Modeling the reactive properties of tandemly activated tRNAs†

Maria Duca, Shengxi Chen and Sidney M. Hecht*

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Tandemly activated tRNAs, bearing amino acid moieties at both the 2′- and 3′-positions of the 3′-terminal adenosine moiety (A_{76}), have been shown to participate efficiently in protein synthesis [B. Wang, J. Zhou, M. Lodder, R. D. Anderson, III and S. M. Hecht, *J. Biol. Chem.*, 2006, **281**, 13865]. The mechanism by which such activated tRNAs are able to donate both amino acids to the growing polypeptide chain is not well understood. Here we report the chemical behavior and participation in protein synthesis of new bisaminoacyl derivatives of pdCpA and tRNA. Both amino moieties of the aminoacyl groups are shown to be important to enable participation in protein synthesis; paradoxically, they also confer an unanticipated chemical stability toward different nucleophiles. The results obtained suggest a model for participation of bisaminoacylated tRNAs in protein synthesis.

Introduction

While ribosomal protein synthesis ordinarily involves (mono)aminoacylated tRNAs, Stepanov *et al.* have described the formation of bis-(2′,3′-*O*-phenylalanyl)-tRNAs by phenylalanyl-tRNA synthetase from *Thermus thermophilus*.[1,2] This enzyme was shown to form a tandemly activated tRNA using *T. thermophilus* $tRNA^{Phe}$ as substrate, as well as *Escherichia coli* $tRNA^{Phe}$. Recently, we reported that it is possible to use such tandemly activated tRNAs as participants in protein synthesis in spite of the fact that they exhibit surprising chemical stability.[3,4]

The steps involved in protein synthesis with bisaminoacylated tRNAs are outlined in Fig. 1. The bisaminoacylated tRNA is capable of binding with the prokaryotic elongation factor complex EF-TuGTP, which enables its introduction to the ribosomal A (aminoacyl) site. It has been demonstrated that transfer of the peptidyl moiety from the tRNA bound in the P (peptidyl) site involves the aminoacyl moiety at the 3′-position of the bisaminoacylated tRNA in the A- site; this was demonstrated by analysis of the amino acid incorporated into firefly luciferase in the presence of a bisaminoacylated tRNA having different amino acids at the 2′- and 3′-positions.[3,5]

The steps outlined in Fig. 1 suggest that following peptide acceptance and donation, the bisaminoacylated tRNA utilized initially must be lost from the ribosome as a monoacylated tRNA. Consistent with this interpretation is the finding that under protein synthesis conditions limiting for bisaminocylated tRNA, twice as much full-length protein was formed in the presence of a bisaminoacylated tRNA as the corresponding monoaminoacylated tRNA.[3]

An alternative mechanism, involving initial hydrolysis to a monoacylated tRNA, was also excluded by the finding that the yield of full-length protein doubled in the presence of bisaminoacylated tRNA.[3] Also excluded by this finding was the possible rearrangement of a bisaminoacylated tRNA to a dipeptidyl-tRNA, the latter of which inserted two amino acids simultaneously into the growing polypeptide chain. In fact, authentic dipeptidyl-tRNAs were shown not to function in protein synthesis.[3]

Earlier studies of protein synthesis have documented the requirement for a free 2′-OH group on peptidyl-tRNAs to support their function as peptide donors.[6–9] The absence of a 2′-OH group in bisaminoacylated tRNAs does not preclude their participation in protein synthesis, suggesting that the 2′-*O*-aminoacyl moiety must somehow function in lieu of the 2′-OH group normally present. To define the nature of the involvement of the 2′-*O*-aminoacyl group in protein synthesis, this functionality has been modified to permit identification of the functional groups required to support protein biosynthesis.

Transfer RNAs modified at the 3′-end are accessible by T4 RNA ligase-mediated condensation of aminoacylated pCpA or pdCpA derivatives with tRNAs or tRNA transcripts lacking the 3′-terminal dinucleotide pCpA (Fig. 2).[10–13] While mainly employed for the elaboration of proteins containing non-natural amino acids at predetermined sites,[14,15] they have also been used to study the mechanism of peptide bond formation.[11,12,16,17] In the present study, this strategy was employed to prepare modified bisaminoacyl-tRNAs.

Presently, we describe the preparation and evaluation of bisacylated 3′-*O*-alanyl-$tRNA_{CUA}$s bearing alanine (**I**), *N*-acetylalanine (**II**) or desaminoalanine (propionic acid) (**III**) at the 2′-position of A_{76} in the tRNAs (Fig. 3). The effects of these 2′-substituents on the ability of 3′-*O*-alanyl-tRNA to function in protein synthesis are discussed, as are the effects of the 2′-substituents on the chemical reactivity of the alanine moiety. These findings support a model which enables the participation of bisaminoacylated tRNAs in protein biosynthesis.

