ALA-HIS MEDIATED PEPTIDE BOND FORMATION REVISITED

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Abstract. The historical origin of the translation machinery remains unresolved. Although the large 23S ribosomal RNA (rRNA) is almost certainly the catalytic component of the peptidyl transferase center in the modern ribosome, it is likely that greatly simplified systems were initially employed in the late stages of the prebiotic world. In particular, it has been suggested that small RNAs carrying amino acids were important for the genesis of protein synthesis. Consistent with this, a dipeptide, Ala-His, was previously claimed to be a prebiotically feasible catalyst mediating peptide bond formation in the presence of aminoacylated tRNA and cognate mRNA template, in the absence of other ribosomal components (Shimizu, 1996). We herein report a detailed study of putative dipeptide formation by Ala-His and RNAs carrying leucine. Based on the results presented here, it is unlikely that the dipeptide, Ala-His, catalyzes significant levels of Leu-Leu dipeptide formation in solution. A product is produced which can be readily mistaken for a dipeptide in the TLC separation systems employed in earlier work. We offer explanations for the formation of this product as well as another unexpected product. The results presented here are consistent with the notion that the translation machinery was likely based on catalytic RNA from its very inception.

Keywords: minimalist RNA, origin of translation, peptide bond synthesis, peptidyl transferase, RNA World, small peptide catalysts

List of Abbreviations

unk1 – unknown product 1
unk2 – unknown product 2
tRNA – transfer RNA
rRNA – ribosomal RNA
[^{14}C]-leu-tRNA^{leu} – *in vitro* transcribed tRNA^{leu} (UAA) aminoacylated with [^{14}C]-leucine
HPLC – high-performance liquid chromatography
MS – mass spectrometry
Ad-Leu – 2′(3′)-aminoacyl-ester (adenosine-leucine)
aa-AMP – 5′-aminoacyl adenylate
Leu-OEt – leucyl-ethyl-ester.

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1. Introduction

Ribosome-mediated protein biosynthesis occurs as components of the ribosomal complex coordinate to bind and orient two aminoacylated tRNA molecules in the peptidyl transferase center, which is located in the 50S subunit of the ribosome. Peptide bond formation occurs when two amino acid moieties are correctly juxtaposed. Recent biochemical investigations (Noller *et al*., 1992; Green and Noller, 1996) implicated the 23S rRNA itself as being the catalytic moiety. In addition, it has been shown that *in vitro* selected RNAs catalyze peptide bond formation (Zhang and Cech, 1997). Thus, it was not surprising that the recent publication of a three-dimensional structure of the entire 50S subunit convincingly confirmed that the catalytic center of the ribosome was comprised exclusively of RNA (Nissen *et al*., 2000; Ban *et al*., 2000). The strong conservation of the relevant regions of the 23S rRNA and the 50S subunit strongly suggests that RNA catalyzed translation almost certainly occurs in all extant forms of life.

Comparative data from various genomic studies make it clear that the translational machinery was highly developed by the time the last ancestor of extant life had arisen, as defined by 16S rRNA phylogeny (Woese, 1998; Kyrpides and Woese, 1998). However, the complex translation machinery likely present in this ancestor is far more complex than that which must have existed initially. Thus, several investigators have independently proposed models suggesting that primordial peptide synthesis most likely relied on discrete sub-domains of what ultimately comprises the modern apparatus (Dick and Schamel, 1995; Noller, 1999; Schimmel *et al*., 1993, 1995, 1998). At the extreme it might be argued that a minimal translation system might be one in which an aminoacylated RNA alone could participate in peptide bond formation in the absence of ribosomal components. If this view were correct, RNA based catalysis of the modern ribosome may have been a later addition to the evolving process and some other catalytic mechanism may have been employed originally.

