Traut, R. R., & Monro, R. E. (1964) *J. Mol. Biol. 10*, 63–72.
Victorova, L. S., Kotusov, V. V., Azhaev, A. V., Krayevsky, A. A., Kukhanova, M. K., & Gottikh, B. P. (1976) *FEBS Lett. 68*, 215–218.
Vogel, Z., Zamir, A., & Elson, D. (1969) *Biochemistry 8*, 5161–5168.
von Ehrenstein, G., Weisblum, B., & Benzer, S. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 669–675.

Articles

Anticodon Loop of $tRNA^{Phe}$: Structure, Dynamics, and Mg^{2+} Binding

W. Bujalowski,[†,§] E. Graeser,[‖] L. W. McLaughlin,[‖,⊥] and D. Porschke*[,‡]

Max-Planck-Institut für biophysikalische Chemie and Max-Planck-Institut für experimentelle Medizin, D-3400 Göttingen, FRG

Received April 7, 1986; Revised Manuscript Received June 12, 1986

ABSTRACT: The structure, dynamics, and Mg^{2+} binding reactions of the isolated anticodon hairpin loop from $tRNA^{Phe}$ (yeast) have been analyzed by fluorescence-detected temperature-jump relaxation, melting experiments, and equilibrium sedimentation. Most of the measurements were performed at an ionic strength of 0.15 M and at temperatures below 25 °C, where the hairpin loop proved to be stable. A relaxation effect with a time constant of ~100 μs, indicated by the Wye base fluorescence, is attributed to a conformational change of the anticodon loop and is very similar to a corresponding transition observed previously for the whole $tRNA^{Phe}$ molecule. A Mg^{2+} binding site reflected by an inner-sphere relaxation process and associated with a strong increase of the Wye base fluorescence closely resembles a corresponding site observed in the complete $tRNA^{Phe}$ and is attributed to a site in the anticodon loop identified by X-ray analysis. In addition to the Mg^{2+} site in the loop, which is associated with a binding constant of 2×10^3 M^{-1}, the existence of sites with a higher affinity is demonstrated by an unusual relaxation effect, showing a minimum in the reciprocal time constant with increasing Mg^{2+} concentration. The experimental data can be described by a transition between two states and Mg^{2+} binding to both states resulting in a reaction cycle, which is extended by an additional Mg^{2+} binding reaction to one of the states. The unusual effect has not been observed for the complete $tRNA^{Phe}$ and is also not observed when Ca^{2+} is added instead of Mg^{2+}. This result indicates the existence of a conformational change involving Mg^{2+} inner-sphere complexation. None of the relaxation effects is observed for a hexamer, which is excised from the anticodon loop and contains the Wye base but does not form the loop structure. Thus, the presence of the hairpin loop is necessary for the anticodon loop transition, the Mg^{2+} inner-sphere complexation in the anticodon loop, and the special transition coupled to the Mg^{2+} sites with high affinity; apparently, the hairpin loop structure is required for a specific arrangement of molecular contacts.

Loops are essential elements of nucleic acid structures in general. The most important example appears to be the anticodon loop of tRNA molecules because of its function in the translation process. Apparently, loop structures have been selected for this crucial function because of various favorable properties. It has been shown, for example, that a nucleic acid sequence enclosed in a loop structure has an unusually high affinity to a complementary sequence (Eisinger et al., 1970; Uhlenbeck et al., 1970; Högenauer, 1970; Eisinger & Gross, 1974; Grosjean et al., 1976). This seems to be partly due to the special conformation of the loop residues (Grosjean et al., 1976). Another reason appears to be the existence of special binding sites for ions in the loop, especially for Mg^{2+} ions (Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978). The folded structure of a loop provides more contacts to a ligand than a simple linear structure. Finally, loop structures may exist in different conformations, which may be converted to each other in a relatively short period of time (Urbanke & Maass, 1978; Labuda Porschke, 1980). Such different anticodon loop conformations are probably important for ribosomal translation (Woese, 1970) and also for recognition by proteins (Ehrlich et al., 1980; Bujalowski & Porschke, 1984).

Most of these properties have been characterized for loops as integral parts of whole tRNA molecules, and it remains to be established to which degree these properties are dependent upon coupling of the loop to the rest of the tRNA structure. For an investigation of this effect, a hairpin loop is required with a sufficiently stable helix stem, which is not readily converted to the single-stranded state and also does not form a dimer of two nucleotide strands. Furthermore, a spectroscopic label is required for a convenient characterization of reactions. All these conditions appear to be fulfilled by a pentadecamer representing the anticodon loop of $tRNA^{Phe}$ (yeast) with seven residues in the loop and four relatively stable base pairs in the stem. We have characterized the structure of this anticodon loop by measurements of its thermal stability,

[‡] Max-Planck-Institut für biophysikalische Chemie.
[§] On leave from the Institute of Biology, Department of Biopolymer Biochemistry, Poznan University, 61-701 Poznan, Poland. Present address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128.
[‖] Max-Planck-Institut für experimentelle Medizin.
[⊥] Present address: Department of Chemistry, Boston College, Chestnut Hill, MA 02167.

0006-2960/86/0425-6365$01.50/0

by equilibrium sedimentation, and by chemical relaxation detected by the fluorescence of the Wye base. The Wye base fluorescence has also been used to characterize the binding of Mg^{2+}, the degree of its inner-sphere complexation, and its influence on the loop conformation.