Results and discussion

Synthesis of bisaminoacylated pdCpA derivatives and tRNAs

Aminoacylated pdCpA derivatives **1–6** were prepared as outlined in the ESI†. They were used to study the relative reactivities of

Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia, 22904, USA. E-mail: sidhecht@virginia.edu; Fax: +1 434 924 7856; Tel: +1 434 924 3906

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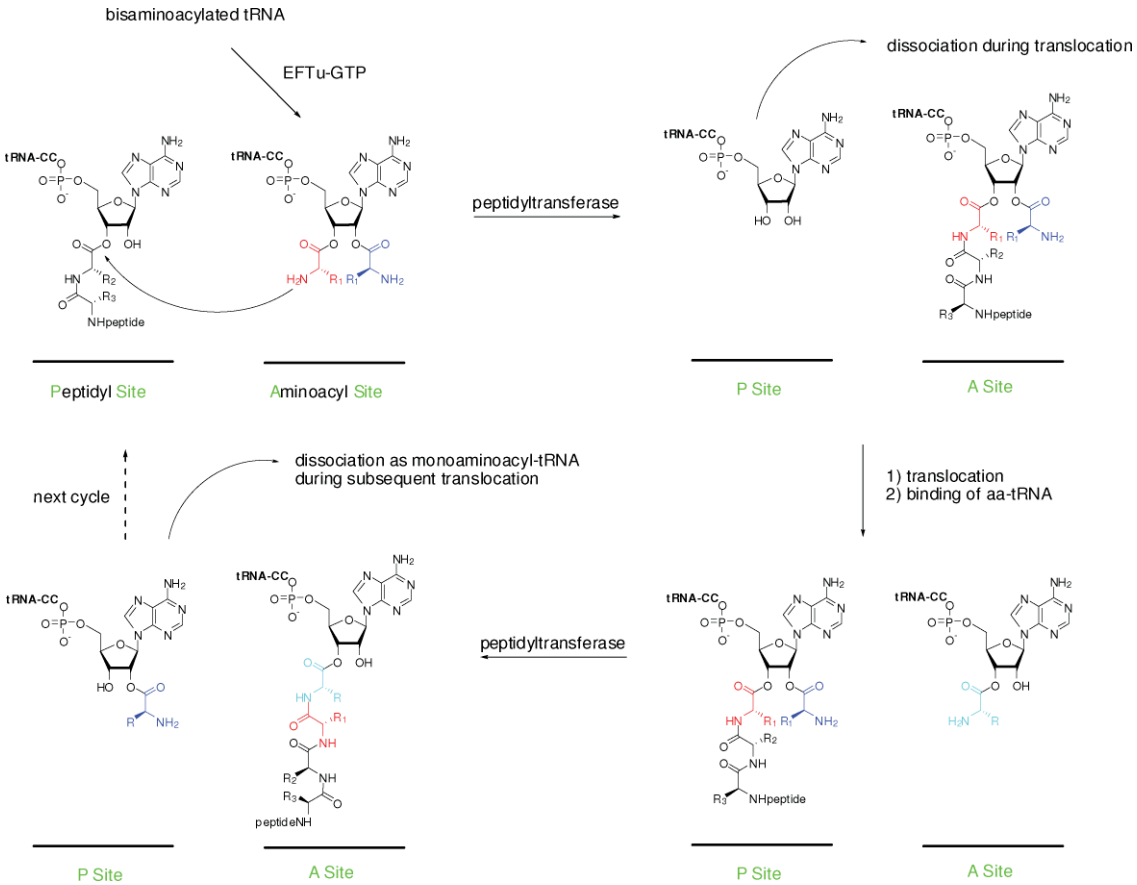

Fig. 1 Participation of bisaminoacylated tRNAs in the partial reactions of protein synthesis.

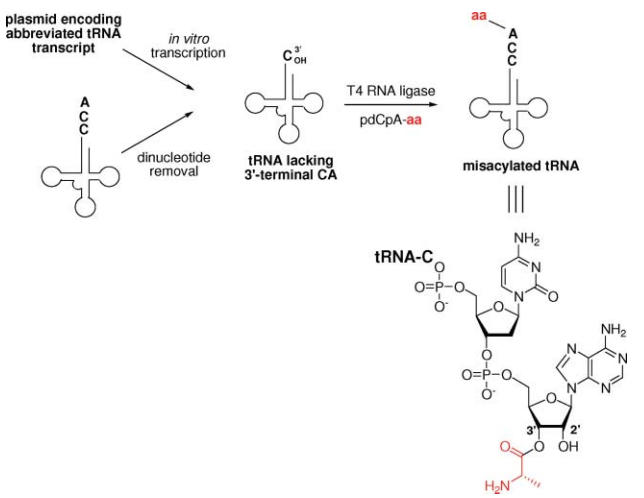

Fig. 2 Preparation of misacylated tRNAs by "chemical aminoacylation".

these species toward representative N nucleophiles, and also to prepare bisaminoacylated tRNAs (Fig. 3). All pdCpA derivatives were purified by C_{18} reversed phase HPLC using a gradient of 0→63% acetonitrile in 50 mM NH_4OAc, pH 4.5.

The *N*-pentenoyl protected bisaminoacylated pdCpA derivatives were each ligated to abbreviated suppressor $tRNA_{CUA}$ transcripts lacking the cytidine and adenosine moieties normally present at the 3′-terminus of all tRNAs (Fig. 2).[13,18] The tRNA transcript was obtained by *in vitro* run-off transcription of a *Fok1*-linearized plasmid encoding the abbreviated $tRNA_{CUA}$ (Fig. 2).[19] The ligation reaction was catalyzed by T4 RNA ligase.[10–13] The completion of the ligation reaction was verified by polyacrylamide gel electrophoresis at pH 4.5.[20] The formed *N*-pentenoyl bisacylated tRNAs were deprotected by treatment with aqueous iodine as described.[21–23]

The ability of the bisacylated tRNAs to participate in protein synthesis was investigated in a prokaryotic protein biosynthesizing system programmed with the mRNA for a dihydrofolate reductase construct in which a nonapeptide was fused at the N-terminus of wild-type DHFR. A stop codon (UAG) was introduced in lieu of the codon for Val10.[19] As shown (Fig. 4), in the presence of the deprotected monoalanyl- and bisalanyl-$tRNA_{CUA}$s, full length fusion protein was obtained by suppression of the UAG codon at position 10 of dihydrofolate reductase. The suppression yields for monoalanyl- and bisalanyl-tRNAs were 22 and 26%, respectively. In contrast, the bisaminoacylated tRNAs bearing one modified amino acid instead of alanine (*N*-acetyl-*S*-alanine or propionic acid) were not able to effect suppression of the UAG codon to a significant extent.

