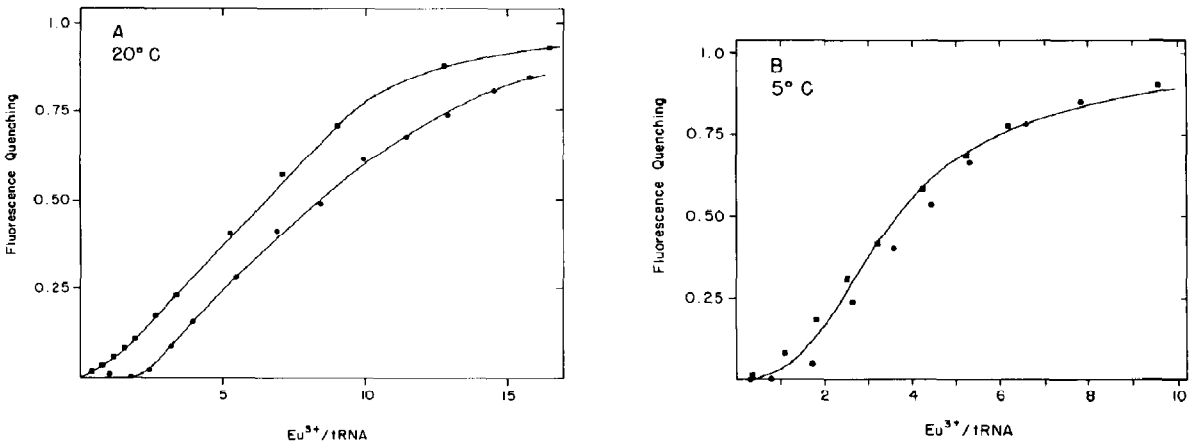

Fig. 6. Titrations of $tRNA^{phe}$ with $EuCl_3$ in 0.2 M NaCl, 5 mM Mes (pH 6.0). Quenching of Y base fluorescence (excitation 314 nm, emission 430 nm) is reported relative to maximum quenching at high Eu^{3+}/tRNA (64 to 70%). (●) 2.5 μM tRNA, (■) 10 μM. (A) 20°C, (B) 5°C. The curve in B is calculated assuming $K_1 = 6\times10^7\ M^{-1}$ (2 sites), $K_2 = 6\times10^6\ M^{-1}$ (1 site) and $K_3 = 3\times10^5\ M^{-1}$ (25 sites).

The Mg^{2+} binding affinities deduced from these competition data agree very well with the Stein and Crothers Mg^{2+} binding data if three classes of Mg^{2+}-binding sites are assumed. It appears that Mg^{2+} binds to the same sites as Eu^{3+}, and maintains about a 10^4 weaker affinity. A significant finding of Stein and Crothers [22] is that the strongly bound Mg^{2+} preferentially stabilizes tRNA tertiary structure against denaturation. This strong, highly dehydrated binding site may thus have an important structural role.

3.5. Eu^{3+} influences a conformational transition in $tRNA^{phe}$

An attempt was made to follow the binding of Eu^{3+} to $tRNA^{phe}$ by monitoring the fluorescence of Y base, located in the anticodon loop at position 37. Substantial quenching of the Y base fluorescence is observed, and the curves are distinctly sigmoid (fig. 6A). In contrast to $tRNA_f^{met}$ the lag in the binding curve varies with the tRNA concentration. It appears that at a free Eu^{3+} concentration of about 5 μM a cooperative uptake of ions occurs. At tRNA concentrations lower than about 1 μM the binding curve is complicated by an initial enhancement (up to 50%) of the Y base fluorescence, followed by quenching at higher Eu^{3+} concentrations (not shown).