In fact, Shimizu (1996) has presented evidence that suggests non-ribosomal peptidyl transfer can occur between aminoacylated phenylalanyl, lysyl, prolyl, or glycyl tRNAs. These reactions were dependent on the presence of a specific dipeptide catalyst (Ala-His), Mg^{2+}, and an appropriate RNA template that could anneal to the anticodon. Reaction products were exclusively characterized by TLC mobility and it was not entirely clear from the published reports whether aminoacyl-tRNA synthetases were removed from the reaction mixture. As described herein, we have examined these claims within the context of an analogous system for leucine. We found that significant amounts of Leu-Leu dipeptide synthesis did not occur in the presence of Ala-His. Moreover, we did discover that two by-products were formed during the reaction. One of these could be readily mistaken for the dipeptide product and may have accounted for the results obtained by Shimizu.

2. Materials and Methods

2.1. Materials

Plasmid ptDNA^{leu} was kindly provided by Drs. John Abelson and Giuseppe Tocchini-Valentini (California Institute of Technology). Oligonucleotides were purchased from MWG Biotech, Inc. (High Point, NC). T7 RNA polymerase was purified from *E. coli* (BL21) harboring the PAR1219 plasmid according to Grodberg and Dunn (1988). *E. coli* leucyl-tRNA synthetase containing a six-histidine N-terminal fusion was purified as described by Martinis and Fox (1997). [^{14}C]-Leucine was purchased from Amersham with a specific activity of 159 Ci/mmol. Dipeptides, amino acids, nucleotides, and inorganic pyrophosphatase were purchased from Sigma. Ala-His dipeptide was obtained from either Sigma or Bachem (Torrance, CA). The Leu-OEt standard was acquired from Bachem. Commercial Ala-His was purified on a TLC plate and recovered by extraction with water in order to remove traces of ethanol. Chroma Spin + TE-10 size exclusion spin columns were purchased from Clonetech Laboratories, Inc. Centricon YM-3 centrifugal filter devices with a membrane molecular weight cut-off of 3000 Daltons were acquired from Millipore.

2.2. Expression and purification of tRNA molecules

E. coli tRNA^{leu} (UAA) and the mRNA template (poly-AUUU) were made by *in vitro* T7 RNA polymerase run-off transcription (Sampson and Uhlenbeck, 1988) using a specifically designed ptDNA^{leu} template and a synthetic oligonucleotide, respectively. The DNA template was prepared by digesting the plasmid with *Bst*N I at 60 °C for 4 hr. Approximately 50 μg of template were incubated for 4 hr at 42 °C in a 1 ml reaction buffer optimized for the specific template. The buffer contained 40 mM Tris buffer (pH 8.1), 80 mg ml^{-1} PEG 8000, 5 mM DL-dithiothreitol (DTT), 30 mM $MgCl_2$, 2 mM spermidine, 50 μg ml^{-1} BSA, 0.01% Triton X-100, and 0.003 units ml^{-1} inorganic pyrophosphatase. Reagents were made with diethyl pyrocarbonate-treated water to inhibit potential RNase activity. It was empirically determined that a final concentration of 7.5 mM for each nucleotide and 0.1 mM GMP optimized RNA production. Approximately 100 μg of T7 RNA polymerase (460 μg ml^{-1} stock solution determined by the Lowry method using a BSA standard) were added to the RNA synthesis mixture followed by a second aliquot after 2 hr incubation.

Subsequent to *in vitro* transcription, the RNA product was ethanol precipitated, washed with 70% ethanol, and dried in a speed-vac. RNA pellets were rehydrated directly in 8 M urea/50 mM Tris (pH 8) buffer and loaded onto a 10 or 16% polyacrylamide [19:1] and 8 M urea denaturing gel. Once separated, RNA bands were visualized by UV shadowing on Whatman TLC plates, cut out of the gel, and eluted twice in an equal volume of 0.5 M NH_4OAc/1 mM EDTA (Sampson and Uhlenbeck, 1988). RNA was concentrated to 400 μl through a series of butanol extractions and precipitated with ethanol at –80 °C using 1 μl of a 25 mg ml^{-1}

glycogen stock as a carrier. The reaction and purification process yielded approximately 1 mg of RNA product per 5 ml reaction as determined by UV spectroscopy at 260 nm.