Stability of bisacylated pdCpA derivatives in the presence of nucleophiles

For the investigation of the chemical stability of bisacylated-pdCpAs, the susceptibility of compounds **1** and **2** to two different nucleophiles, namely *n*-butylamine and imidazole, was studied.

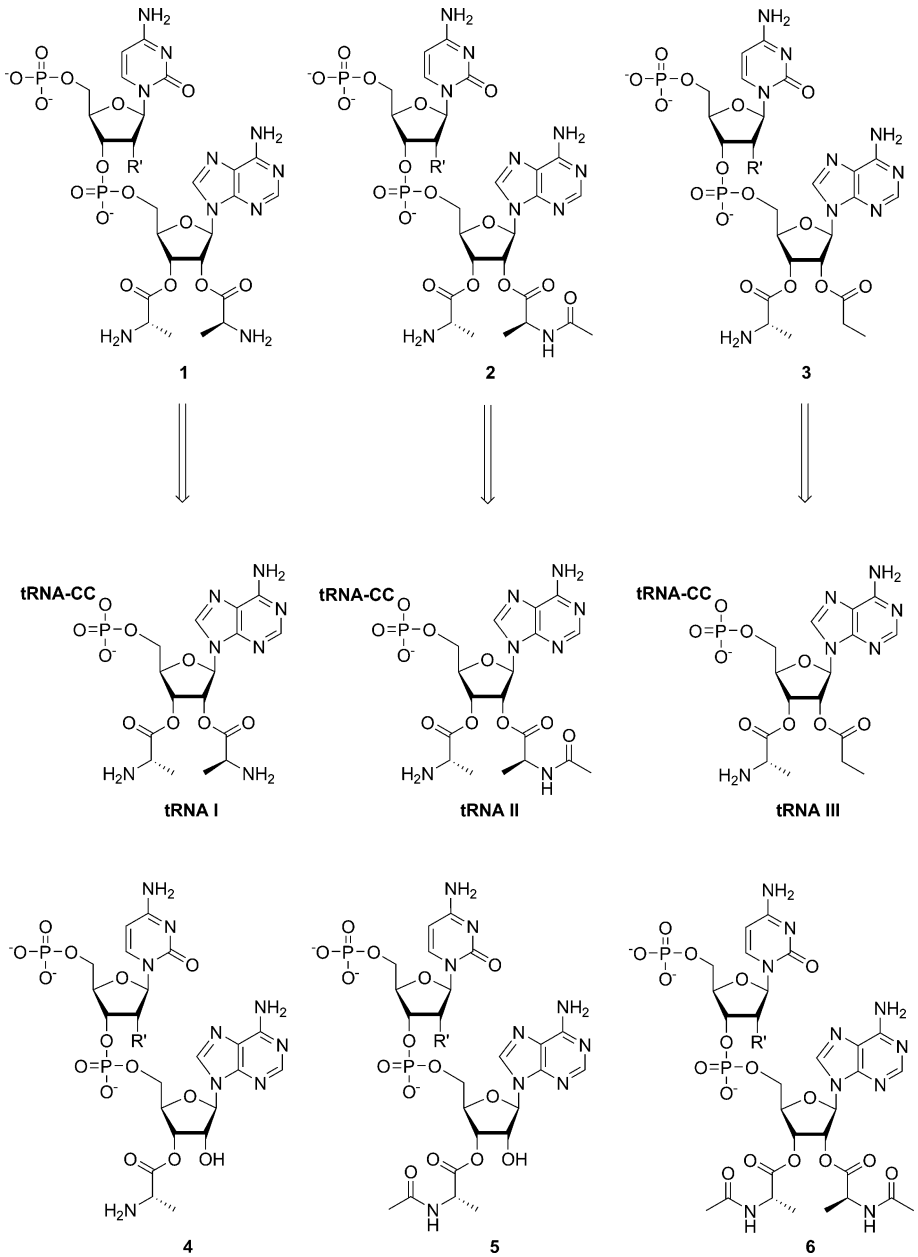

Fig. 3 Aminoacyl-pdCpAs used for this study. Compound **1** is bis-alanyl-pdCpA, compound **2** is 2-*O*-(*N*-acetylalanyl)-3-*O*-(alanyl)-pdCpA and compound **3** is 2-*O*-propionyl-3-*O*-alanyl-pdCpA. The latter two compounds lack one free amino group normally present in tandemly substituted pdCpAs. Also shown are the tRNAs (**I–III**) derived from these bisaminoacylated pdCpAs. Additionally, aminoacylated pdCpA derivatives **4–6** were used to study the time course of deacylation by imidazole and *n*-butylamine.

Initially, pdCpA was tested as a substrate to determine whether any reaction with the nucleobases, phosphate groups or sugar moieties would occur. No reaction with pdCpA was observed, even after 24 h or with a large molar excess of nucleophile. Accordingly, the reactivities of mono-2′(3′)-*O*-alanyl-pdCpA (**4**) and bis-2′,3′-*O*-alanyl-pdCpA (**1**) to *n*-butylamine and imidazole were studied. The kinetics of the hydrolysis of these compounds over 2 h is shown (Fig. 5). As expected, compound **4** was completely absent after 120 min in the presence of *n*-butylamine or imidazole. The only product was pdCpA, as confirmed by HPLC retention time and molecular weight analysis by mass spectrometry. When compound **1** was treated with the same two nucleophiles under the same conditions, the majority of the starting material was still present.

