

# Activity of 3'-thioAMP derivatives as ribosomal P-site substrates

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## ABSTRACT

The ribosome is a large RNP complex but its main enzymatic activity, the peptidyl transferase, is a ribozyme. As many RNA enzymes use divalent metal ions in catalysis, one of the hypotheses put forward proposed that metal ions might aid peptide bond formation. To be able to test a possible coordination of a metal ion to the 3'-bridging oxygen of P-site substrates, a 3'-thioAMP was synthesized. Its chemical acylation with *N*-acetyl-L-leucine yielded both mono and diaminoacylated 3'-thioAMP. These thioated substrates were tested for peptide bond formation in an optimized fragment reaction in comparison with their unmodified counterparts. As the amino acid was predominantly linked to the unproductive 2'-OH in AcLeu-thioAMP (5), this substrate was barely active and not used for further analysis. In contrast, Di(AcLeu)-thioAMP (4) was more active than Di(AcLeu)-AMP (2) which is in line with the higher energy of thioesters. Both activities were slightly enhanced when Mn<sup>2+</sup> containing buffers were employed in the assay. These data show that thioated P-site substrates are active in peptide bond formation and can in principle be used for metal-ion-rescue experiments in a full translation system.

## INTRODUCTION

The precise mechanism of ribosomal peptide bond formation was always a matter of controversial debates starting from the assumption that a ribosomal protein of the 50S subunit catalyses protein synthesis. The pH dependence of the reaction suggested an ionizable group with a pK<sub>a</sub> value between 7 and 8,

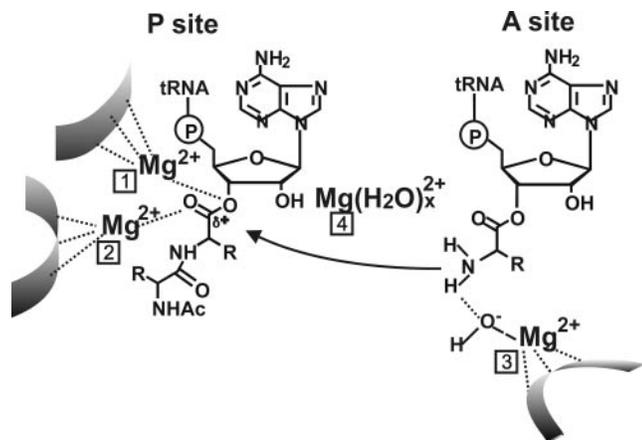
thus a histidine residue was thought to participate in peptide bond formation (1). As long suspected by biochemical evidence (2–4), X-ray structure analysis of ribosomal subunits has revealed that the ribosome is indeed a ribozyme as no protein component could be detected in the near vicinity of the peptidyl transferase center (5). RNA has only a few possibilities to catalyze chemical reactions: general acid/base catalysis and/or electrostatic stabilization of the transition state can be achieved by a nucleobase or by divalent metal ions. Indeed, based on the X-ray structure of a 50S complex with a putative intermediate state analog (5), a model was proposed that attributed an important catalytic function to A2451 (*Escherichia coli* numbering), a conserved nucleotide in the active site. Although this hypothesis entailed many genetic and biochemical experiments, it is still heavily discussed (6–10).

Metal ions are crucial for the structure and function of the ribosome and neither monovalent metal ions nor polyamines can substitute for a certain amount of divalent metal ions (11,12). Metal ions can carry out at least two distinct functions in RNA enzymes: divalent cations may aid structural stabilization of folded RNA and/or might be required for the chemistry of a reaction. In many RNA-catalyzed reactions, specific metal ions have been shown to contribute significantly to catalysis (13–17). The variety of possibilities of how a metal ion might promote RNA catalysis have been extensively reviewed (18,19). As evidence was accumulating early on that the ribosome is indeed a ribozyme (2,3), we had previously proposed that divalent metal ions coordinated by RNA might be involved in the catalysis of the peptidyl transfer reaction (4). There are at least four possibilities of how a metal ion may aid peptidyl transfer (Figure 1): one variant type involves direct metal-ion coordination to the bridging oxygen (metal ion 1) or to the oxygen of the carbonyl group (metal ion 2), thereby either stabilizing the developing negative charge on the leaving group or rendering the carbon center more susceptible to the nucleophilic attack. A third