Materials and Methods

A dodecamer containing both 5′- and 3′-terminal phosphates and including base residues A31 to G42 of $tRNA^{Phe}$ (yeast) was prepared as described elsewhere (McLaughlin & Graeser, 1982). The pentadecamer was prepared from the phosphorylated dodecamer and the trinucleoside diphosphate CpApG as catalyzed by T_4 RNA ligase. A typical reaction mixture of 1.25 mL contained 48 A_{260} units (0.5 mM) of the dodecamer, 30 A_{260} units (0.78 mM) of CpApG, ATP (4.0 mM), $MgCl_2$ (20 mM), dithiothreitol (3 mM), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 8.4 (50 mM), bovin serum albumin (10 μg/mL), and T_4 RNA ligase (160 μg/mL). After incubation of the mixture at 17 °C for 18 h, the product pentadecamer was isolated by high-pressure liquid chromatography (HPLC) with a 9.4 × 250 mm column of ODS-Hypersil (5 μm) and using 20 mM KH_2PO_4, pH 5.5, and a gradient from 0% to 70% methanol in 100 min at 35 °C. Under these conditions with a flow rate of 4 mL/min, the product eluted with a retention time of 25.1 min. After desalting (Sephadex G-10) and lyophilization, 37 A_{260} units of the pentadecamer were obtained. The pentadecamer was dialyzed first against 100 mM $NaClO_4$ and 20 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0, then against water, and finally against several changes of a standard buffer containing 100 mM $NaClO_4$ and 50 mM tris(hydroxymethyl)aminomethane–cacodylate (Tris–cacodylate), pH 7.1.

Fluorescence titrations were recorded with an SLM fluorometer equipped with a double monochromator in the excitation path (wavelength of excitation usually 312 nm), whereas a simple cutoff filter (GG385 from Schott, Mainz, FRG) was used in the emission path in order to avoid loss of fluorescence intensity in the monochromator. Equilibrium sedimentation was measured with a Spinco Model E analytical ultracentrifuge equipped with collimator optics according to Flossdorf and Schillig (1979), a photoelectric scanner, a multiplexer, and electronic speed control. The sedimentation was recorded in 12-mm double-sector cells at a wavelength of 313 nm. The data were first stored in a personal computer, which was used to control for complete equilibrium and for a first evaluation. Then the data were transferred for final evaluation to the computer center of the Gesellschaft für wissenschaftliche Datenverarbeitung GmbH, Göttingen.

The fluorescence-detected temperature-jump experiments were performed as described (Labuda & Porschke, 1982). The data were stored initially by a Biomation 1010 transient recorder and then transferred to the computer center mentioned above for evaluation of time constants and amplitudes. The concentration dependence of time constants and amplitudes was simulated according to various reaction models by the program SIMULA (Avery, 1982; modified by R. Clegg, J. Reiter, and M. Jung). The error bars given in the figures have been estimated from the deviations observed upon repetition of the experiments. In general, temperature jumps for a given solution were repeated 5–7 times. Some of the whole titration series have also been repeated several times, in particular, the pentadecamer + Mg^{2+} titration.

Results and Discussion

Thermal Stability of the Helix Stem. For most of the conclusions obtained from various types of measurements, it is important to known about the stability of the loop structure. Thus, we have characterized the stability of the helix stem by measurements of the absorbance as a function of the temperature. As shown in Figure 1, the helix stem melts in the temperature range from 25 to 65 °C with a midpoint of the transition at 44.5 °C (standard buffer, without Mg^{2+}). Least-squares fitting of the experimental data according to a simple two-state model with a linear temperature dependence of the absorbance below and above the transition [cf. Porschke & Jung (1982)] provides a transition enthalpy of 134 kJ/mol. Thus, the melting enthalpy calculated per base pair (ΔH = 34 kJ/mol) is in the expected range. Addition of 10 mM Mg^{2+} leads to an increase of the melting temperature to 55.6 °C and also to an increase of the total enthalpy change to 172 kJ/mol. The increase of the enthalpy change is mainly attributed to a decrease in the degree of single-strand stacking with increasing temperature.

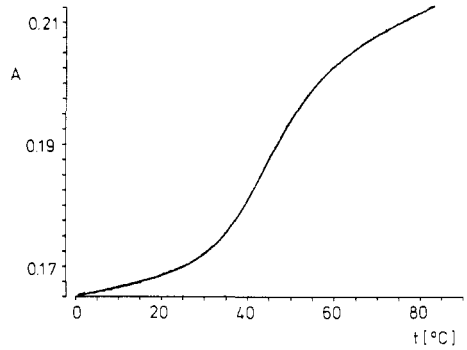


FIGURE 1: Absorbance of the pentadecamer at 260 nm as a function of temperature. A fit according to a simple two-state model (for details, cf. text) provides a t_m value of 44.5 °C and an enthalpy change of −134 kJ/mol (standard buffer).

The results obtained from the present melting experiments are consistent with recent melting experiments recorded by nuclear magnetic resonance (Clore et al., 1984). It may be concluded that the hairpin loop in the standard buffer is stable up to about 25 °C.

Equilibrium Sedimentation. The pentadecamer may form two different types of helical structures: (a) the hairpin loop with four base pairs and (b) a dimer with two double-helical segments of four base pairs each separated by an interior loop of seven residues for both strands. It should be relatively simple to distinguish between these possibilities by analytical ultracentrifugation. Measurements of the equilibrium sedimentation in the standard buffer at 7 °C provided a molecular weight of 5200 for the pentadecamer, in perfect agreement with a hairpin loop structure. The observed linear dependence of the pentadecamer concentration upon the square of the radius demonstrates the absence of aggregates. A corresponding result has been obtained in the standard buffer containing different Mg^{2+} concentrations up to 10 mM. Thus, it is established that the pentadecamer forms a hairpin loop structure in the standard buffer both in the absence and in the presence of Mg^{2+} (provided that the temperature does not exceed ~25 °C in the absence of Mg^{2+} and ~35 °C in the presence of 10 mM Mg^{2+}).