The appearance of a peak in the HPLC trace corresponding to pdCpA was observed in 10–20% yield after 2 h. Thus, the bisacylated dinucleotide was clearly more stable than its analogous monoacylated derivative toward nucleophiles. To better understand the increased and unanticipated stability of the bisalanyl-pdCpA, compound **2** (2′-*O*-(*N*-acetylalanyl)-3′-*O*-alanyl-pdCpA) was studied under the same conditions. In this case, mono-2′(3′)-*O*-(*N*-acetylalanyl)-pdCpA (**5**) was used for comparison. As shown (Fig. 6), compound **2** was much less stable than **5** in the presence of the nucleophiles, undergoing 90% conversion to pdCpA after

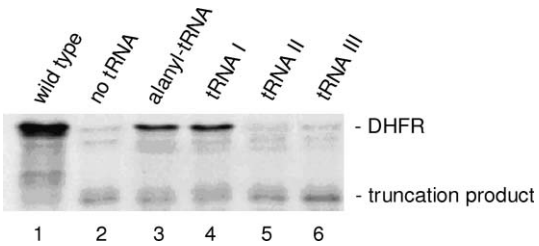

Fig. 4 Incorporation of alanine into position 10 of DHFR from mono- and bisalanyl-tRNAs (by suppression of a UAG codon). Lane 1, wild-type DHFR; lane 2, no tRNA; lane 3, alanyl-$tRNA_{CUA}$; lane 4, bisalanyl-$tRNA_{CUA}$ (**I**); lane 5, 2-*O*-(*N*-acetylalanyl)-3-*O*-(alanyl)-$tRNA_{CUA}$ (**II**); lane 6, 2-*O*-propionyl-3-*O*-alanyl-$tRNA_{CUA}$ (**III**).

120 min incubation with *n*-butylamine and 40% conversion to pdCpA after treatment with imidazole. In contrast, compound **5** underwent less than 20% conversion to pdCpA after treatment with the nucleophiles. The behavior of **5** was expected since the presence of the acetyl group should diminish the electrophilicity of the ester moiety. In contrast, the greater lability of **2** to nucleophiles, as compared with **5** (and **1**), was unanticipated. The conversion of **2** to pdCpA presumably involves the intermediacy of a monoacylated pdCpA (*i.e.*, either **4** or **5**). As is clear from Fig. 6, aminoacylated dinucleotide **5** is converted to pdCpA less quickly than **2**, and is thus not kinetically competent to be the intermediate on the pathway from **2** to pdCpA. In contrast, initial conversion of **2** to alanyl-pdCpA (**4**) would afford a species whose subsequent conversion to pdCpA is faster than the overall rate of conversion of **2** to pdCpA. The presumed conversion **2** → **4** → pdCpA is fully consistent with the absence of any observed intermediate during the conversion of **2** to pdCpA. An analogous observation was made for compound **3**, which underwent 65% conversion to pdCpA after 2 h of treatment with *n*-butylamine, and 43% conversion after treatment with imidazole for 2 h (data not shown). Finally, the pdCpA derivative containing two *N*-acetylalanine moieties (**6**) was subjected to *n*-butylamine and imidazole under the same conditions. As shown (Fig. 7), this compound underwent conversion to pdCpA very slowly.

Bisaminoacylated tRNAs as participants in protein synthesis

Our first study of bisacylated tRNAs demonstrated that these compounds are able to participate efficiently in protein synthesis.[3] It was established that bisacylated tRNAs bound to the ribosomal A-site must act as peptide acceptors at the 3′-position and then donate the extended peptide chain from this same position following translocation (Fig. 1). Both amino acid moieties of bisacylated tRNAs can be incorporated into protein. Thus, it is logical to conclude that the tRNA dissociated from the ribosome after the initial utilization of a bisacylated tRNA must be a monoaminoacylated tRNA able to participate in a subsequent round of peptide bond formation. Interestingly, in contrast to their obvious reactivity in prokaryotic and eukaryotic protein-synthesizing systems these bisacylated tRNAs, as well as the corresponding pdCpAs, exhibited unusual and unanticipated chemical stability when incubated under different conditions (pH 3.0–7.0, 25 °C, 3–12 h). Thus, the chemical structure and behavior of these compounds seemed worthy of study to define