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**Figure 1.** Model for possible interactions of metal ions during peptide bond formation.

possibility (metal ion 3) would be coordination of a metal-ion hydroxyl-group, which could enhance the nucleophilicity of the attacking amino group by hydrogen bonding. The fourth possibility (metal ion 4) could be stabilization of the developing negative charge on the 2'-OH group in case its proton migrates during reaction over to the 3'-oxygen. In addition, this metal ion might donate a proton from its water shell, thereby restoring the 2'-OH group. An important role of the 2'-OH for activity has been recently proposed by several reports (20–23). Each of these described interactions might occur singly or in various combinations.

One way of testing a predicted metal-ion coordination in RNA catalysis is to substitute oxygen by sulfur at the coordination site that is involved in the reaction. The substituted site has a lower affinity for  $Mg^{2+}$ , which results in decreased activity. However, it has enhanced affinity for divalent ions, such as  $Mn^{2+}$  and  $Cd^{2+}$ , which can consequently rescue the activity.

These metal-ion-rescue experiments have been widely used to predict metal-ion binding sites in RNA for both structure and catalysis (14,16,19). Therefore, we started out to synthesize model P-site substrates to investigate whether metal ions might aid peptide bond formation. A prime candidate for a metal coordination was the 3'-bridging oxygen of the P-site substrate because a metal is coordinated to the equivalent position in group I introns (14). In order to test whether a 3'-thioated substrate would be active as P-site substrate, we took advantage of our recently established fragment reaction system in which the P-site substrate is an 2'(3')-O-(N-acylaminoacyl)adenosine-5'-phosphate and [ $^{14}C$ ]Phe-tRNA<sup>Phe</sup> is the A-site substrate. The detailed characterization of this reaction has been published elsewhere (22). Here, we report the synthesis of 3'-thioAMP, which was further chemically aminoacylated with N-acetyl-L-leucine. The synthesis yielded predominately the diaminoacylated P-site substrate Di(AcLeu)-thioAMP (4). Its peptidyl transferase activity was 2–3 times higher than that of the oxygen containing control substrate, Di(AcLeu)-AMP (2), but both activities were considerably lower than the monoaminoacylated AcLeu-AMP (1). When tested in the presence of  $Mn^{2+}$  ions, all substrates are slightly stimulated. Competition experiments indicate that binding of the P-site substrates appear similar under all conditions employed. Therefore, metal-ion-rescue experiments

in a translation system using tRNAs with a 3'-thioAMP at their 3' end seem feasible.

## MATERIALS AND METHODS

For chemical synthesis, all solvents, except triethyl phosphate, phosphoryl chloride and 1-bromocarbonyl-1-methylethylacetate, were distilled and DMF was stored over molecular sieves (4 Å). Reagents were purchased from Sigma-Aldrich, Fluka or Merck. The preparation of AcLeu-AMP (1), DiAcLeu-AMP (2) AcLeu-dAMP (3), ribosomes and [ $^{14}C$ ]Phe-tRNA<sup>Phe</sup> and reaction conditions were as described previously (22).