2.3. AMINOACYLATION AND ISOLATION OF LEU-tRNA^{LEU}

Gel-purified tRNA^{leu} transcript was diluted in H_20 depending on the anticipated final reaction volume and denatured at 90 °C for 2 min. Addition of 1 mM $MgCl_2$ facilitated RNA folding under quick cooling conditions. The purified tRNA^{leu} was aminoacylated with [^{14}C]-leucine using *E. coli* leucyl-tRNA synthetase. A 50 μl reaction mixture contained 100 nM leucyl-tRNA synthetase, 5 μM purified tRNA^{leu}, 20.5 μM [^{14}C]-leucine (159 Ci/mmol), 2 mM ATP, 0.5 mM DTT, 10 mM $MgCl_2$, and 60 mM Tris buffer (pH 7.5). Aminoacylated RNAs were stabilized by acidification to pH 5 with 10% acetic acid (Schreier and Schimmel, 1972), phenol-extracted to remove protein, and ethanol precipitated. The radiolabeled leu-tRNA^{leu} was rehydrated in 50 mM KH_2PO_4 buffer (pH 5) and used directly or further purified using a Clonetech size-exclusion spin column equilibrated with the buffer. The latter step removes small molecules and stabilizes the labile amino acid-nucleic acid ester bond. Extensive desalting washes using Centricon YM-3 centrifugal filter devices with 10 ml of 50 mM KH_2PO_4 buffer (pH 5) were employed to remove excess Mg^{2+} ions from the charged tRNA^{leu}.

2.4. REACTION MIXTURES CONTAINING ALA-HIS

[^{14}C]-leu-tRNA^{leu} (4 μM) was incubated with 250–500 mM Ala-His at 37 °C for 30 min to 1 hr. Alternative histidine derivatives or dipeptide catalysts were also added in 250–500 mM concentrations under the same experimental conditions. For the indicated reactions, aminoacylated-RNA was hydrolyzed by treatment with 0.5 N KOH for 1–2 hr at 37 °C. Between 8–10 μl reaction aliquots (2 μl each followed by drying) were spotted onto a Whatman 60 Å silica gel TLC plate and developed in a mobile phase of either Solvent 1 (butanol/acetic acid/water (12:3:5)) or Solvent 2 (chloroform/methanol/32% acetic acid (5:3:1)). Dry plates were sprayed with ninhydrin and baked at 110 °C to detect migrated reaction components including potential catalyst, Ala-His, and various leucine standards. Standards were superficially spotted with a radiolabel to mark their position and the TLC plate was visualized using a Fuji BAS1000 phosphoroimager. Reactions mimicking those carried out by Shimizu's studies were done according to his described procedure (1996).

2.5. HPLC SEPARATION AND ELECTROSPRAY API-MASS SPECTROMETRY

In order to separate excess Ala-His from possible Leu-Leu dipeptide product, large-scale reaction mixtures containing 250 mM or 500 mM of Ala-His and approximately 100 μM leu-tRNA^{leu} were directly loaded (10 μl injection) onto a Microsorb

C8 reversed-phase HPLC column (100 Å pore size) using a Hewlett Packard 1090 LC. A gradient of acetonitrile and 0.1% trifluoroacetic acid (TFA) in H_2O was established by increasing the concentration of acetonitrile from 5 to 60% over 15 min at a flow rate of 0.5 ml min^{-1}. A standard curve for the Leu-Leu dipeptide standard was generated by correlating known concentrations (0.058 mg ml^{-1} to 1.16 mg ml^{-1}) to the area of each HPLC-eluted peak. The average Leu-Leu dipeptide yield (0.11 mg ml^{-1}) detected from the experimental reactions was derived using the standard curve and calculating the area under the reaction product peak.