Mg^{2+} Binding Constant from Fluorescence Quenching. The fluorescence of the Wye base is known to be very sensitive to changes in its environment (Beardsley et al., 1970; Robinson & Zimmerman, 1971; Eisinger et al., 1971) and thus may be used to characterize the binding of Mg^{2+} ions. Addition of Mg^{2+} to the pentadecamer induces a strong increase in the fluorescence intensity (Figure 2). Quantitative analysis of these data according to a simple one-step binding mechanism

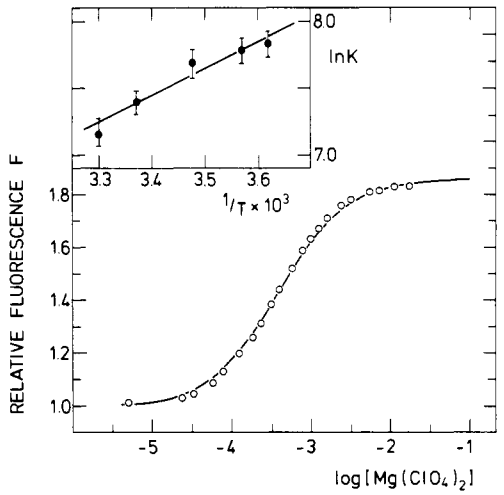


FIGURE 2: Relative fluorescence of the pentadecamer as a function of the logarithm of $Mg(ClO_4)_2$ concentration. The continuous line shows a fit according to a one-step binding reaction with a stability constant of 2600 M^{-1} (standard buffer, 3.4 °C, 0.26 μM pentadecamer). The insert shows a van't Hoff plot: $\Delta H = -16.3$ kJ·mol^{-1}; $\Delta S = 5.0$ J mol^{-1} deg^{-1}.

provides a stability constant of 2600 M^{-1} (at 3.4 °C). Both the fluorescence increase and the stability constant are very similar to that observed previously for the case of the complete tRNA^{Phe} molecule. The binding constant of Mg^{2+} to the hairpin loop decreases with increasing temperature; a van't Hoff plot (cf. Figure 2) provides an enthalpy change of −16.3 kJ/mol and an entropy change of 5.0 J/(mol·deg).

Anticodon Loop Transition and Its Coupling to Ca^{2+} Binding. In general, the emission intensity of fluorophores decreases with increasing temperature. This effect, which is due to deactivation of the excited state by solvent molecules, is also observed for the fluorescence of the Wye base in the anticodon loop and is reflected in temperature-jump (T-jump) experiments by a relatively large amplitude with a time constant beyond the time resolution of currently available T-jump instruments. In addition to this fast "physical" quenching effect, T-jump experiments with the pentadecamer reveal a process with a time constant of about 100 μs which is associated with a further decrease of the fluorescence intensity. A corresponding effect has been observed previously for the complete tRNA^{Phe} (Urbanke & Maass, 1978; Labuda & Porschke, 1982) and has been attributed to a conformational change of the anticodon loop. The present observation of the same transition in the isolated anticodon hairpin loop confirms that the conformational change can be attributed to the loop itself and demonstrates that the rest of the tRNA molecule is not required for this transition.

Addition of Ca^{2+} to the pentadecamer leads to a decrease of the transition amplitude together with a decrease of the reciprocal time constant (cf. Figure 3). The experimental data are very similar to those obtained previously for the complete tRNA^{Phe} and thus can be described by the same mechanism. According to this mechanism, the bivalent ions bind preferentially to one of the loop conformations:

$$A \underset{k_a^-}{\overset{k_a^+}{\rightleftharpoons}} B$$

$$B + Me \underset{k_m^-}{\overset{k_m^+}{\rightleftharpoons}} C \qquad (1)$$

A fit of both time constants and amplitudes provides values for the rate constants, $k_a^+ = 6200$ s^{-1} and $k_a^- = 9500$ s^{-1}, indicating that the difference of the free energy between the

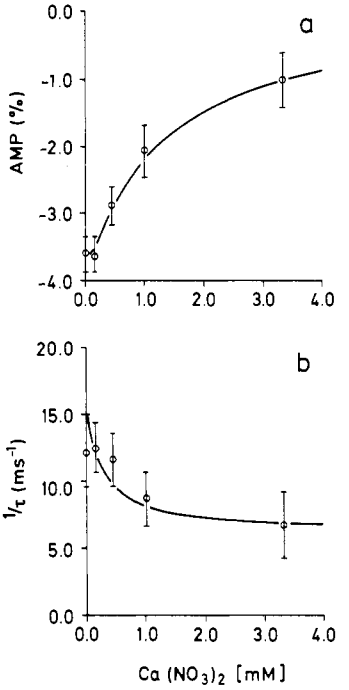


FIGURE 3: Fluorescence-detected temperature-jump relaxation of the pentadecamer as a function of Ca^{2+} concentration (standard buffer, 7.2 °C, 5.1 μM pentadecamer). (a) Relative amplitudes; (b) reciprocal relaxation time constant. Solid lines represent a least-squares fit according to the two-step reaction (eq 1): $k_a^+ = 6200$ s^{-1}; $k_a^- = 9500$ s^{-1}; $K_b = 3.8 \times 10^3$ M^{-1}; $\Delta H_a = -24.3$ kJ/mol; $\Delta H_b = +9.8$ kJ/mol; relative quantum yields of (A) 1.00, (B) 2.08, and (C) 2.08.

two loop conformations in the absence of Ca^{2+} is relatively small. A corresponding result has been obtained previously for the complete tRNA^{Phe} (Labuda & Porschke, 1982). Since the rate of Ca^{2+} binding is very fast compared to the rate of the anticodon loop transition, the rate constants of Ca^{2+} binding cannot be determined, but only the equilibrium constant, $k_m^+/k_m^- = 3.8 \times 10^3$ M^{-1}.