### Fragment reaction conditions

The 50S ribosomes (6 pmol) were preincubated for 15 min at 37°C in reaction buffer containing 50 mM HEPES-KOH, 100 mM KCl (pH of the buffer was adjusted to pH 7.5 at 0°C in the presence of KCl), 20 mM magnesium acetate and 10 mM cytidine. After cooling for 10 min to 0°C, acylaminoacylated mononucleotide substrate at 1 mM f.c. and 6 pmol [ $^{14}C$ ]Phe-tRNA<sup>Phe</sup> (~1000 d.p.m./pmol) premixed with 0.3 pmol internal standard (Ac[ $^{14}C$ ]Phe, ~1100 d.p.m./pmol) were added (final volume of 25 µl). The reaction was initiated by the addition of 25 µl cold methanol; however, all concentrations were calculated for the reaction mixture before the addition of methanol. In competition experiments, AcLeu-dAMP (3) was added to the reaction after the addition of P-site substrate, but before the addition of Phe-tRNA<sup>Phe</sup>. After 2 h at 0°C, the reaction was terminated by the addition of 25 µl of 3 M NaOH (f.c. 1 M) and incubated for 30 min at 37°C to hydrolyse the ester bonds. The mixture was acidified with 100 µl of 32% HCl and AcLeuPhe product was extracted with 1 ml ethyl acetate. The ethyl acetate was evaporated in the speedvac, the remaining substance was dissolved in ethyl acetate and subjected to thin layer chromatography on silica gel 60 TLC plates (Merck). The TLC plates were developed for 20 min in  $CHCl_3/MeOH/AcOH$  96% = 10/2/1 and exposed overnight with tritium-sensitive PhosphorImager screens. Screens were scanned with a Molecular Dynamics Storm 840 PhosphorImager (Amersham Pharmacia) and quantitative data were obtained in the ImageQuant software version 5.0. Under these conditions, 30–40% of the Phe-tRNA<sup>Phe</sup> had reacted to AcLeuPhe.

### Preparation of 3'-thioadenosine

The preparation of 3'-thioadenosine was based on a synthesis route outlined by (24) with the addition of several purification steps that were essential to obtain highly purified 3'-thioadenosine. The synthetic sequence started from adenosine that was transformed into the 2',3'-D-ribo-epoxide derivative. The epoxide was then activated by a Lewis acid catalyst and opened by a nucleophilic attack at C-3' with iodide ion. Treatment with thiobenzoate in DMF installed the sulfur at the 3' carbon by  $S_N2$  displacement of the iodo group to afford the protected (benzoylated) 3'-thioadenosine derivative. Removal of the benzoyl group with methanolic sodium methoxide yielded 3'-thioadenosine. The 5'-OH group was phosphorylated with phosphoryl chloride, termination of this reaction resulted in copious salt and its removal would have resulted in significant loss of product. Therefore, unpurified

3'-thioAMP was used without prior removal of the salt, *N*-acetyl-L-leucine was attached to its thiol group using 1,1'-Carbonyldiimidazole (CDI) in H<sub>2</sub>O/DMF (25). The reaction afforded diaminoacylated 3'-thioAMP (4) and depending on the gradient conditions during reversed-phase high-performance liquid chromatography purification we could obtain diaminoacylated (2' and 3') or monoaminoacylated (2') thioAMP (5). For detailed description of the synthesis, see Supplementary Material. Each step was monitored by NMR and mass spectroscopic analysis.

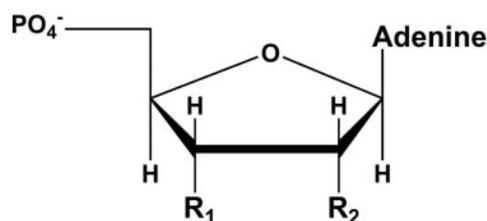
## RESULTS AND DISCUSSION

To be able to test the hypothesis that a magnesium ion might electrostatically stabilize the developing negative charge of the leaving group in peptide bond formation (Figure 1, metal 1), we first synthesized 3'-thioAMP, which was further chemically aminoacylated with *N*-acetyl-L-leucine (see Material and Methods). The synthesis yielded Di(AcLeu)-thioAMP (4) in high purity, but no monoaminoacylated 3'-S-AcLeu-3'-thioAMP could be isolated. This is due to the efficient migration of the amino acid from the 3'-SH to the 2'-OH and subsequent reacylation of the 3'-SH with a second amino acid. However, applying stringent workup procedures resulted in the preparation of 2'-O-AcLeu-3'-thioAMP (5); in these preparations, the majority of the amino acid group was located at the 2'-position as judged by NMR analysis.