At lower temperatures (5°C) the Eu^{3+} titration of $tRNA^{phe}$ becomes nearly independent of tRNA concentration and has the same appearance as the titrations of $tRNA_f^{met}$ (fig. 6B). Enhancement of Y base fluorescence is not observed at any tRNA concentration. The complex behavior observed at 20°C is thus altered, indicating that a conformational transition in the tRNA has taken place between 5 and 20°C. These titrations again suggest three classes of binding sites, with only the intermediate-affinity class affecting the Y base fluorescence. The affinities and stoichiometries of the sites can be estimated by a similar procedure as used with the $tRNA_f^{met}$ titrations. In this case the affinity of Mg^{2+} for the anticodon site was determined first, by observing Y base fluorescence enhancement with addition of Mg^{2+} [23], and then the Eu^{3+} affinity for the same site determined by competition with bound Mg^{2+}. A value of $4\times10^6\ M^{-1}$ was found. The strong and weak site affinities and stoichiometries were then varied to give an optimum fit to the curve. A stoichiometry of two for the strong class generally gave the best fit. It is possible to fit both the $tRNA^{phe}$ and $tRNA_f^{met}$ titration data with the same

set of binding parameters, if three sites distinguishable from the non-specific, screening ions are assumed: one site of approx. 10^8 M^{-1}, one site of approx. 2×10^7 M^{-1} near position 8 in the sequence, and one site of approx. 5×10^6 M^{-1} in the anticodon loop.

4. Discussion

4.1. Properties of the classes of Eu^{3+} binding tRNA

The experiments presented here distinguish three classes of Eu^{3+} interacting with tRNA. The weakest class is similar to the single class of ions observed with DNA, and has properties expected for Debye-Hückel screening ions: the number of apparent sites approaches one-third the number of phosphates, and very little dehydration accompanies binding. The intermediate-affinity ion must be localized near the 4-thiouracil in $tRNA_f^{met}$. The lifetime experiments do not resolve the hydration of this ion.

The strongest Eu^{3+}-binding site on $tRNA_f^{met}$ has properties quite different from a screening ion: only one such site exists on a tRNA molecule; the binding is to some extent localized in a particular region of the RNA; the binding is tighter by several orders of magnitude than predicted for a screening ion; and the bound ion is highly dehydrated with no phosphate in the inner coordination sphere. The hydration number suggests that four or five tRNA ligands are coordinating to the ion, although the possibility that the ion is in an unusual environment with as few as two tRNA ligands (for a total coordination number of six) cannot be ruled out. (The higher coordination seems more likely, since two nonphosphate ligands probably would not create a site with such high affinity.) The competition experiment reported suggests that this high-affinity site corresponds to the single, high-affinity Mg^{2+} site found by Stein and Crothers [21]. Mg^{2+} binding at this site has been shown to stabilize preferentially tRNA tertiary structure [22]. The evidence thus seems to favor strongly the idea that metal ions may stabilize particular RNA conformations by direct coordination. The view that metal ions interact with tRNA entirely by Debye-Hückel screening [12] is not consistent with the properties of the single high-affinity Eu^{3+}-binding site.

4.2. Comparison of results with tRNA crystal structure

The properties of the Eu^{3+}-binding sites determined here differ in important ways with some of the assumptions that have been made about tRNA-metal ion interactions. First, 'hard' metal ions such as Mg^{2+} and the rare earths are known to interact more strongly with phosphates than with nucleic acid bases, and so it has seemed reasonable to rank the metal ion sites observed in the $tRNA^{phe}$ crystal structure in order of the number of coordinating phosphates [8]. The finding that the strongest Eu^{3+} site involves no inner sphere phosphate contradicts this. Although it is surprising to find a hard metal coordinated to tRNA without making direct use of phosphate, it is not unprecedented: one of the five Sm^{3+} sites in the $tRNA^{phe}$ crystal has the O6 of G45 as its only inner sphere ligand (ref. 5; two phosphates are in the outer sphere as well). It is perhaps possible to rationalize the observation by noting that four or five base ligands arranged in a favorable geometry may well have a higher affinity for ions than a site involving only one or two phosphate ligands. The absence of phosphate ligands in the high-affinity site also accounts for the failure of ^{31}P NMR experiments to detect the site [12].