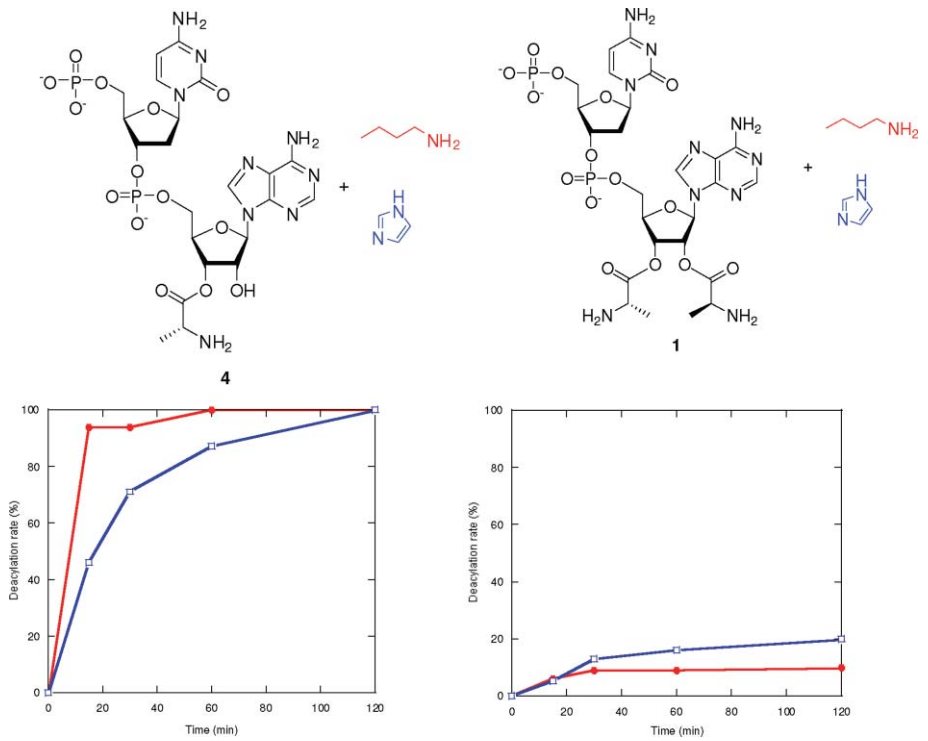

Fig. 5 Time course of the deacylation of alanyl-pdCpA (**4**) (0.2 mg, left) and bis-2,3-*O*-alanyl-pdCpA (**1**) (0.2 mg, right) in the presence of 2 eq. of *n*-butylamine (red) or imidazole (blue). The percent formation of pdCpA after treatment is shown over a period of 120 min as judged by analytical C_{18} reversed phase HPLC (250 × 4.6 mm, 5 μm column). The column was washed with 0→63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 1 mL min^{-1} (monitoring at 260 nm). HPLC analyses were made after 15, 30, 60 and 120 min.

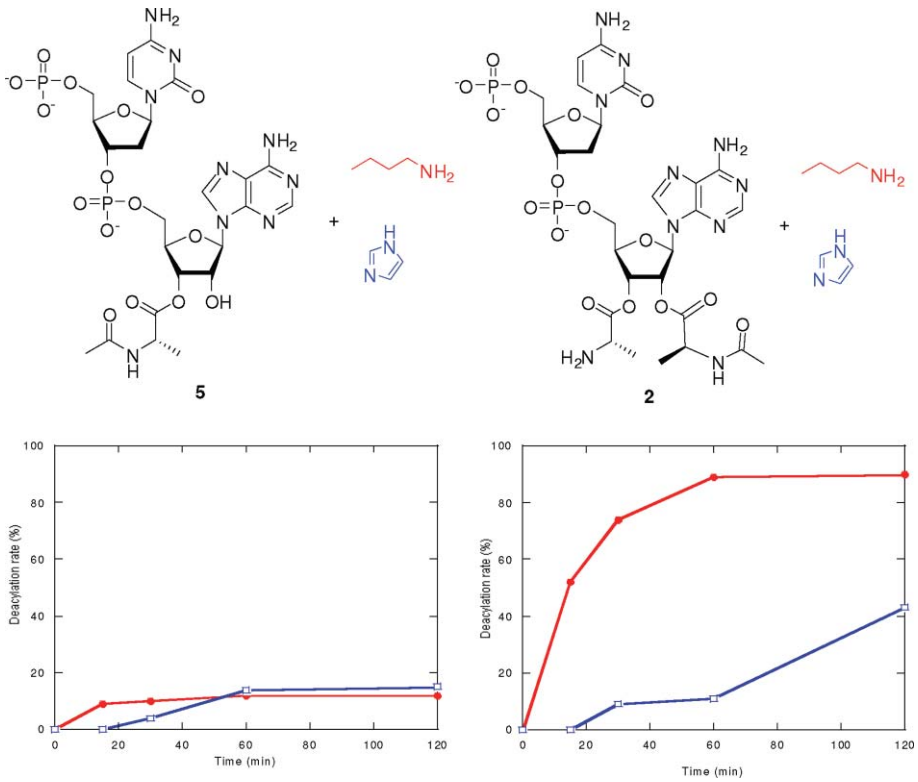

Fig. 6 Time course of the deacylation of 2′(3′)-*O*-(*N*-acetylalanyl)-pdCpA (**5**) (0.2 mg, left) and 2-*O*-(*N*-acetylalanyl)-3-*O*-alanylpdCpA (**2**) (0.2 mg right) in the presence 2 eq. of *n*-butylamine (red) and imidazole (blue). The percent formation of pdCpA after treatment is shown over a period of 120 min as judged by analytical C_{18} reversed phase HPLC (250 × 4.6 mm, 5 μm column). The column was washed with 0→63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 1 mL min^{-1} (monitoring at 260 nm). HPLC analyses were made after 15, 30, 60 and 120 min.

how their intrinsic stability could be compatible with their ability to participate in protein synthesis.