To test whether the 3'-thio AMP derivatives are active as P-site substrate in peptide bond formation and to investigate the effect of the thio-substitution, control substrates AcLeu-AMP (1) and Di(AcLeu)-AMP (2) were synthesized (22) and used to compare activities in parallel reactions. As the use of mononucleotide substrates instead of whole tRNAs makes the chemical synthesis of thio substrates more reasonable, we previously had established a simplified version of the fragment reaction for testing these substrates in peptide bond formation. In this system, acylaminoacylated mononucleotides are active as P-site substrates, provided they are used at high concentrations. Furthermore, [<sup>14</sup>C]Phe-tRNA<sup>Phe</sup> is used as A-site substrate and an internal standard allows quantification of the

reactions. The reaction products are extracted by ethyl acetate, separated by thin layer chromatography and quantified by PhosphorImager analysis. Previously, AcLeu-AMP (1) and Di(AcLeu)-AMP (2) have been thoroughly characterized and standard reaction conditions (1 mM P-site substrate and 2 h incubation time) have been defined (22). We also showed that the addition of 10 mM cytidine stimulated the reaction up to 30-fold. This is most probably caused by base pairing to the 23S RNA, thereby simulating binding of the -CCA end of a tRNA, which results in a better positioning of the aminoacylated mononucleotides. Under these conditions using AcLeu-AMP (1), ~30–40% of [<sup>14</sup>C]Phe-tRNA<sup>Phe</sup> had reacted to AcLeuPhe (22). Di(AcLeu)-AMP (2) had only ~10% of the activity of AcLeu-AMP (1) (see also Figure 3A), which suggested a significant contribution of the 2'-OH to activity [see Discussion in (22)]. Interestingly, Di(AcLeu)-thioAMP (4), showing a similar kinetic performance, was ~2–3 times more active than Di(AcLeu)-AMP (2) with ~30% of the activity of the monoacylated substrate (1) (Figure 3A and B). Both activities are equally well inhibited by a competitive but inactive inhibitor AcLeu-dAMP (3) (Figure 3C), indicating similar binding modes. Compared with oxoesters, thioesters are more reactive toward amino nucleophiles (26) and thiols are better leaving groups than alcohols. It is, therefore, conceivable that the higher activity of Di(AcLeu)-thioAMP (4) most probably reflects the higher energy of the thioester linkage in peptide bond formation. A possible magnesium coordination to the sulfur group should have reduced the activity compared with the ester substrate; however, this effect might have been masked by the better leaving group properties of the thiol group.

When testing AcLeu-thioAMP (5) in this system, the activity was barely detectable (Figure 3B, lane 4), which was expected of a substrate with an aminoester linkage predominantly at the unproductive 2'-position. In order to promote migration of the amino acid group to the 3'-position, AcLeu-thioAMP (5) was preincubated for 10 min at 37°C before the reaction at 0°C. Indeed, as shown in Figure 3D, lanes 3 and 4, thermal activation of this substrate slightly enhanced the activity, which is in contrast to Di(AcLeu)-thioAMP (4) where activation showed no effect (lanes 1 and 2) as one



