Ribosome-Catalyzed Formation of an Abnormal Peptide Analogue†

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ABSTRACT: The peptidyl-tRNA analogue *N*-(chloroacetyl)phenylalanyl-tRNA^{Phe} was prepared by chemical aminoacylation and prebound to the P site of *Escherichia coli* ribosomes in response to poly(uridylic acid). Admixture of phenylalanyl-tRNA^{Phe} to the A site resulted in the formation of two "dipeptides", one of which was formed by displacement of chloride ion from the peptidyl-tRNA. This constitutes the first example of ribosome-mediated formation of a peptide of altered connectivity and suggests a need for revision of the current model of peptide bond formation. Also suggested by the present finding is the feasibility of utilizing tRNAs to prepare polypeptides of altered connectivity in an in vitro protein biosynthesizing system.

Peptidyltransferase is an integral part of the 50S bacterial ribosomal subunit that catalyzes the formation of peptide bonds during protein biosynthesis (Allen & Zamecnik, 1962; Nathans, 1964; Traut & Monro, 1964; Monro, 1967; Harris & Symons, 1973a,b; Symons et al., 1979). Primarily through the use of modified tRNAs,[1] or aminoacylated oligonucleotides resembling the 3′ end of tRNAs, it has been possible to demonstrate peptide bond formation between certain amino acids of altered structure. Although a variety of peptide analogues have been formed in this fashion including polyesters (Scolnik et al., 1970; Fahnestock et al., 1970; Fahnestock & Rich, 1971a,b) and a thioamide (Victorova et al., 1976), the accumulated evidence has suggested that "peptide bond" formation is efficient only when the tRNAs in the ribosomal A and P sites have aminoacyl (peptidyl) moieties attached to the appropriate position at the 3′-terminus of tRNA (Hecht, 1977a,b) and only when the formed peptide bond analogue has the normal connectivity.[2]

Reported herein is the first example of ribosome-mediated formation of a peptide analogue having altered connectivity, an observation with important implications for the mechanism of ribosomal peptide bond formation.

Experimental Procedures

Materials. *Escherichia coli* tRNA^{Phe} (sp act. 1270 pmol/A_{260} unit) and BD-cellulose were purchased from Boehringer Mannheim. T4 RNA ligase (RNase free; sp act. 1400 units/mg of protein) was obtained from PL-Pharmacia; 1 unit was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphatase-resistant ^{32}P from 5′-[^{32}P]oligo(rA) in 30 min at 37 °C. DEAE-cellulose (coarse mesh), poly(uridylic acid) (M_r >10^5), bovine serum albumin, and L-phenylalanyl-L-phenylalanine were from Sigma Chemical Co. [^3H]- and [^{14}C]phenylalanines (sp act. 37 and 520 Ci/mol, respectively) were purchased from Amersham and purified by chromatography on BD-cellulose. HPLC analysis was carried out on a Rainin Microsorb C_{18} column (3 μm; 0.46 × 10 cm).

E. coli phenylalanyl-tRNA synthetase and *E. coli* ribosomes were isolated as described (Pezzuto & Hecht, 1980). *E. coli* tRNA^{Phe}-CC_{OH} was prepared from intact *E. coli* tRNA^{Phe} as described (Alford et al., 1977) and then converted to tRNA^{Phe}-C_{OH} by successive treatments with periodate, lysine (pH 8.0), and alkaline phosphatase, by modification of a published procedure (Neu & Heppel, 1964). When incubated in the presence of ATP and yeast CTP(ATP):tRNA nucleotidyltransferase (Alford et al., 1977), the reconstituted tRNA had a sp act. of 1000 pmol/A_{260} unit, reflecting retention of biochemical integrity during the modification procedures. 2′(3′)-*O*-[*N*-(Chloroacetyl)-L-phenylalanyl]-pCpA was prepared essentially as described for related dinucleotides (Heckler et al., 1984b).