Two Separate Relaxation Processes Induced by Mg^{2+} Addition. The mechanism of Ca^{2+} binding to the anticodon loop is probably not important for biological function because of the low intracellular concentration of Ca^{2+}. However, knowledge of the Ca^{2+} binding mechanism is very useful as a reference for the Mg^{2+} binding reaction. It has been shown for many ligands that the thermodynamics of Ca^{2+} and Mg^{2+} binding are very similar, whereas the kinetics are quite different: formation of Mg^{2+} inner-sphere complexes is a relatively slow reaction with a characteristic rate constant of 10^5 s^{-1}, whereas the corresponding reaction of Ca^{2+} is much faster with a rate constant of about 3×10^8 s^{-1} (Eigen, 1963; Eigen & Maass, 1966). Thus, the formation of Ca^{2+} inner-sphere complexes usually is not reflected by a separate relaxation process, and the observation of an additional slow relaxation process upon exchange of Ca^{2+} against Mg^{2+} provides strong evidence for the formation of Mg^{2+} inner-sphere complexes.

Temperature-jump experiments with fluorescence detection on solutions of the pentadecamer in the presence of Mg^{2+} clearly show an additional slow chemical relaxation, which may be attributed to inner-sphere complexation of Mg^{2+}. In this respect, the pentadecamer resembles the complete tRNA^{Phe}. However, a detailed analysis demonstrates a more complex relaxation for the pentadecamer, where two processes are induced by addition of Mg^{2+} compared to only a single one in the case of the whole tRNA^{Phe} molecule. The separation and assignment of the two relaxation effects for the pentadecamer in the presence of Mg^{2+} would be very difficult if

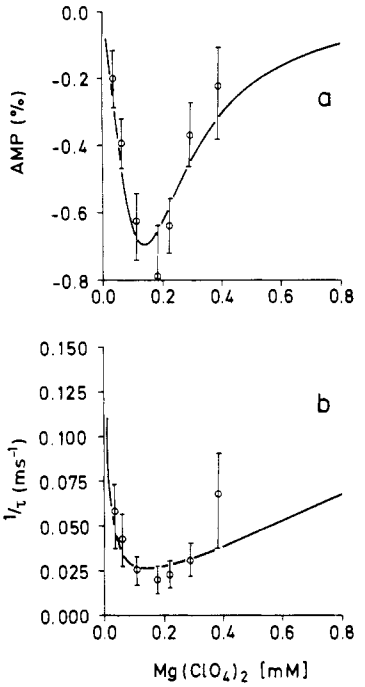


FIGURE 4: (a) Relative amplitudes and (b) reciprocal relaxation times of the pentadecamer low-Mg^{2+} effect as a function of Mg^{2+} concentration (standard buffer, 3.4 °C, 3.3 μM pentadecamer). The continuous lines represent a fit according to eq 3 with the following parameters: $k_1^+ = 2.2\ s^{-1}$; $k_1^- = 2200\ s^{-1}$; $k_2^+ = 2 \times 10^4\ s^{-1}$; $k_2^- = 40\ s^{-1}$; $K_3 = 4\ M^{-1}$; $K_4 = 2 \times 10^5\ M^{-1}$; $K_5 = 2 \times 10^4\ M^{-1}$; $\Delta H_1 = \Delta H_2 = -135$ kJ/mol; $\Delta H_3 = \Delta H_4 = 0$; relative quantum yields of (R) 1.0, (T) 1.0, (R·Mg) 1.05, (T·Mg) 1.05, and (T·2Mg) 1.05. The larger error bar for the measurement at the highest Mg^{2+} concentration results from the relatively small amplitude and from partial overlap with relaxation due to the onset of the high-Mg^{2+} effect (cf. text).

these effects would appear together in the same range of Mg^{2+} concentrations. Fortunately, the analysis is facilitated by the separate appearanceof a "low-Mg^{2+} effect" observed at Mg^{2+} concentrations from 50 to 400 μM and a "high-Mg^{2+} effect" at concentrations from ~0.5 to 10 mM Mg^{2+}. The amplitude of the low-Mg^{2+} effect decays to values which are virtually undetectable at the Mg^{2+} concentrations where the high-Mg^{2+} effect appears. Correspondingly, the high-Mg^{2+} effect is not detected at low Mg^{2+} concentrations. Thus, the two relaxation effects reflect separate normal modes of coupled reactions [cf. Eigen & DeMaeyer (1963)], and the high-Mg^{2+} effect should not be regarded as a continuation of the low-Mg^{2+} effect in the range of high Mg^{2+} concentrations. Owing to the clear separation on the concentration scale, it is justified to describe the processes independently.

Special Mg^{2+}-Dependent Conformational Change of the Anticodon Loop. The amplitude of the low-Mg^{2+} effect shows a concentration dependence with a maximum at about 150 μM Mg^{2+} as may be expected for a usual association reaction with a binding constant of about $10^4\ M^{-1}$ (cf. Figure 4a). However, the minimum in the concentration dependence of the reciprocal time constant (cf. Figure 4b) demonstrates that the relaxation does not reflect a simple elementary step of binding. A minimum of $1/\tau$ with increasing reactant concentration is quite unusual. Similar effects have been observed only for relatively complex coupled reactions like the α-helix–coil transition of polypeptides (Schwarz, 1965) and the B–Z-helix transition of poly(dG-dC) (Pohl & Jovin, 1972). In the present case, there is not evidence for any large change of the pentadecamer conformation upon addition of Mg^{2+}. The magnitude of the circular dichroism observed for the pentadecamer increases when Mg^{2+} is added (cf. Figure 5): however, the shape of the

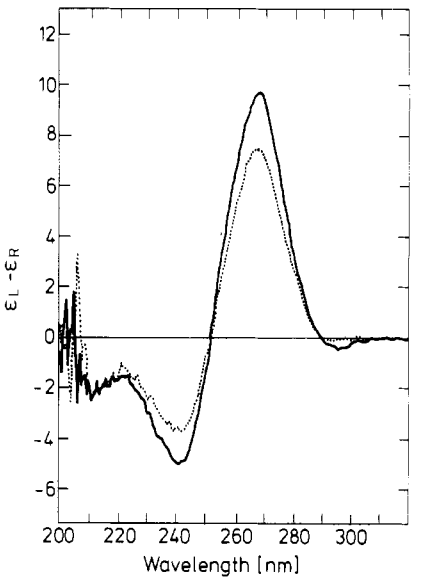


FIGURE 5: Circular dichroism of the pentadecamer in standard buffer at 0 (···) and 17.3 mM Mg^{2+} (—) (3.4 °C, 3.4 μM pentadecamer).