### AcLeu-AMP (1):

R1: -O-(*N*-Acetyl)-L-Leu

R2: -OH

### DiAcLeu-AMP (2):

R1: -O-(*N*-Acetyl)-L-Leu

R2: -O-(*N*-Acetyl)-L-Leu

### AcLeu-dAMP (3):

R1: -O-(*N*-Acetyl)-L-Leu

R2: -H

### DiAcLeu-thioAMP (4):

R1: -S-(*N*-Acetyl)-L-Leu

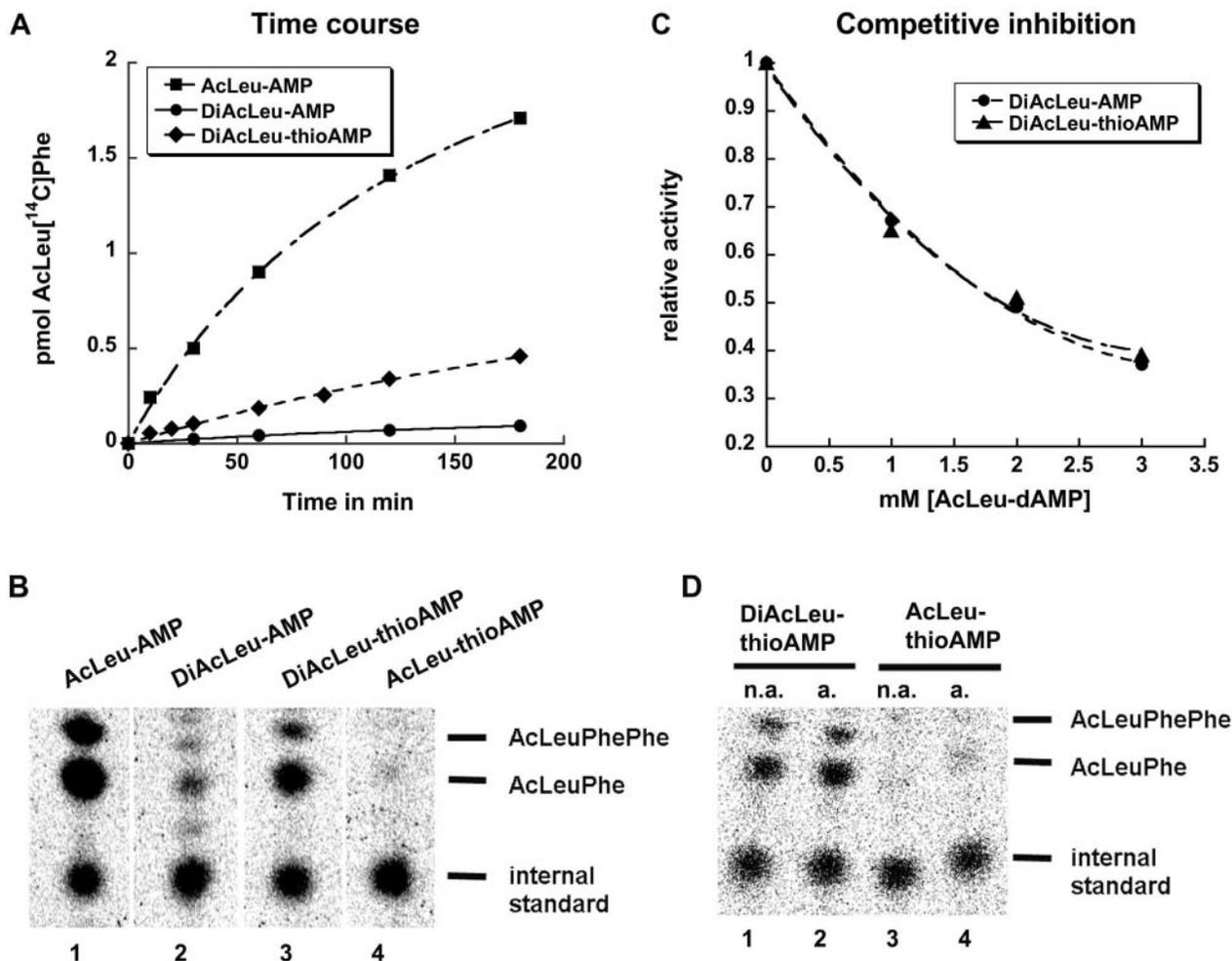
R2: -O-(*N*-Acetyl)-L-Leu

### AcLeu-thioAMP (5):

R1: -SH

R2: -O-(*N*-Acetyl)-L-Leu

Figure 2. Structure of acylaminoacylated mononucleotides used as P-site substrates.