N-(Chloroacetyl)-L-phenylalanyl-tRNA^{Phe}. *E. coli* tRNA^{Phe}-C_{OH} (0.7 A_{260} unit) was ligated to 0.4 A_{260} unit of 2′(3′)-*O*-[*N*-(chloroacetyl)-L-phenylalanyl]-pCpA. The reaction mixture (70 μL total volume; 10% final Me_2SO concentration) contained 100 mM NaHepes buffer, pH 7.0, 30 mM $MgCl_2$, 4 μg/μL bovine serum albumin, 600 μM ATP, and 15 units of T4 RNA ligase. The reaction mixture was incubated at 4 °C for 16 h and then applied directly to a 1-mL DEAE-cellulose column (Cl^- form) for isolation of the ligated tRNA. The column was washed with 50 mM NaOAc buffer, pH 4.5, containing 1 mM $MgCl_2$ and 0.25 M NaCl to effect elution of unreacted dinucleotide, and then with the same buffer containing 1 mM $MgCl_2$ and 1 M NaCl to remove tRNA from the column. The ligated product was separated from tRNA^{Phe}-C_{OH} by chromatography on a 1-mL BD-cellulose column (Cl^- form). The column was washed with 50 mM NaOAc, pH 4.5, containing 1 mM $MgCl_2$ and 1 M NaCl, to effect elution of tRNA^{Phe}-C_{OH}; subsequent washing with the same buffer containing 25% ethanol permitted the isolation of *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe}. The tRNA was dialyzed against 1 mM KOAc, pH 4.5, prior to use in the peptidyltransferase reaction; typical yields for the preparation of *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe} were in the range 30–35%.

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[1] Abbreviations: tRNA-CC_{OH}, tRNA missing the 3′-terminal adenosine moiety; tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; BD-cellulose, benzoylated (diethylaminoethyl)cellulose; NaHepes, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NaPipes, sodium 1,4-piperazinediethanesulfonate; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

[2] That is, when a bond is formed between a heteroatom (N or O) attached to C^α of the aminoacyl moiety of the A-site tRNA and the (thio)carbonyl group that connects the peptidyl moiety to the P-site tRNA.

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Alternatively, *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe} was prepared by activation of *E. coli* tRNA with phenylalanine via the agency of *E. coli* phenylalanyl-tRNA synthetase, followed by acylation with *N*-(chloroacetoxy)succinimide. The aminoacylation reaction (400-μL total volume) was carried out in 90 mM NaPipes buffer, pH 7.8, containing 100 mM KCl, 25 mM $MgCl_2$, 0.5 mM EDTA, 10 mM ATP, 40 μM [^{3}H]phenylalanine (2 Ci/mmol), 80–100 A_{260} units of *E. coli* tRNA, and 7.5 μL of *E. coli* phenylalanyl-tRNA synthetase (Pezzuto & Hecht, 1980). The aminoacylation reactions were carried out at 25 °C for 60 min; isolation of the aminoacylated tRNA was carried out following phenol extraction by successive additions of 0.1 volume of 1 M NaOAc, pH 4.5, and 2 volumes of cold ethanol. The precipitated tRNA was isolated by centrifugation and then redissolved in 400 μL of 20 mM triethanolamine, pH 8.0. This solution was treated with 5 mg (26 μmol) of *N*-(chloroacetoxy)succinimide [prepared in analogy with other *N*-(acyloxy)succinimide derivatives (Schmidt et al., 1972)] dissolved in 400 μL of DMF. The combined solution was maintained at 25 °C for 20 min and then treated with 0.4 M NaOH to readjust the pH to 8.0. The reaction mixture was acidified to pH 4.5 with 1.0 M acetic acid and treated with 2 volumes of cold ethanol. *N*-(Chloroacetyl)-L-[^{3}H]phenylalanyl-tRNA^{Phe} was purified following deacylation of any unacylated phenylalanyl-tRNA with 10 mM $CuSO_4$ in 100 mM NaOAc, pH 5.5, for 30 min at 25 °C (Schofield & Zamecnik, 1968). Purification was effected by chromatography on a 1-mL column of BD-cellulose (Cl^- form). Following elution with 50 mM NaOAc, pH 4.5, containing 1 mM $MgCl_2$ and 1 M NaCl, the desired tRNA was recovered by washing with the same buffer containing 25% ethanol. The isolated *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe} was obtained in typical yields of 30–50%; dialysis against 1 mM KOAc, pH 4.5, was carried out prior to use in the peptidyltransferase reaction.