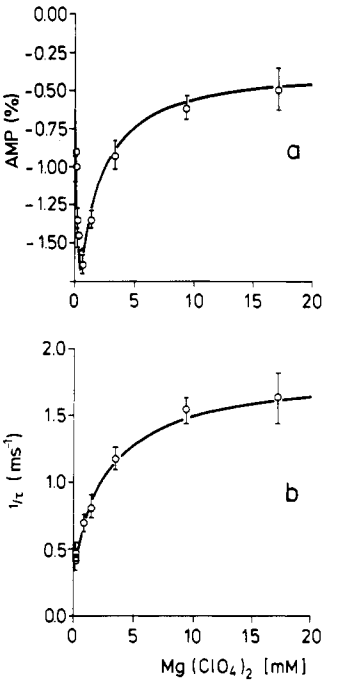
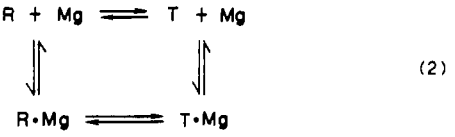


FIGURE 6: (a) Relative amplitudes and (b) reciprocal relaxation time of the pentadecamer high-Mg^{2+} effect as a function of Mg^{2+} concentration (standard buffer, 3.4 °C, 3.3 μM pentadecamer). The continuous lines represent a fit according to eq 4 with the parameters $k_a^+ = 3500\ s^{-1}$, $k_a^- = 5000\ s^{-1}$, $K_0 = 10^3\ M^{-1}$, $k_i^+ = 1500\ s^{-1}$, and $k_i^- = 380\ s^{-1}$.

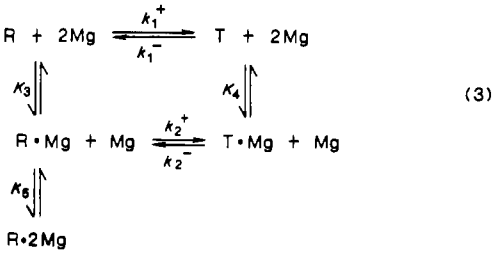
CD spectrum is not affected, and the change remains very small in the range of Mg^{2+} concentrations, where the low-Mg^{2+} effect is observed. Addition of Ca^{2+} to the pentadecamer induces changes of the circular dichroism very similar to those induced by Mg^{2+}.

In the absence of direct information on the nature of the low-Mg^{2+} effect, we tried to design the most simple reaction mechanism consistent with our observations. The components of the mechanism should be some conformational change of the pentadecamer and some Mg^{2+} binding reaction. Since a simple two-step mechanism would not be sufficient to describe our data, we started model calculations on a mechanism involving a conformation change with Mg^{2+} binding to both conformations:



(2)

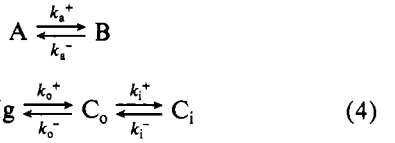
For simplicity, it is assumed that Mg^{2+} binding is fast compared to the conformation change. Under these conditions, the cyclic reaction is reflected by a single slow relaxation process associated with the conformation change and two fast processes associated with Mg^{2+} binding. It could be demonstrated that the reciprocal time constant associated with the conformation change passes a minimum value for increasing Mg^{2+} concentration, provided that the parameters of the cyclic scheme are selected to drive the conformation from one side to the other by addition of Mg^{2+}. Model calculations with many combinations of parameters showed, however, that the minimum obtained for the simple cyclic reaction scheme is not yet sufficient for a satisfactory description of our experimental data. Since the pentadecamer certainly has more than a single Mg^{2+} binding site, it is justified to extend the cyclic mechanism by adding another Mg^{2+} binding reaction. Of course, it cannot be excluded that a second Mg^{2+} can be bound to both conformations, but for simplicity, we added a second Mg^{2+} binding step only to one of the two conformations:



(3)

Again it is assumed that Mg^{2+} binding is fast compared to the conformation change and thus the reaction is reflected by a single slow process associated with the conformation change and three fast processes associated with Mg^{2+} binding. The reciprocal relaxation time associated with the conformation change passes a distinct minimum for increasing Mg^{2+} concentration, provided that Mg^{2+} binding drives the transition from T to R. A trial and error procedure was used to fit the parameters of the extended cyclic reaction scheme to the experimental data. The result shown in Figure 4 demonstrates that this reaction scheme is sufficient to describe the data within the limits of experimental accuracy. The parameters used for this description (cf. legend to Figure 4) demonstrate that one of the conformations is strongly favored in the absence of Mg^{2+} whereas the other one with two Mg^{2+} binding sites is much more stable at high Mg^{2+} concentration. The data obtained for the low-Mg^{2+} effect demonstrate the existence of strong Mg^{2+} sites with binding constants in the range 2×10^4 to 2×10^5 M^{-1}. Since these Mg^{2+} binding processes are not detected in the fluorescence curve (cf. Figure 2), it is apparent that the overall fluorescence change associated with these processes remains relatively low and the observation of the amplitude associated with the low-Mg^{2+} effect is mainly due to a relatively high enthalpy change associated with the conformation change.