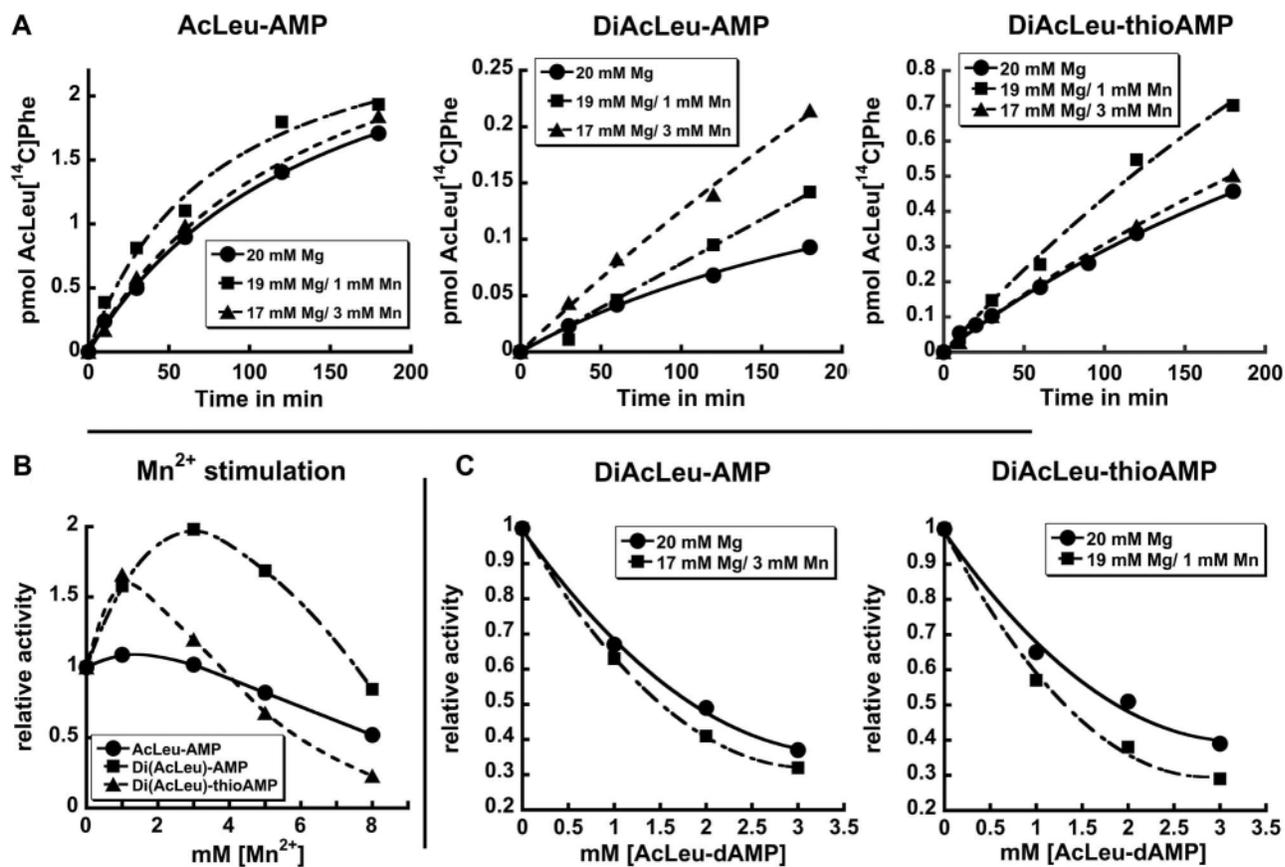
**Figure 3.** Analysis of thioated P-site substrates in a fragment reaction with [ $^{14}\text{C}$ ]Phe-tRNA<sup>Phe</sup> as A-site substrate. (A) Time course of AcLeu[ $^{14}\text{C}$ ]Phe formation. (B) Thin layer chromatography of AcLeu[ $^{14}\text{C}$ ]Phe formation after 2 h incubation. (C) Competitive inhibition of the activity with increasing amounts of inactive AcLeu-dAMP (3). An aliquot of 0.5 mM P-site substrate was used under standard reaction conditions. (D) Thin layer chromatography of fragment reactions with P-site substrates DiAcLeu-thioAMP (4) or AcLeu-thioAMP (5). Substrates were used without any prior treatment (n.a.; lanes 1 and 3) or after thermal activation at 37°C for 10 min (a.; lanes 2 and 4). Data in (A) and (C) represent an average of 3–5 independent series.

would predict. Consequently, AcLeu-thioAMP (5) was not used for further analysis as the amino acid could not be shifted to the active 3'-position in sufficient quantity. Furthermore, as the thioester linkage is more labile in Di(AcLeu)-thioAMP (4), simple hydrolysis during the long incubation would produce predominantly AcLeu-thioAMP (5), which does not contribute significantly to the activity. These experiments demonstrate that Di(AcLeu)-thioAMP (4) is the active component and consequently this substrate was used for further analysis.