Transfer tRNAs are key participants in the translation process.[24] Each of them is initially recognized by the cognate aminoacyl-tRNA synthetase, which attaches the corresponding amino acid to the 3′-end of its cognate tRNA species. In prokaryotic systems, the aminoacyl-tRNA is then presented to the ribosomal A-site by EF-TuGTP, where the aminoacyl-tRNA subsequently acts as a peptide acceptor. The formed peptidyl-tRNA is translocated by EF-G to the ribosomal P-site, where it serves as a peptide donor before finally moving to the E-site and being released.[24] The A_{76} ribose sugar at the 3′-terminus of tRNA has two free hydroxyl groups on adjacent carbons which are available for the formation of an ester linkage to an amino acid, namely the 2′-OH and the 3′-OH groups.[7,25,26] This 2′,3′ *cis*-diol has important functions in every step of translation. An amino acid is activated for translation by ester linkage to the 2′ or the 3′-OH group, and each regioisomer is readily converted to the other *via* a 2′,3′-transacylation reaction. However, there is now a significant body of data indicating that peptide bond formation involves tRNAs in both the A-site and P-site containing exclusively 3′-*O*-aminoacyl (peptidyl) moieties.[3,6–9,27–31]

Further, for monoaminoacylated tRNAs, the remaining 2′-OH group is essential during the peptidyltransferase reaction: it has been suggested that this hydroxyl group is directly involved in proton transfer by serving as a "proton shuttle" and mediating concerted proton transfers from the A-site α-amino group (the nucleophile) and to the P-site 3′-OH (the leaving group).[27,32,33] It has been shown that the absence of 2′-OH or its substitution with other residues precludes the tRNA from being able to act as a P-site donor.[6–9]

The requirement for a free 2′-OH group on A_{76} of aminoacyl-tRNAs to enable their participation in protein synthesis argues that the 2′-*O*-aminoacyl moiety in bisaminoacylated tRNAs must be able to function in lieu of the 2′-OH group. The present study was intended to define those structural features in the 2′-*O*-aminoacyl group essential to support protein synthesis, and also to develop an understanding of the chemical behavior of such bisacylated pCpAs and tRNAs. To permit this study, new bisacylated pdCpA derivatives were prepared for the study of their chemical properties, and for incorporation into modified bisaminoacylated tRNAs whose function in protein synthesizing systems could be studied. The simple bis-2′,3′-*O*-alanyl-pdCpA derivative (**1**) had been already synthesized and described[3,4] and was chosen for the present study. Since it seemed likely that the amino group of the second aminoacyl residue was involved in the biochemical mechanism, enabling participation in protein synthesis, other analogues were prepared in which this amino group was protected or absent. For this reason 2′-*O*-(*N*-acetylalanyl)-3′-*O*-alanyl-pdCpA (**2**) and 2′-*O*-propionyl-3′-*O*-alanyl-pdCpA (**3**) were synthesized. The synthetic methodology used to prepare these compounds is analogous to that reported previously reported.[4,5] In particular, the monoacylated pdCpA derivative was isolated and converted to its tetra-*n*-butylammonium salt in order to perform a second coupling with the desired amino acid derivative, different from the one already present. As reference compounds, we used pdCpA as well as dinucleotides **4** and **5**,

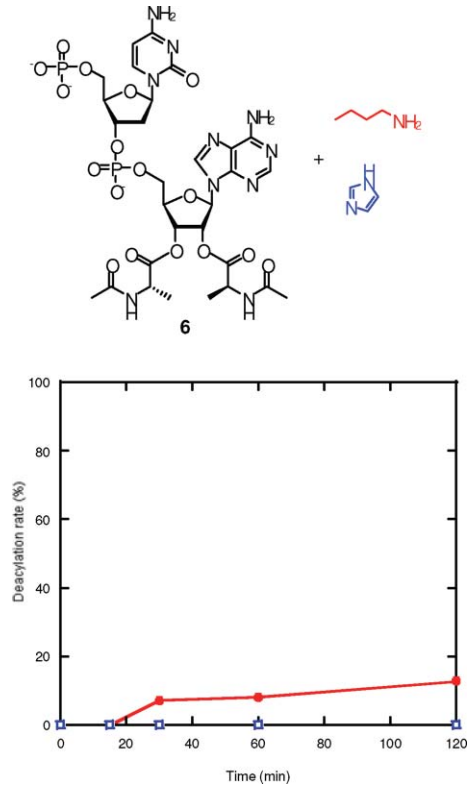

Fig. 7 Time course of the deacylation of bis-2,3-*O*-(*N*-acetylalanyl)-pdCpA (**6**) in the presence of 2 eq. of *n*-butylamine (red) or imidazole blue). The percent formation of pdCpA after treatment is shown over a period of 120 min as judged by analytical C_{18} reversed phase HPLC (250 × 4.6 mm, 5 μm column). The column was washed with 0→63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 1 mL min^{-1} (monitoring at 260 nm). HPLC analyses were made after 15, 30, 60 and 120 min.

both of which were synthesized using the methodology described previously.[4]

Bisaminoacylated tRNAs **I**, **II** and **III** (Fig. 3) were prepared by T4 RNA ligase-mediated condensation of the appropriate dinucleotides with $tRNA_{CUA}$-C_{OH}, and then employed in a prokaryotic protein synthesizing system. Unmodified alanyl-$tRNA_{CUA}$ was also tested in comparison. Only tRNA **I** and alanyl-$tRNA_{CUA}$ were able to participate in protein synthesis (lanes 3 and 4, Fig. 4), while tRNAs **II** and **III**, in which no free amino group was available on the "second" amino acid, failed to support the synthesis of full length protein (lanes 5 and 6, Fig. 4). This result indicates that this amino group is required to support the chemical reactions that lead to peptide transfer from the bisaminoacylated tRNA in the ribosomal P-site, as was the 2′-OH group present in monoacylated tRNAs. While tRNA **I** was at least as efficient as "wild-type" alanyl-tRNA, tRNAs **II** and **III**, lacking the second amino group, were incapable of supporting protein synthesis.