Inner-Sphere Complexation by the Anticodon Loop. The high-Mg^{2+} effect observed in the millimolar concentration range of Mg^{2+} can be described without much problem, since the concentration dependence of both time constants and amplitudes (cf. Figure 6) follows the simple scheme observed previously for the complete tRNA^{Phe}. Fortunately, the transition reflected by the low-Mg^{2+} effect can be regarded as complete at the Mg^{2+} concentration where the onset of the high-Mg^{2+} effect is observed. Thus, the two Mg^{2+}-induced relaxation effects can be treated separately. The reaction scheme used previously for the complete tRNA^{Phe} included (a) the intramolecular anticodon loop transition A to B, (b) preferential formation of a Mg^{2+} outer-sphere complex (C_o) with one of the loop conformations, and (c) conversion of the outer-sphere complex to an inner-sphere complex (C_i) (Labuda & Porschke, 1982):



(4)

The outer-sphere complex C_o is formed from B and Mg^{2+} in a relatively fast reaction, which cannot be observed directly and is described as a diffusion-controlled process, as expected for this type of reaction. The anticodon loop transition A ↔ B and the formation of the inner-sphere complex C_i are reflected by separate slow relaxation processes. Least-squares fitting of the experimental data according to the reaction scheme 4 using the independent information available from the equilibrium titration curves [cf. Labuda & Porschke (1982)] provides the following parameters: (1) rate constants for the anticodon loop transition, k_a^+ and k_a^-; (2) the equilibrium constant for the formation of outer-sphere complexes, K_o; and (3) the rate constants for the formation of inner-sphere complexes, k_i^+ and k_i^-. A set of these constants together with the enthalpy changes and the optical parameters is given in the legend to Figure 6.

The equilibrium and rate constants of inner-sphere complexation have been determined as a function of temperature in the range from 3.4 to 25 °C. The van't Hoff and Arrhenius plots are given both for the pentadecamer and for the complete tRNA^{Phe} in panels a and b, respectively, of Figure 7.

Some comments should be added to explain the relation of the species defined by eq 3 and 4. Since the coupled reactions reflected by eq 3 are driven toward species R·2Mg at the saturation of the low-Mg^{2+} effect, the species A defined by eq 4 should be identical with R·2Mg. However, this does not mean that after the transition of R·2Mg ≡ A to B a third Mg^{2+} ion is bound to the same site. Certainly, the Mg^{2+} ions bind to separate sites: binding of the first two Mg^{2+} ions detected by the low-Mg^{2+} effect is probably to the double-helix stem, whereas the Mg^{2+} binding reflected by the high-Mg^{2+} effect should be very close to the Wye base as indicated by the larger fluorescence change (cf. Conclusions).

Mg^{2+} binding to the hexamer $G_mAAYA\psi$ has been analyzed as a reference, because this hexamer contains the nucleotide sequence with the main contact sites observed for the Mg^{2+} inner-sphere complex in the anticodon loop of tRNA^{Phe}. The fluorescence of the Wye base in this hexamer was used again to characterize the interaction with Mg^{2+}. Addition of Mg^{2+} leads to an increase of the fluorescence intensity, which can be described quantitatively by a simple one-step binding reaction with an equilibrium constant of 490 M^{-1} and an increase of the fluorescence intensity by a factor of 1.23 (standard buffer, 7.2 °C). Thus, both the binding constant and the fluorescence change are lower for the binding of Mg^{2+} to the hexamer than to the pentadecamer or to the complete tRNA^{Phe}.

The fluorescence of the hexamer has been studied previously as a function of the Mg^{2+} concentration: Beardsley et al. (1970) reported that the fluorescence is independent of magnesium, whereas Maelicke et al. (1973) found a much

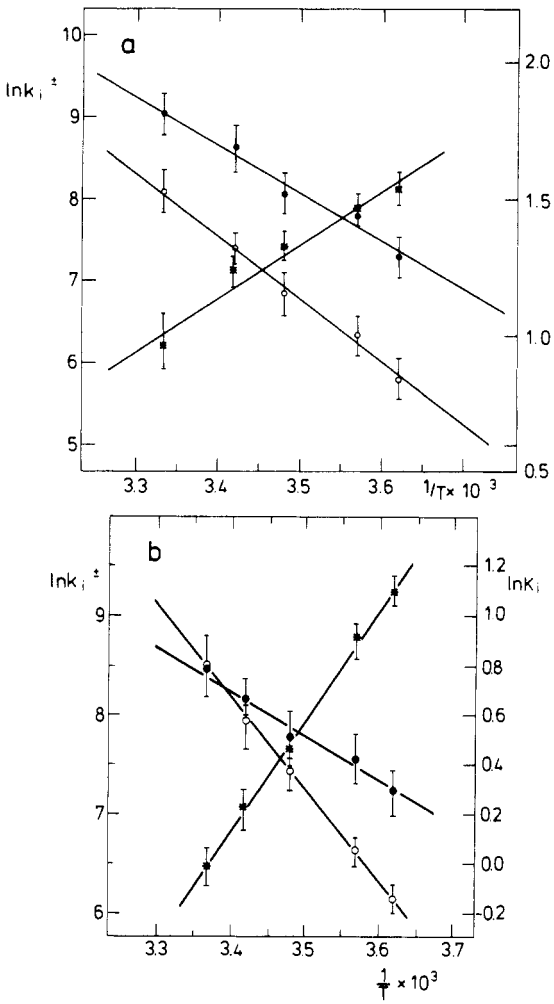


FIGURE 7: Parameters of the Mg^{2+} inner-sphere complexation with (a) the pentadecamer and (b) the complete $tRNA^{Phe}$ (yeast) as a function of the reciprocal absolute temperature (standard buffer): k_i^+ (●); k_i^- (○); $K_i^+ = k_i^+/k_i^-$ (asterisks). According to these data, Mg^{2+} inner-sphere complexation to the pentadecamer is associated with an enthalpy change $\Delta H_i = -15$ kJ mol^{-1}, an entropy change $\Delta S_i = -43$ J mol^{-1} deg^{-1}, and an Arrhenius activation enthalpy $\Delta E_i^+ = +49$ kJ mol^{-1}; the corresponding parameters for the whole $tRNA^{Phe}$ are $\Delta H_i = -36$ kJ mol^{-1}, $\Delta S_i = -124$ kJ mol^{-1} deg^{-1}, and $\Delta E_i^+ = 43$ kJ mol^{-1}.

larger increase of the fluorescence intensity and a higher Mg^{2+} affinity than observed in the present experiments. Unfortunately, we do not have any simple explanation for these differences.