As already discussed in the introduction, metal-ion coordination to an oxygen group can be tested by metal-ion-rescue experiments, which take advantage of the different coordination abilities of oxygen and sulfur. As divalent ions, such as  $\text{Mn}^{2+}$  and  $\text{Cd}^{2+}$ , possess a higher affinity to sulfur than  $\text{Mg}^{2+}$  ions, their presence could stimulate the activity of thio substrates.

Although the activity of Di(AcLeu)-thioAMP (4) was enhanced compared with Di(AcLeu)-AMP (2), we reasoned that a stimulation of the activity of Di(AcLeu)-thioAMP (4) by  $\text{Mn}^{2+}$  and  $\text{Cd}^{2+}$  would suggest that a metal ion was coordinated

to the bridging sulfur. Hence, the activity of all three substrates (1,2,4) was tested under various ionic conditions.  $\text{Mg}^{2+}$  ions were continuously substituted by  $\text{Mn}^{2+}$  by keeping the total concentration of divalent ions at 20 mM (Figure 4A). All substrates showed stimulated activity in the presence of  $\text{Mn}^{2+}$ ; however, the extent and optimal  $\text{Mn}^{2+}$  concentration varied (Figure 4A and B). Interestingly, Di(AcLeu)-AMP (2) always showed the best stimulation of all substrates with an optimum at 3 mM  $\text{Mn}^{2+}$ . AcLeu-AMP (1) and Di(AcLeu)-thioAMP (4), however, had their optimal activity in the presence of 1 mM  $\text{Mn}^{2+}$ . At their optimal concentrations, AcLeu-AMP (1) was stimulated 1.1-fold, Di(AcLeu)-thioAMP (4) 1.3-fold and Di(AcLeu)-AMP (2) 2.1-fold (Figure 4B). The slight stimulation of AcLeu-AMP (1) by  $\text{Mn}^{2+}$  is coherent with early experiments, which showed that  $\text{Mn}^{2+}$  can substitute  $\text{Mg}^{2+}$  ions when testing ribosomal activities by completely substituting  $\text{Mg}^{2+}$  with 1 mM  $\text{Mn}^{2+}$  (27). However, so far we cannot explain the better stimulation of Di(AcLeu)-AMP (2) by 3 mM  $\text{Mn}^{2+}$ . One possible explanation would be that the 2 h reaction time in  $\text{Mn}^{2+}$  containing buffer could lead to the hydrolysis of one amino acid ester,



**Figure 4.** Mn<sup>2+</sup> ion dependence of the fragment reaction: mononucleotide P-site substrates were reacted with [<sup>14</sup>C]Phe-tRNA<sup>Phe</sup> in different ionic conditions. (A) Time course of AcLeu[<sup>14</sup>C]Phe formation. (B) Manganese dependence of AcLeu[<sup>14</sup>C]Phe formation using different P-site substrate. (C) Competitive inhibition of the activity with increasing amounts of inactive AcLeu-dAMP (3). An aliquot of 0.5 mM P-site substrate was used and the formation of AcLeu[<sup>14</sup>C]Phe was followed under different ionic conditions. (A–C) Data represent the average of at least three independent experimental series.