The LC was connected to a Hewlett Packard 59987A Electrospray API interface and HP 5989B MS engine. Positive ionization of each component eluted from the LC was achieved by post-separation addition of 2% propionic acid to counteract the ion suppression caused by TFA present during chromatographic separation. The sample next entered a 50 μl injector loop (Rheodyne injector) and was analyzed at a temperature of 300 °C in nitrogen nebulization and drying gas. The LC and MS analysis were initiated simultaneously in order to correlate the retention time of the product on the LC (11.7 min) with the ionized species on the LCMS chromatogram (mass data extracted on an average time between 11–12 min). The spectra were recorded simultaneously from 190 nm – 600 nm for each eluted peak. The peak of the putative Leu-Leu dipeptide (mass 243) was detected using a diode array detector set at 211 nm, which corresponded to the Leu-Leu dipeptide standard as well.

3. Results

3.1. Incubation of [^{14}C]-LEU-tRNA^{LEU} with ala-his

We established a leucine-based experimental system, using protocols that were originally described by Shimizu and previously applied to phenylalanyl, lysyl, prolyl, or glycyl systems (1996). Specifically, micromolar concentrations of the activated tRNA substrate ([^{14}C]-leu-tRNA^{leu} with the UAA anticodon) were incubated with reaction components excluding the final addition of KOH. Although we did not detect Leu-Leu dipeptide formation, two unknown spots were observed in TLC separations (Figure 1). The complete reaction mixture consisting of [^{14}C]-leu-tRNA^{leu}, poly-AUUU mRNA template, Ala-His and Mg^{2+} produced both spots (Figure 1, Lane 4). Unknown product 1 (unk1) was barely visible and migrated near the Leu-Leu dipeptide standard. Unknown product 2 (unk2) migrated above the free leucine standard. A reaction containing [^{14}C]-leu-tRNA^{leu}, mRNA template, and Ala-His, but lacking Mg^{2+} also produced unk2 (Figure 1, Lane 2). Two reactions containing only [^{14}C]-leu-tRNA^{leu} with (Figure 1, Lane 5) or without (Figure 1, Lane 6) mRNA template reveal that neither produces an unknown spot or a Leu-Leu dipeptide product, ruling out the possibility that background formation of product species occurs with activated tRNA substrate even in the presence of mRNA template.