Temperature-jump experiments performed with solutions of the hexamer in the standard buffer did not reveal any relaxation effect apart from the usual fast physical quenching effect. Addition of Mg^{2+} up to concentrations of 140 mM also did not induce any detectable relaxation effect. Thus, the Mg^{2+}-induced conformation change and the Mg^{2+} inner-sphere complexation observed for the pentadecamer are dependent upon the existence of the loop structure.

Conclusions

The results obtained in the present investigation for the isolated hairpin loop may be directly compared to those obtained previously for the same anticodon loop as an element of the complete $tRNA^{Phe}$ (yeast) (Labuda & Porschke, 1982). In both cases, structure and dynamics have been analyzed by temperature-jump measurements detected by the fluorescence of the Wye base located at the anticodon. Two of the three relaxation processes detected for the isolated hairpin loop—the "anticodon loop transition" and the high-Mg^{2+} effect—have been found in a closely corresponding form for the complete $tRNA^{Phe}$, whereas the third process—denoted low-Mg^{2+} effect—could only be detected for the isolated loop and not for the complete $tRNA^{Phe}$.

The anticodon loop transition has been assigned tentatively to a conversion between a "3′-stack" and a "5′-stack" conformation of the loop (Urbanke & Maass, 1978; Labuda & Porschke, 1982). In the 3′-stack conformation, the anticodon triplet together with the nucleotides adjacent to its 3′ side form a single-stranded helix, which continues base stacking and helicity of the 3′ strand of the anticodon stem double helix. The stacking pattern in the 5′-stack conformation is simply converted toward the 5′ side. The time constant of this transition (~100 μs) is too slow to be assigned to a simple stacking rearrangement, since the helix–coil transition of single-stranded polynucleotides is observed in the time range below 1 μs (Porschke, 1973, 1976; Dewey & Turner, 1980). The relatively low rate of the anticodon loop transition suggests that the rearrangement involves several nucleotide residues as required for a 3′–5′-stack transition. The present observation of this process in the isolated anticodon loop and its absence in the hexamer provides further evidence for an assignment to the 3′–5′-stack transition.

The two additional relaxation effects observed for the isolated hairpin loop are dependent upon the presence of Mg^{2+}. The hairpin loop with 15 nucleotide residues provides a rather large number of sites for Mg^{2+} binding. In the case of complete tRNA molecules, the Mg^{2+} sites have been classified according to their affinity [cf. Schimmel & Redfield (1980) and Teeter et al. (1980)]: up to 5 Mg^{2+} ions are associated with relatively high binding constants in the range of 2×10^4 to 5×10^6 M^{-1}, depending upon the solvent; an "intermediate" class of about 10 Mg^{2+} ions are associated with binding constants of 5×10^2 to 10^4 M^{-1}, and an additional class of "weak" sites is characterized by even lower binding constants. Teeter et al. (1980) suggested that the strong binding sites provide two phosphate groups for a direct coordination of Mg^{2+} ions, whereas intermediate sites are characterized by contacts of Mg^{2+} ions with a single phosphate and some base residue(s).

As discussed previously for the case of the complete $tRNA^{Phe}$ (Labuda & Porschke, 1982), the Mg^{2+} binding site characterized in solution by the strong increase of the Wye base fluorescence can be readily assigned to the site identified by X-ray analysis in the center of the anticodon loop (Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978). According to the criteria described above, this site belongs to the intermediate class, since it is characterized by a binding constant of about 2×10^3 M^{-1} and provides a single, direct contact to a phosphate residue. As shown by the present results, the same binding site observed for the complete $tRNA^{Phe}$ exists in the isolated anticodon hairpin loop but not in the hexamer. Although most of the contact sites are available in the hexamer, there is no indication for Mg^{2+} inner-sphere complexation by this oligonucleotide. Apparently, the hairpin loop structure is essential for formation of the inner-sphere complex, since the hairpin loop provides a unique arrangement of many contact sites to the Mg^{2+} ion. This is clearly illustrated by the structure of the anticodon loop found in the crystal by X-ray analysis (Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978). In addition to the direct contact of the Mg^{2+} to phosphate residue 37, which explains the slow inner-sphere relaxation effect observed in solution, there are six hydrogen bonds via water molecules from the Mg^{2+} inner hydration sphere to residues Y37, A38, ψ39, A31,

C32, and phosphate residue 37. The contact to Y37 provides an explanation for the strong change of the Wye base fluorescence. An additional and probably even more important contribution to the fluorescence change seems to be due to the change of the conformation induced by Mg^{2+} binding. The preferential binding of Mg^{2+} to one of two loop conformations deduced from the relaxation data can be easily explained by the large number of contacts formed by Mg^{2+}, which requires a special conformation of the loop.