Peptidyltransferase Reactions. The peptidyltransferase reactions were carried out in 90 μL (total volume) of 100 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 150 mM NH_4Cl, 0.05 mg/mL poly(U), and 4 A_{260} units of *E. coli* 70S ribosomes. Approximately 25 pmol of *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe} was added to the reaction mixture and allowed to prebind to the ribosomes for 15 min at 4 °C. The extent of binding was determined at this point in a control reaction by filtration of the reaction mixture through a nitrocellulose filter and measurement of filter-bound radioactivity. Approximately 60 pmol of L-[^{3}H]phenylalanyl-tRNA^{Phe} that had been activated via the agency of phenylalanyl-tRNA synthetase was added to the reaction mixture, which was incubated at 25 °C for an additional 15 min prior to termination of the reaction by treatment with 90 μL of 1.0 M NaOH for 30 min. This solution was neutralized with 120 μL of 1.2 M acetic acid and then diluted to 1.5 mL with 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl and 5% ethanol, and applied to a 1-mL BD-cellulose column that had been equilibrated with the same buffer solution. The column was washed with the same buffer solution, and 1-mL fractions were collected until the radioactivity of the eluate had reached a base-line value; 12–15 mL of buffer was typically required. Elution with 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl, 30% ethanol, and 32% formamide, permitted recovery of the peptide products. The appropriate fractions were pooled and evaporated to dryness under diminished pressure.

Purification and Analysis of Products of the Peptidyltransferase Reaction. The material isolated from the BD-cellulose column was dissolved in ~150 mL of 5 mM NaOAc, pH 4.5, and applied to a 1-mL DEAE-cellulose column (acetate form). The eluate from the column was saved for analysis, and the column was washed successively with 10 mL of 5 mM NaOAc, pH 4.5, and then with a linear gradient of triethylammonium bicarbonate, pH 7.5 (100-mL total volume, 0.1–1.0 M, 2-mL fractions), at a flow rate of 0.25 mL/min. A portion of each fraction was used for determination of radioactivity; product(s) eluted in the void volume and as a single peak in the salt gradient.

HPLC analysis was carried out on a C_{18} reverse-phase column with the solvent mixtures indicated in the figure legends. Fractions (0.5 mL or 0.33 mL) were collected at a flow rate of 1.0 mL/min and analyzed for the presence of ^{3}H or ^{14}C radiolabel as appropriate. Prior to the analysis of each ribosomally generated sample, authentic synthetic samples of the putative products were admixed; their subsequent elution from the column was monitored by A_{254} measurement.

Synthesis of Authentic Samples of Peptide Analogues. The preparation of *N*-(chloroacetyl)-L-phenylalanyl-L-phenylalanine (**1**) was accomplished via the acylation of L-phenylalanine with *N*-(chloroacetoxy)succinimide (dimethylformamide/dimethyl sulfoxide/triethylamine, 8 h, 45 °C). The product was crystallized from water as colorless microcrystals: mp 217–219 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.74 (dd, 1 H), 2.92 (dd, 1 H), 3.00 (dd, 1 H), 3.08 (dd, 1 H), 3.98 (s, 2 H), 4.42 (m, 1 H), 4.57 (m, 1 H), 7.0–7.35 (m, 10 H), 8.31 (d, 1 H), and 8.45 (d, 1 H); mass spectrum (CI, positive ion) m/z 389 $(M + H)^+$.

The preparation of *N*,*N*′-(oxoethylene)bis(phenylalanine) (**2**) was accomplished essentially as described by Fones (1952); the identity of the product was verified by ^{1}H and ^{13}C NMR and by mass spectrometry.