producing the much higher active AcLeu-AMP (1). However, preincubation of Di(AcLeu)-AMP (2) in Mn<sup>2+</sup> ion buffer before the reaction did not enhance the activity significantly (data not shown). In addition, inhibition studies with the inactive AcLeu-dAMP (3) under different ionic conditions do not indicate different binding properties for these substrates (2,4) in Mn<sup>2+</sup> buffer compared with Mg<sup>2+</sup> buffer (Figure 4C). Using Cd<sup>2+</sup> for metal-ion-rescue experiments, basically the same results were obtained: Di(AcLeu)-thioAMP (4) is only marginally better stimulated by Cd<sup>2+</sup> than AcLeu-AMP (1) and even lower stimulated than Di(AcLeu)-AMP (2) (C. Panuschka and A. Barta, unpublished data). Taken together, these metal-ion-rescue experiments do not show a stimulation of the peptidyl transferase activity of thioated substrates by Mn<sup>2+</sup> ions. However, it is not clear yet whether this means that no metal ion is coordinated to the bridging oxygen of the P-site substrate as the fragment reaction is so much slower under our conditions (50% methanol, 0°C, endpoint is 30–40% of Phe-tRNA<sup>Phe</sup> reacted after 2 h). Nevertheless, in this system AcLeu-dAMP (3) was found to be completely inactive (22) and an equivalent substrate was recently shown to be also inactive in a system using full-size tRNAs and pre-steady state kinetics (23). Therefore, it is still possible that the coordination of a metal ion to the bridging sulfur might be masked in our system. Only a full-size tRNA with a 3'-thioadenosine used as P-site substrate in a system

using pre-steady state kinetics of peptide bond formation could provide evidence for a metal-ion coordination to the 3'-oxygen group of the P-site substrate. However, as sulfur is also a much better leaving group than oxygen, changing the ester to a thioester bond might not only change metal-ion coordination, but might make the reaction independent of the presence of metal-ion interaction. Therefore, also in this experiment, we cannot completely exclude metal-ion binding to the bridging oxygen in the case of the unmodified substrate.

There is still an ongoing debate about how peptide bond formation is catalyzed by the ribosome. Biochemical and structural analyses have shown that the invariant CCA ends of both tRNA substrates carrying either the growing peptide chain or the next amino acid bind via RNA interactions to 23S RNA (5,28–30) and this precise positioning is an important contribution to catalysis (31). However, it also became quite clear that the peptidyl transferase region is very flexible, changing structure depending on the substrates that are used in the X-ray analysis (32,33). In addition, chemical probing has shown that the peptidyl transferase center undergoes pH-dependent structural rearrangements (34–36), which could in principle be the cause for the observed pH-dependency of the reaction. This makes a prediction which chemical groups at the active site might be involved in a catalytic function (such as in general acid/base catalysis, electrostatic stabilization of the transition state) quite imprecise. *In silico* reconstructions

of a composite of several structures (33), theoretical considerations and the results of several experiments do not unambiguously support the proposed base-mediated catalysis by A2541 in peptide bond formation (8,10). Interestingly, experiments with model P-site substrates with modifications in the 2'-OH of the ultimate A76 suggested a significant influence of this group on the activity leading to a model proposing that this 2'-OH might help in aligning the amino nucleophile (21,22). This model is supported by the composite computer model that places the 2'-OH of the terminal A within 2.5 Å of the nucleophile (33) as well as by a chemical study showing the importance of the vicinal OH group for amide synthesis (20). While this manuscript was under revision, the Strobel and Green lab have published additional proof for the significance of the 2'-OH for peptide bond formation (23). Nevertheless, the apparent dependence of peptide bond formation on divalent metal ions led to the proposal that RNA coordinated  $Mg^{2+}$  might function in catalysis [(4), see Figure 1]. This has prompted a series of experiments to identify divalent metal-ion binding sites in both rRNAs by metal-ion-induced RNA cleavage. Although these experiments provided evidence for metal ions close to the peptidyl transferase center (37,38), only the X-ray structure of the 50S subunit from *Deinococcus radiodurans* (32) but not from *Haloarcula marismortui* (5) shows density that could be attributed to two hydrated  $Mg^{2+}$  ions. Therefore, we started experiments to determine whether  $Mg^{2+}$  ions might coordinate to either of the oxygen groups of P-site substrates (Figure 1). To this end, we have synthesized model thioated P-site substrates, in which the 3'-oxygen has been substituted by sulfur and have shown that these substrates can in principle be used as P-site substrate. In the context of a whole tRNA and using pre-steady state kinetics, these thioated substrates might provide evidence for a metal-ion coordination to the bridging oxygen of P-site substrates.

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*Conflict of interest statement.* None declared.

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