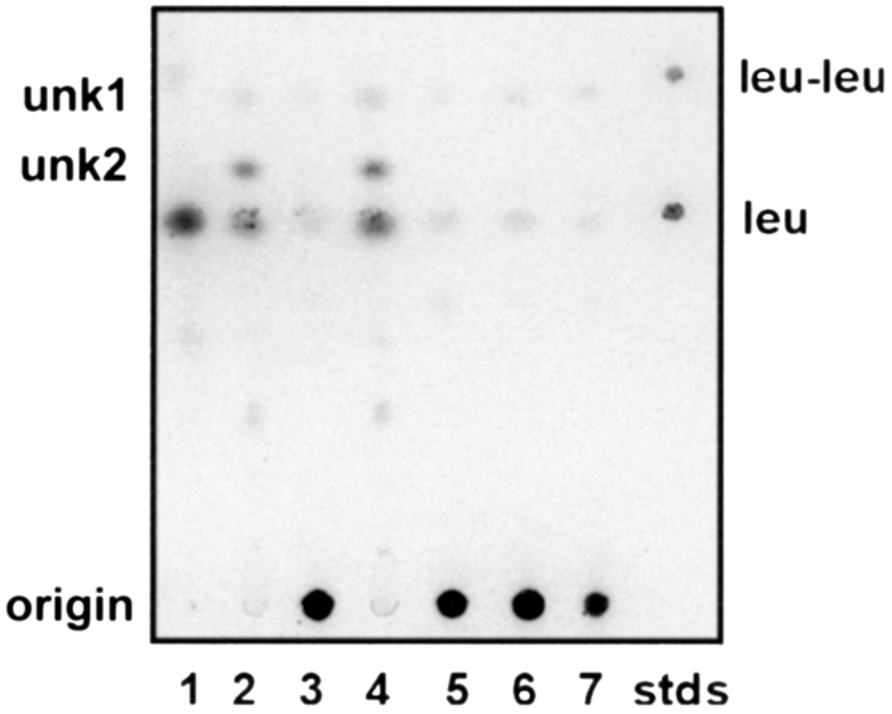

Figure 1. Ala-His, in the presence of purified [^{14}C]-leu-tRNA^{leu}, produces two unknown product species. Various reactions containing a mixture of [^{14}C]-leu-tRNA^{leu}, 278 mM Ala-His, 17 mM $MgCl_2$ and cognate mRNA template (repeating AUUU sequence) at pH 7 were prepared as described by Shimizu (1996). Certain reagents were substituted with water for the various control reactions described below. Products were chromatographed in butanol/acetic acid/water (12:3:5) on Whatman 60 Å silica gel TLC plates and visualized using a Fuji BAS1000 phosphoroimager. Lane 1 shows a [^{14}C]-leucine free amino acid standard. The reactions in Lanes 2–5 contain the cognate mRNA template as well as [^{14}C]-leu-tRNA^{leu} and Ala-His (Lane 2), [^{14}C]-leu-tRNA^{leu} and Mg^{2+} (Lane 3), the complete reaction including [^{14}C]-leu-tRNA^{leu}, Ala-His and Mg^{2+} (Lane 4), and [^{14}C]-leu-tRNA^{leu} only (Lane 5). Lane 6 contains only [^{14}C]-leu-tRNA^{leu} incubated in the absence of mRNA template. Lane 7 contains [^{14}C]-leu-tRNA^{leu} spotted directly onto the TLC plate. The free leucine and Leu-Leu dipeptide standards (Lane 8) were detected by ninhydrin and then superficially spotted with radioactivity in order to visualize by phosphoroimaging. Unknown 1 (unk1) is a faint spot migrating near the Leu-Leu dipeptide standard. It is also noteworthy that unknown product 2 (unk2) appears only in the reactions containing Ala-His.

3.2. HPLC-MS EXAMINATION OF THE REACTION MIXTURE

Despite the fact that Leu-Leu dipeptide was never detected in the TLC separations, a very small amount (0.11 mg ml^{-1}) was found when large-scale reactions were subjected to HPLC separation and electrospray ionization (ESI) mass analysis, as shown in Figure 2. The standard spectrum for Leu-Leu dipeptide (Figure 2A) stored in a library database matched the spectrum from the sample chromatogram

peak (Figure 2B) with a high score of 929 out of 1000. Repetitive analysis was performed to confirm reproducibility of the Leu-Leu dipeptide peak and mass spectrometry data. Several controls including leu-tRNA^{leu} alone, Ala-His alone, and blank runs were also analyzed for possible Leu-Leu mass detection. None of the controls were positive. Figure 2C shows the chromatogram of leu-tRNA^{leu} incubated without Ala-His, in which a mass peak for Leu-Leu dipeptide (243) is not detected. The control reactions indicate that the presence of Ala-His is necessary for a dipeptide producing reaction to occur. Although it is not likely that Ala-His is acting as a catalyst in the reaction mixture, it remains to be explained why Leu-Leu dipeptide formation occurred during the HPLC/MS analysis.

3.3. IDENTIFICATION OF UNKNOWN SPOT 1

Since Shimizu's TLC plate analysis indicated substantial quantities of Phe-Phe, Pro-Pro, Lys-Lys, and Gly-Gly dipeptide product generated in his experimental system, we initiated an investigation to elucidate why Leu-Leu dipeptide was not detected by our TLC analysis. The most obvious explanation was the possibility that unk1 is a species readily misinterpreted to be a dipeptide product because it migrates so closely to the Leu-Leu standard. Khaitovich *et al*. (1999) observed that the presence of residual ethanol resulted in the formation of a by-product species that migrated near dipeptide standards and caused a great deal of confusion when interpreting results using TLC plate analysis. We suspected that a similar explanation might account for the products that were interpreted to be dipeptides by Shimizu (1996).