Hydrolysis of (Putative) N,N′-(Oxoethylene)bis(phenylalanine). Putative *N*,*N*′-(oxoethylene)bis(phenylalanine) was dissolved in concentrated hydrochloric acid and heated at reflux for 6 h to effect conversion to *N*-(carboxymethyl)-phenylalanine (**3**) as described by Fones (1952). An authentic sample of **3** was obtained from synthetic **2** via the same hydrolysis procedure.

Results and Discussion

In this laboratory we devised a general method ("chemical aminoacylation") for the introduction of amino acid analogues onto the 3′-terminus of tRNAs (Hecht et al., 1978; Heckler et al., 1983, 1984a,b). This methodology has been used to help define certain of the requisite chemical and spatial features that control participation of the donor substrate in the peptidyltransferase reaction. In the present case, the technique has been used to prepare *E. coli N*-(chloroacetyl)-L-phenylalanine-tRNA^{Phe} (Figure 1), a peptidyl-tRNA analogue containing two electrophilic centers.

When *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe} was added to an incubation mixture containing *E. coli* ribosomes, poly(uridylic acid), and [^{3}H]phenylalanyl-tRNA^{Phe}, a dipeptide-like product was formed.[3] Fractionation of the product on DEAE-cellulose resolved it into two components, only one of which was retained on the column when the sample was applied in 5 mM NaOAc buffer (data not shown). Repetition of the experiment utilizing *N*-(chloroacetyl)-L-[^{14}C]phenylalanyl-tRNA^{Phe} and [^{3}H]phenylalanyl-tRNA^{Phe} gave the same

[3] *N*-(Chloroacetyl)-L-phenylalanyl-tRNA^{Phe} was prepared both via chemical aminoacylation and by acylation of phenylalanyl-tRNA^{Phe} with the *N*-hydroxysuccinimide ester of chloroacetic acid. Mixtures of the same two dipeptides were obtained from both tRNAs, although experiments using chemically aminoacylated tRNA gave better results as solvolysis of the chloroacetyl moiety was minimized.

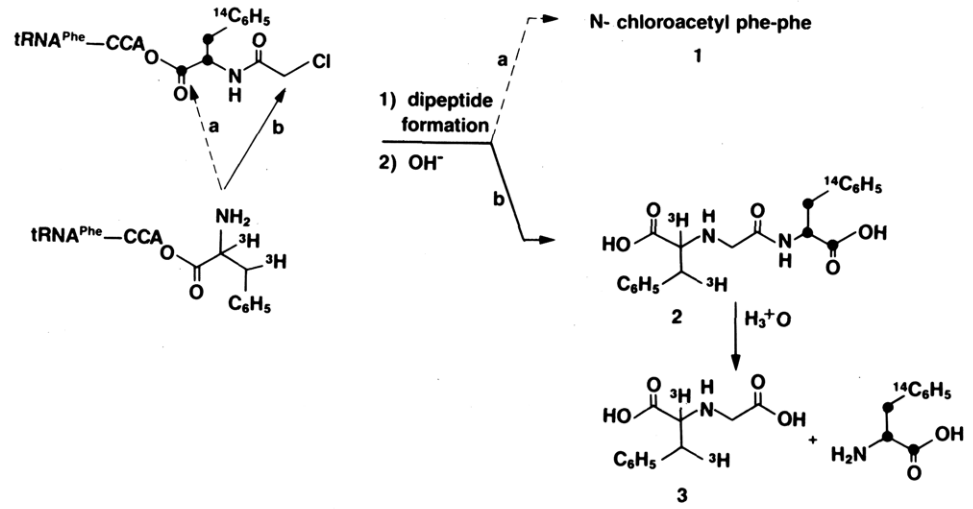

FIGURE 1: Formation of dipeptide analogues from *N*-(chloroacetyl)phenylalanyl-tRNA^{Phe}.