In an effort to understand the role played by the amino group of the 2′-*O*-aminoacyl moiety in protein synthesis, dinucleotide derivatives **1** and **2** were subjected to treatment with the N nucleophiles *n*-butylamine and imidazole, in order to model the nucleophilic N^{α} group of the aminoacyl moiety in the A-site tRNA during the peptidyltransferase reaction. Initially, we sought to confirm our earlier finding that bisacyl-pdCpAs, as well as their corresponding tRNAs, have much greater stability *in vitro* (pdCpAs) and in cell-free protein-synthesizing systems (tRNAs) than the corresponding monoacylated derivatives.[3] This result was confirmed when the deprotected mono-2′(3′)-*O*-alanyl-pdCpA (**4**) and bis-2′,3′-*O*-alanyl-pdCpA (**1**) were treated with *n*-butylamine and imidazole (red and blue respectively, Fig. 5). While compound **4** was converted to pdCpA within 2 h at 25 °C, compound **1** was only modestly affected. The degradation product was identified as pdCpA both by HPLC retention time and by mass spectrometry. Only 10–20% pdCpA was observed after treatment of compound **1** for 2 h and 30–50% after 24 h treatment (data not shown).

From the perspective of protein synthesis, a key step is the transformation that results in formation of the new (peptide) bond. This step involves one *aminoacyl*-tRNA (in the A-site) and one *peptidyl*-tRNA (in the P-site). Studies with tRNAs lacking a 2′-OH group on A_{76} suggest that it is the peptidyl-tRNA that critically requires activation by the 2′-OH group.[6–9,27] Thus the experiment summarized in Fig. 6 represents a simple chemical model comparing the effects of vicinal O-alanine and OH groups on the electrophilicity of the *O*-(*N*-acetylalanyl) ester (*i.e.* on the ability to "donate" this group to a N-nucleophile. As shown in Fig. 6, the "peptidyl" (*i.e. N*-acetylalanyl) moiety was dramatically more labile when adjacent to an alanine ester than when adjacent to an OH group. Thus, unlike aminoacyl-tRNA, which is rendered less electrophilic when acetylated on N^{α} of the amino acid (*cf.* **4** and **5**, Figs. 5 and 6),[34] acetylation of one (but not both, *cf.* **2** and **6**, Figs. 6 and 7) of the aminoacyl moieties of a bisalanyl-pdCpA significantly increases its ability to react with nucleophiles. It seems highly likely that this activation forms the basis for the unanticipated ability of bisaminoacylated tRNAs to participate in protein synthesis.[3]

The model suggested in the literature for the peptidyltransferase reaction is reported in Fig. 8A.[27,35] The amino group in the 3′-position of the A-site tRNA acts as a nucleophile toward the carbonyl group of the amino acid in the 3′-position of the P-site tRNA. The 2′-OH group of the latter tRNA is essential in acting as a "proton shuttle" and coordinating the 3′-carboxylate oxygen, thus activating the 3′-carboxyl group toward nucleophilic attack. Further, it also coordinates the amino group which acts as a nucleophile, following addition of the amino group to the carboxylate of the P-site tRNA.[27] Fig. 8B shows the model that arises from the data for bisaminoacyl-tRNA. In this case the amino group in the 2′-position of A_{76} of the P-site tRNA probably acts similarly to the 2′-OH of the monoacyl-tRNA by coordinating the 3′-carboxylate oxygen attached to the 3′-position of the P-site tRNA.

Not yet resolved by the present experiments is the molecular basis for the exceptional chemical stability of bisaminoacylated pdCpA derivatives and tRNAs (*cf.* Figs. 5 and 6). It seems likely that specific interactions of the second (2′-*O*-) aminoacyl moiety with the 3′-*O*-aminoacyl group will be found to contribute importantly to the observed chemical behavior.

Conclusion

Bisaminoacylated tRNAs bearing amino acid esters at both the 2′- and 3′-OH groups of the 3′-terminal adenosine moiety of tRNA were shown to participate in ribosomally mediated protein synthesis. Their ability to participate in protein synthesis was shown to be absolutely dependent on the presence of a free

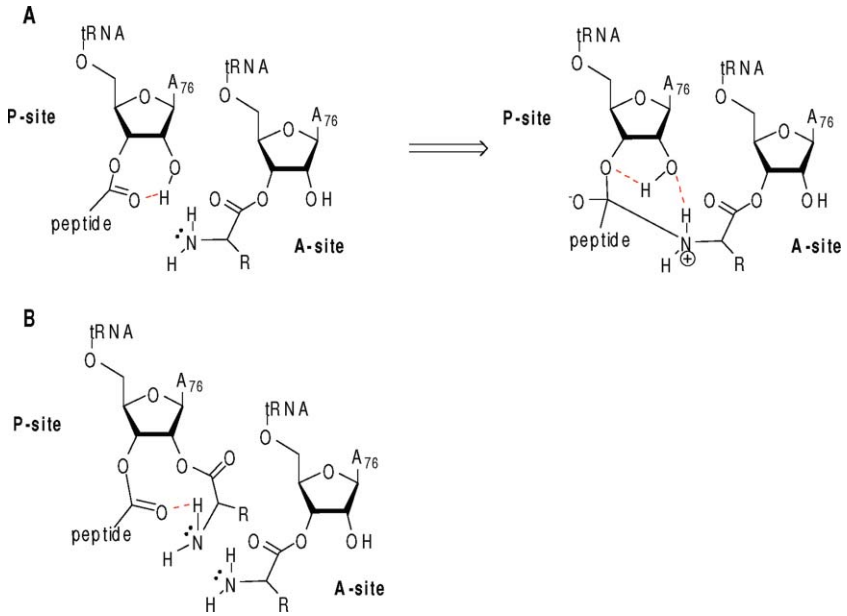

Fig. 8 Proposed model for participation of bisaminoacyl-tRNAs in protein synthesis. A) "Proton shuttle" model previously reported to explain the essential role of 2-OH in P-site tRNA. Shown here is the step leading to the formation of the putative tetrahedral intermediate formed during the peptidyltransferase reaction. B) Suggested model for the peptidyltransferase reaction involving a bisaminoacylated tRNA in the P-site.