Therefore, we sought to determine if unk1 could be a leucyl-ethyl-ester (Leu-OEt), formed due to the presence of ethanol in the commercially produced Ala-His dipeptide (Figure 3A). When Ala-His was purified to remove traces of ethanol, unk1 was not detected (Figure 3A, Lane 2). In contrast, when ethanol was added directly to [^{14}C]-leu-tRNA^{leu} in the absence of Ala-His, the intensity of unk1 increased (Figure 3A, Lane 3). We also examined both Leu-OEt and Leu-Leu dipeptide standards (Figure 3A, Lanes 4 and 5, respectively), which migrate together when separated in a mobile phase of butanol/acetic acid/water (12:3:5) on 60 Å silica gel TLC plates as described by Shimizu. Unk1 migrates with both standards such that it was difficult to distinguish whether the product was Leu-Leu or Leu-OEt by this TLC separation method.

We next developed duplicate reactions in a mobile phase of chloroform/methanol/32% acetic acid (5:3:1) as shown in Figure 3B. Leu-OEt and Leu-Leu standards are clearly distinguishable under these conditions (Figure 3B, Lanes 4 and 5, respectively). Unk1 migrates with the Leu-OEt standard, well above the Leu-Leu standard, for both the complete reaction mixture (Figure 3B, Lane 1) and when ethanol is added exogenously in place of Ala-His (Figure 3B, Lane 3). In addition, when various concentrations of Ala-His ranging from 0–500 mM were added to the reaction, unk1 did not appear until the Ala-His concentration reached 100 mM