Table I: Effect of Chloramphenicol on Dipeptide Formation in the Presence of *N*-(Chloroacetyl)phenylalanyl-tRNA^{Phe} [a]

[^{3}H]peptidyl-tRNA^{Phe}	ribosomal binding (pmol of tRNA/A_{260} unit ribosome)	dipeptide (%)[b]
N-acetylphenylalanine	2.8	100
N-acetylphenylalanine (+4 mM chloramphenicol)	2.3	10
N-(chloroacetyl)phenylalanine	3.2	40[c]
N-(chloroacetyl)phenylalanine (+4 mM chloramphenicol)	2.7	3[c]
N-(chloroacetyl)phenylalanine (no ribosomes)		0

[a] The dipeptide-forming reaction, as well as analysis of ribosome binding and the dipeptide synthesized, was carried out as described under Experimental Procedures. [b] Yield of dipeptide relative to amount of peptidyl-tRNA bound to the ribosomal P site. [c] Total product obtained following chromatography on BD-cellulose.

two products and indicated that both contained ^{3}H and ^{14}C in the expected ratio (data not shown). The two products were formed in approximately equal amounts [20–25% yields for each, based on the amount of *N*-(chloroacetyl)-L-phenylalanyl-tRNA^{Phe} bound to the ribosomal P site].[4]

Desalting and HPLC analysis of the material that eluted at low salt indicated that it contained the product of "normal" dipeptide bond formation [i.e., *N*-(chloroacetyl)phenylalanylphenylalanine (**1**) and species derived therefrom]. The component that eluated from the DEAE-cellulose column at higher ionic strength was also analyzed by HPLC on a C_{18} reverse-phase column in comparison with an authentic synthetic sample of dipeptide analogue **2** (Fones, 1952). As shown in Figure 2 for an experiment that employed both *N*-(chloroacetyl)-L-[^{14}C]phenylalanyl-tRNA^{Phe} and [^{3}H]phenylalanyl-tRNA^{Phe}, authentic **2** coeluted with (doubly) radiolabeled ribosomal product, strongly suggesting the formation of **2** as a product of the peptidyltransferase reaction.

Further support for the proposed structure was obtained from a "nearest-neighbor" experiment in which the doubly labeled ribosomal product (putative **2**) was hydrolyzed under conditions (concentrated HCl, 100 °C, 6 h) shown to convert authentic **2** cleanly to *N*-(carboxymethyl)phenylalanine and phenylalanine. As illustrated in Figure 1, this would result in net transfer of the two-carbon unit (originally present as a chloroacetyl group) from the ^{14}C-labeled to the ^{3}H-labeled phenylalanine. HPLC analysis of the hydrolysis product (Figure 3) verified the presence of a ^{3}H-labeled product that coeluted with authentic *N*-(carboxymethyl)phenylalanine (Hahn & Litzinger, 1932; Hahn & Endicott, 1938) and lacked any ^{14}C radiolabel. The hydrolysate was also shown to contain [^{14}C]phenylalanine.

[4] Also formed was ribosome-associated material, consistent with earlier observations made for *N*-(bromoacetyl)phenylalanyl-tRNA^{Phe} (Pellegrini et al., 1972, 1974; Oen et al., 1973).

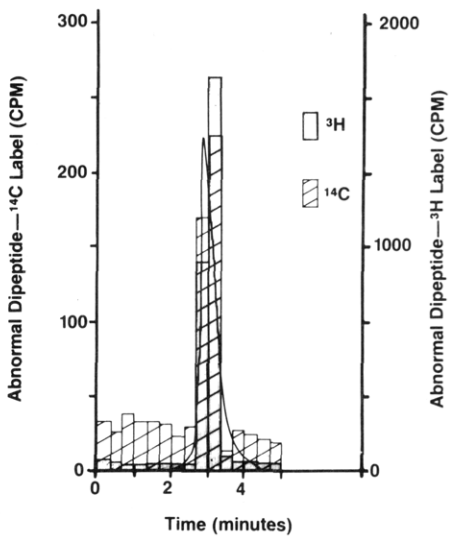

FIGURE 2: HPLC analysis of putative **2** isolated following DEAE-cellulose column chromatography. The analysis was carried out on a C_{18} reverse-phase column, which was washed with 15 mM Et_3N^+H OAc^- buffer, pH 7.0, containing 19.5% acetonitrile.