A second disagreement is that none of the metal ion sites found in either $tRNA^{phe}$ crystal form has the properties determined for the high-affinity Eu^{3+} site, viz., a minimum of two nonphosphate inner sphere ligands. Of all the metal ions located in the monoclinic and orthorhombic $tRNA^{phe}$ crystals (Mg^{2+}, Sm^{3+}, Co^{2+}, Mn^{2+}), only three show as many as two inner sphere ligands and these are all to phosphate [5–7]. There are several possible explanations for this discrepancy: $tRNA^{phe}$ may coordinate ions very differently than does $tRNA_f^{met}$ (though see the discussion of ion binding stoichiometry below); Eu^{3+} may not use the identical sets of tRNA ligands as Sm^{3+} or Mg^{2+} (though Eu^{3+} and Sm^{3+} coordination properties are very similar); or the coordination of ions

in tRNA crystals may not accurately represent the actual interactions in solution. This last possibility deserves serious consideration. Although tRNA molecules in crystals are well solvated and likely to have the same conformation as in solution, the packing of molecules in the crystal could have a strong influence on counterion binding. Ions in the crystal will experience to some extent the electrostatic field from adjacent molecules, and new sites for coordination may be created between molecules. A notable example is the case of two Mg^{2+} found coordinated to both the TψC and D loops in both crystal forms. One interacts with G20, U59, C60 and P19 of one tRNA, and also with C56 of the adjacent molecule in the crystal [8]. A second ion is coordinated between P20 and P21 of one tRNA and D17 of the other. It seems likely that the metal ion interactions in this region will be somewhat different in solution with the perturbing influence of an adjacent tRNA absent.

4.3. Location of the high-affinity Eu^{3+} site

A location with all the properties required for the high-affinity site is an area of nonhydrogen bonded bases between the D and TψC loops. Looking through the D loop in the $tRNA^{phe}$ crystal structure, D16, D17, and G20 of the D loop along with U59 and C60 of the TψC loop line a small pocket [24]. There are sufficient nonphosphate ligands in this pocket to account for the high dehydration of Eu^{3+}. An ion coordinated to these bases would probably help stabilize the RNA tertiary structure. The highest occupancy Mg^{2+}-binding site in the crystal structures is in this region, though the ion is directly coordinated to P19 and only indirectly coordinated to some of the bases [7]. As pointed out above, the ion binding in this region of the crystal may be strongly influenced by the adjacent tRNA molecule. Also, the slightly larger Eu^{3+} may be more likely to show inner sphere coordination than Mg^{2+}. It is interesting to note that at low Mg^{2+}/tRNA ratios the ion catalyzes phosphodiester cleavage uniquely between D16 and D17 [25], suggesting a specific interaction of Mg^{2+} in this region. Measurement of energy transfer to Eu^{3+} from dyes attached to specific residues in the tRNA sequence [26] may be able to provide evidence for the location of the high-affinity site.

4.4. How many metal ions bind 'site-specifically' to tRNA?

The $tRNA^{phe}$ titrations with Eu^{3+} show that the tRNA is not completely folded at 20°C, since a cooperative uptake of added ions occurs at this temperature but not at 5°C. The major transition involving unfolding of the tertiary structure occurs at much higher temperature [27], so the transition detected is probably a minor rearrangement of structure. Coupling of ion binding to tRNA conformational transitions complicates analysis of the binding data, and can lead to a serious overestimation of the number of strong binding sites [10]. Thus, the previous estimate of four to six strong Mg^{2+}-binding sites in $tRNA^{phe}$ is probably high [27], and the three sites distinguishable from nonspecific ions in the Eu^{3+} titrations are probably the only significant sites present.

It may be that the three sites present in $tRNA^{phe}$ are a general feature of tRNA structure: the $tRNA_f^{met}$ binding data can accommodate the same affinity sites, and preliminary data obtained with Eu^{3+} titrating $tRNA^{glu2}$ (monitoring energy transfer from 2-thiouridine at position 34 in the anticodon) or $tRNA^{tyr}$ (energy transfer from 4-thiouridines at positions 7 and 8) can be fitted with the same three affinities. This is very suggestive of a common pattern of metal ion binding in all tRNAs, though the locations of all three sites will have to be determined in several tRNAs before a firm conclusion can be drawn.

4.5. Utility of Eu^{3+} for nucleic acid studies

The results presented here show that the same techniques developed for investigation of Eu^{3+} coordination to proteins can be successfully applied to nucleic acids as well. Solution information about ion coordination to nucleic acids has been obtained almost exclusively by magnetic resonance techniques, which are generally much less sensitive than luminescence measurements. The ability of Eu^{3+} to show energy transfer with dyes or other

metals is also useful for binding studies and distance measurements [13].

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