amino group on each of the aminoacyl moieties. In contrast with monoacylated tRNAs and nucleotides, which exhibit lower reactivity toward nucleophiles when N-acylated, bisaminoacylated pdCpA esters were found to exhibit much greater reactivity toward nucleophiles when N^{α} was acylated. These effects are suggested to be due to H-bonding between the two attached aminoacyl esters in the bisaminoacylated pdCpAs and tRNAs.

Experimental

General methods and materials

E. coli competent cells BL21 (DE3) were obtained from Novagen (Madison, WI). Recombinant RNasin ribonuclease inhibitor and amino acid mixture (complete) were purchased from Promega Corporation (Madison, WI). [^{35}S]-methionine (1000 Ci mmol^{-1}, 10 μCi μL^{-1}) was purchased from Amersham Corporation (Piscataway, NJ). Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). T4 RNA ligase was purchased from New England Biolabs (Beverly, MA). Trizma base, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phosphoenolpyruvate, isopropyl-β-D-thiogalactopyranoside and *E. coli* tRNA were obtained from Sigma Chemicals (St. Louis, MO).

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a Perkin-Elmer Lambda 20 UV/vis spectrometer.

Gels were visualized and quantified by phosphorimager analysis, which was carried out using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.0 software. The pixel density of the image was directly related to the amount of radioactivity present in the sample by using a calibrated phosphorimager screen.

General procedure for the treatment of pdCpA and pdCpA derivatives 1–6 with nucleophiles

Compounds **1–6** (0.2 mg each) were dissolved in 30 μL of water.‡ To this stirred solution was added 30 μL of DMF containing 2 eq. of *n*-butylamine or imidazole; the reaction mixture was stirred for 24 h. After 2 and 24 h, 20 μL aliquots were analyzed by HPLC using a reversed phase column (Alltima RPC_{18} 4.6 × 250 mm, 5 μm). The column was washed with 0→63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 1 mL min^{-1} (monitoring at 260 nm).

Preparation of mono and bis-aminoacyl-tRNAs

Mono and bis-aminoacyl-tRNAs were prepared by a T4 RNA ligase-mediated ligation of the protected, chemically synthesized pdCpA derivatives (**8**, **9**, **14** and **15**, ESI†), with the abbreviated suppressor tRNA-C_{OH}.[13,18,19] The ligation reaction was carried out in 100 μL (total volume) of 100 mM HEPES, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} units of *N*-pentenoyl-protected mono- or bis-aminoacyl-pdCpA derivatives (5- to 10-fold molar excess), 15% DMSO and 200 units of T4 RNA ligase. After incubation at 37 °C for 30 min, the reactions were quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The reaction mixtures were incubated at −20 °C for 30 min and centrifuged at 14 000 × *g* (30 min, 4 °C). The supernatants were carefully decanted. The pellets were washed with 50 μL of 70% ethanol and dissolved in 50 μL of H_2O. The efficiencies of ligation were estimated by gel electrophoresis, pH 5.2 (100 V, 2 h).[20] To a solution containing

‡ Because the deacylation reactions were found not to be especially pH-sensitive, unless extreme pH conditions were employed, we purposefully chose to avoid the use of buffer to preclude the presence of any additional species that could confound the interpretation of our data.

100 μg of aminoacyl-tRNA in 50 μL of water was added 12 μL of 25 mM I_2 (6.3 mg iodine was dissolved in 1 mL of 1 : 1 H_2O–THF). The reaction mixtures were incubated for 10 min at 25 °C, then 6 μL of 3 M NaOAc was added. The tRNAs were precipitated by the addition of 200 μL of ethanol, then centrifuged at 14000 × *g* (30 min, 4 °C) and the supernatants were carefully decanted. The pellets were washed with 50 μL of 70% ethanol and then dissolved in 30 μL of water.

In vitro protein translation reactions

In vitro translation was performed using a bacterial S-30 extract from *E. coli* strain BL21(DE3).[3] The reaction mixture (100 μL total volume) containing 15 μg of mutant DHFR (Asp10) plasmid,[19] 40 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM maganesium acetate, 20 mM phosphoenolpyruvate, 0.8 mg mL^{-1} of *E. coli* tRNA, 0.8 mM isopropyl β-D-thiogalactopyranoside, 20 mM ATP and GTP, 5 mM CTP and UTP, 4 mM cAMP),[36] 250 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-methionine, 10 μg μL^{-1} rifampicin, 30 μL of bacterial S-30 extract from *E. coli* strain BL21(DE3) and 30 μg of aminoacyl-$tRNA_{CUA}$ was incubated at 37 °C for 45 min.[37] As a control, the *in vitro* translation was also carried out in the presence of mono-aminoacyl-$tRNA_{CUA}$. Aliquots (2 μL) of each reaction mixture were taken for analysis by 10% SDS-PAGE (50 V, 3 h).[38] Autoradiography of the gels was carried out to determine the location of ^{35}S-labeled modified DHFR. Quantification of the bands was carried out using a phosphorimager. Suppression efficiency was calculated as the percentage of the protein produced *via* nonsense codon suppression relative to the production of wild-type protein.

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