The peptidyltransferase dependence of product formation was demonstrated (Table I) by showing that neither **1** nor **2** was formed in the absence of ribosomes or in the presence of chloramphenicol, a known inhibitor of peptidyltransferase function (Vogel et al., 1969; Harris & Symons, 1973a; Nierhaus & Nierhaus, 1973; Pestka, 1977).

The accumulated data suggest strongly that in the present case two ribosome-catalyzed reactions occur, one of which leads to a dipeptide analogue of altered connectivity (**2**). This represents the first example of the ribosome-mediated formation of a dipeptide analogue of altered connectivity.[2] The ability of N^α of aminoacyl-tRNA in the ribosomal A site to react with a peptidyl-tRNA at an electrophilic position other

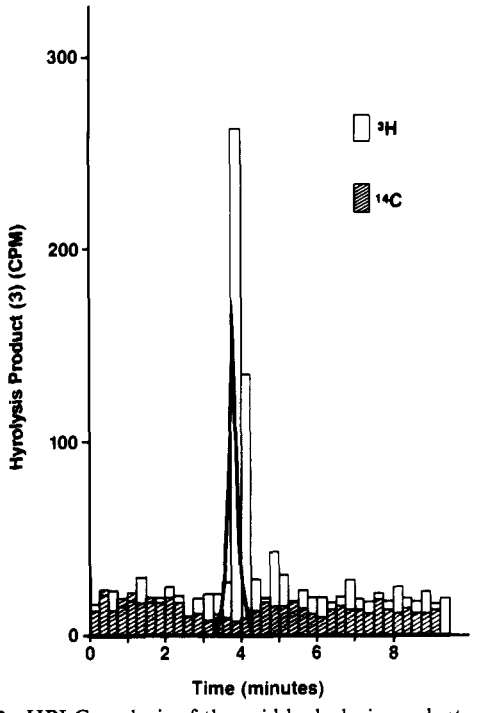

FIGURE 3: HPLC analysis of the acid hydrolysis products obtained from putative **2**. The analysis was carried out as indicated in the legend to Figure 2, except that 9% acetonitrile was employed.

than the carboxylate ester suggests that there is more flexibility in the process of peptide bond formation than is generally assumed. Current models of the 3′ ends of tRNAs bound to the A and P sites invoke a fairly rigid model having multiple recognition sites for the tRNA substrates, including structural domains for aromatic or hydrophobic amino acids on the A-site tRNA and a binding site for the nascent polypeptide (Harris & Symons, 1973a,b; Symons et al., 1979). The existence of these putative binding sites contributes to a view of peptide bond formation in which the A- and P-site tRNAs are bound to the ribosome such that the nucleophilic and electrophilic components responsible for peptide bond formation are in spatial proximity at the peptidyltransferase site. The present results are not readily accommodated by this model; they imply either greater flexibility of the aminoacyl or peptidyl moieties of the bound tRNAs during the peptidyltransferase reaction or else the possibility of chemical reactions between tRNAs in the ribosomal A and P sites that are facile but are not catalyzed by peptidyltransferase.

Implicit in the adaptor hypothesis (Crick, 1958; Hoagland, 1958) is the potential for alteration of protein structure through the use of misacylated tRNAs, an opportunity that has already been realized experimentally in one case (Chapeville et al., 1962; von Ehrenstein et al., 1963). Given current access to misacylated tRNAs of diverse structure (Hecht et al., 1978; Heckler et al., 1983, 1984a,b) and the ready availability of specific mRNAs as a consequence of recombinant DNA technology, the present finding suggests the feasibility of preparing polypeptides having altered *connectivity* via in vitro protein biosynthesis.[5]

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[5] In preliminary experiments *trans*-β-chloroacryloyl-tRNA^{Phe} bound to the P site of *E. coli* ribosomes has been shown to undergo both 1,2 and 1,4 addition during peptidyltransferase-mediated reaction with phenylalanyl-tRNA^{Phe}.

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Articles

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

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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,

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