Compared to the rather simple and unambiguous assignment of the intermediate binding process described above, the assignment of the strong binding sites reflected by the low-Mg^{2+} effect is much more difficult. The unusual concentration dependence of the time constants observed for this process indicates a relatively complex reaction mechanism. We have been able to describe the main attributes of this process by cyclic coupling of conformation change and a Mg^{2+} binding reaction, which is extended by an additional Mg^{2+} binding process. Probably some residual deviation of the experimental data from the curves calculated according to the reaction model is due to the polyelectrolyte nature of the pentadecamer, which has not been considered in the model. In spite of remaining problems with some details, it is obvious that the unusual effect is induced by strongly bound Mg^{2+} ions, which are characterized by binding constants in the range from 2×10^4 to 2×10^5. According to the classification given above, these strongly bound ions may be attached to two phosphate residues simultaneously. However, according to the structure of the hairpin loop expected from X-ray analysis of the complete tRNA^{Phe}, there are no sites with two phosphates close enough to each other. The distance between two adjacent phosphates in the double-helical stem, for example, is about 7 Å, whereas the ion radius of Mg^{2+} is about 0.7 Å. Probably the high affinity of the sites associated with the unusual effect is mainly due to the relatively high electrostatic potential of the helix stem rather than a close approach of two phosphate residues.

The induction of the unusual effect by Mg^{2+} ions and its absence in the presence of Ca^{2+} ions indicate that this effect is associated with inner-sphere complexation by Mg^{2+} ions. However, the minimum in the dependence of the reciprocal time constant upon the Mg^{2+} concentration cannot be explained by any conventional inner-sphere complex formation. The analogy of the experimental data with those obtained previously for the α-helix–coil transition (Schwarz, 1965) and the B–Z-helix transition (Pohl & Jovin, 1972) suggests that Mg^{2+} ions induce a conformation change of the hairpin loop. Evidence for this interpretation also comes from the relatively high enthalpy change associated with the transition, which can hardly be attributed to Mg^{2+} binding itself. However, the absence of any large change in the CD spectrum of the hairpin loop during the transition indicates that the Mg^{2+}-induced change is limited to a subtle rearrangement of the conformation. Thus, the molecular nature of the Mg^{2+}-induced transition remains to be established. As mentioned above, the absence of this transition in the presence of Ca^{2+} indicates the existence of Mg^{2+} inner-sphere complexation during the transition. Probably the slow step in the cyclic reaction scheme includes the formation of an inner-sphere complex. A less conventional, though particularly interesting, interpretation of the Mg^{2+}/Ca^{2+} antagonism would be a conformation change which can only be induced by Mg^{2+} (not by Ca^{2+}) ions. However, there is not direct evidence yet for the existence of sufficiently specific ion binding sites in standard nucleic acids.

Registry No. Mg, 7439-95-4; Ca, 7440-70-2.

References

Avery, L. (1982) *J. Chem. Phys. 76*, 3242–3248.

Beardsley, K., Tao, T., & Cantor, C. R. (1970) *Biochemistry 9*, 3524–3532.

Bujalowski, W., & Porschke, D. (1984) *Nucleic Acids Res. 12*, 7549–7563.

Clore, G. M., Gronenborn, A. M., & McLaughlin, L. W. (1984) *J. Mol. Biol. 174*, 163–173.

Dewey, T. G., & Turner, D. H. (1980) *Biochemistry 19*, 1681–1685.

Ehrlich, R., Lefevre, J. F., & Remy, P. (1980) *Eur. J. Biochem. 103*, 145–153.

Eigen, M. (1963) *Pure Appl. Chem. 6*, 97–115.

Eigen, M., & DeMaeyer, L. (1963) *Tech. Org. Chem. 8*, 895–1054.

Eigen, M., & Maass, G. (1966) *Z. Phys. Chem. (Munich) 49*, 163–177.

Eisinger, J., & Gross, N. (1974) *J. Mol. Biol. 88*, 165–174.

Eisinger, J., Feuer, B., & Yamane, T. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 638–644.

Eisinger, J., Feuer, B., & Yamane, T. (1971) *Nature (London) 231*, 126–128.

Flossdorf, J., & Schilling, H. (1979) *F&M, Feinwerktech. Messtech. 87*, 93–96.

Grosjean, H., Söll, D., & Crothers, D. M. (1976) *J. Mol. Biol. 103*, 499–519.

Högenauer, G. (1970) *Eur. J. Biochem. 12*, 527–532.

Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, S. H. (1977) *Nucleic Acids Res. 4*, 2811–2820.

Jac, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315–318.

Labuda, D., & Porschke, D. (1980) *Biochemistry 19*, 3799–3805.

Labuda, D., & Porschke, D. (1982) *Biochemistry 21*, 49–53.

Maelicke, A., von der Haar, F., & Cramer, F. (1973) *Biopolymers 12*, 27–43.

McLaughlin, L. W., & Graeser, E. (1982) *J. Liq. Chromatogr. 5*, 2061–2077.

Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.

Porschke, D. (1973) *Eur. J. Biochem. 39*, 117–126.

Porschke, D. (1976) *Biochemistry 15*, 1495–1499.

Porschke, D., & Jung, M. (1982) *Nucleic Acids Res. 10*, 6163–6176.

Quigley, G. J., Teeter, M. M., & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64–68.

Robinson, B., & Zimmermann, T. P. (1971) *J. Biol. Chem. 246*, 110–117.

Schimmel, R. R., & Redfield, A. G. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 181–221.

Schwarz, G. (1965) *J. Mol. Biol. 11*, 64–77.

Teeter, M. M., Quigley, G. J., & Rich, A. (1980) in *Nucleic Acid–Metal Ion Interactions* (Spiro, T. G., Ed.) pp 147–177, Wiley, New York.

Uhlenbeck, O. C., Baller, T., & Doty, P. (1970) *Nature (London) 225*, 508–512.

Urbanke, C., & Maass, G. (1978) *Nucleic Acids Res. 5*, 1551–1560.

Woese, C. (1970) *Nature (London) 226*, 817–820.