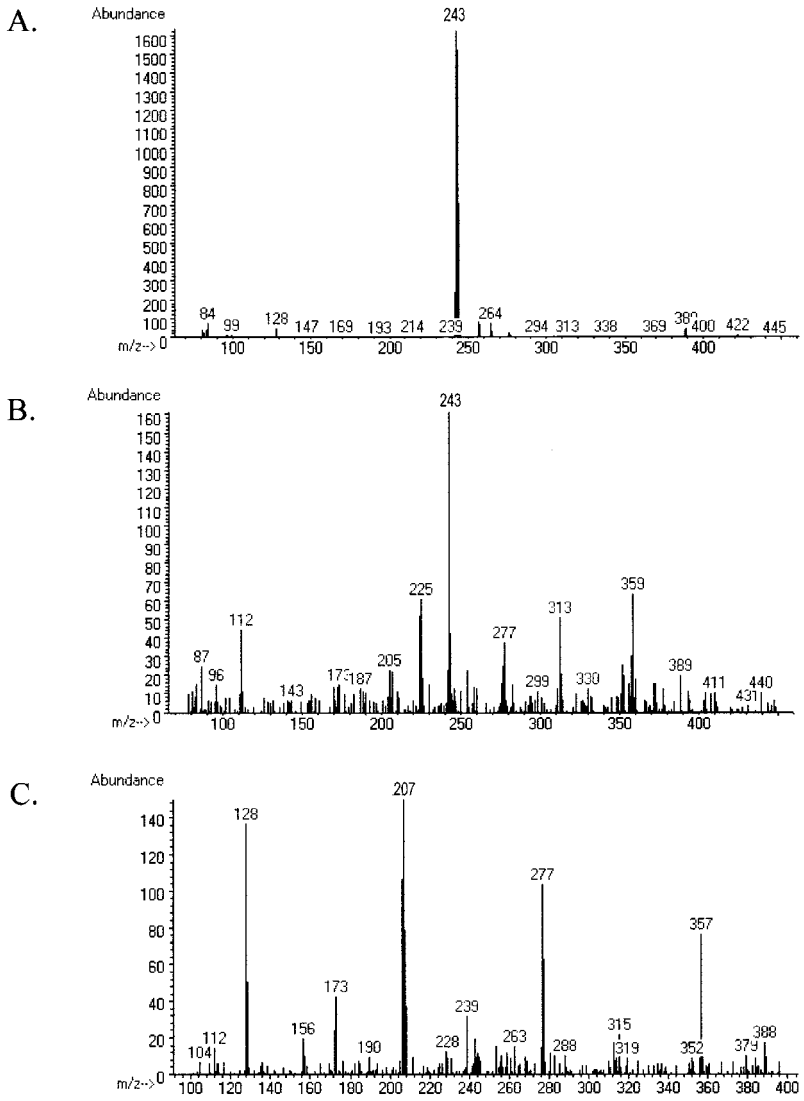

Figure 2. Ala-His and leu-tRNA^{leu} were incubated under various conditions and then injected onto the HPLC reversed-phase column for separation and determination of the Leu-Leu dipeptide product species by MS. **A)** Leu-Leu dipeptide standard (11.6 min) was detected using an LC gradient optimized for separating excess Ala-His from the low yield of dipeptide product present in the reaction. The LC was connected to the MS for analysis of the standard's molecular identity. The mass of Leu-Leu is 243, which is shown in the chromatogram extracted during the same time period (10–12 min) that the Leu-Leu standard peak was observed on the LC. **B)** This chromatogram shows Leu-Leu formed experimentally in the Ala-His dependent reaction in the presence of leu-tRNA^{leu}. The Leu-Leu mass chromatogram was extracted from the time period (11.3–12.2 min) that corresponds to its LC peak (11.7 min). **C)** MS spectra observed for the leu-tRNA^{leu} control during the same time period (11.2–12.2 min) eliminated the possibility that Leu-Leu may be produced in the absence of Ala-His.

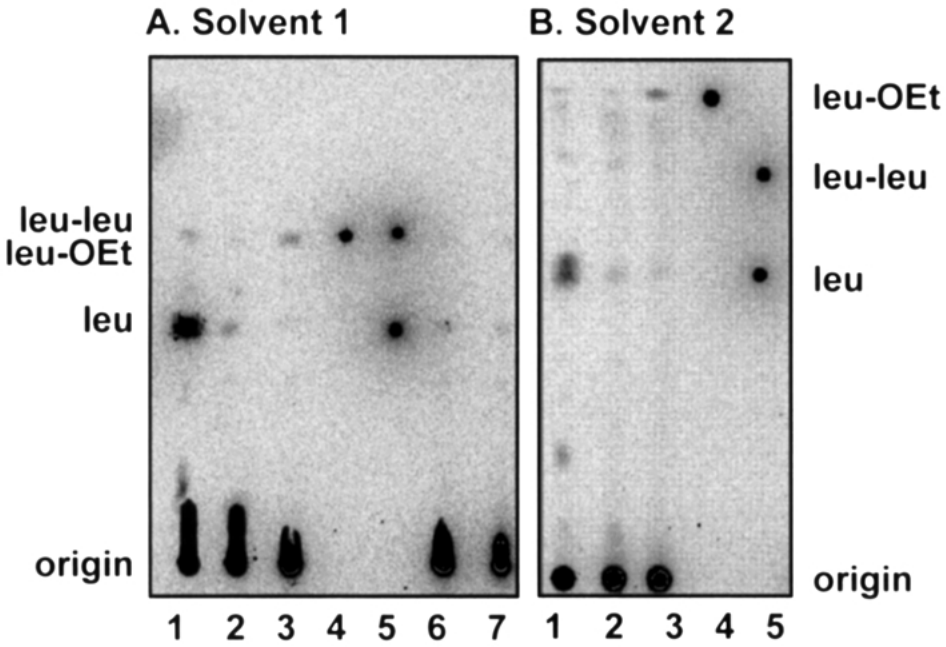

Figure 3. Unk1 forms in the presence of excess ethanol; a leucyl-ethyl-ester (Leu-OEt) caused by ethanolysis of the amino acid. Reaction mixtures were prepared as described by Shimizu (1996) or modified as described in the Materials and Methods section for the control reactions. **A)** After incubation for 30 min at 37 °C, reactions were spotted onto Whatman 60 Å silica gel TLC plates and developed in Solvent 1 (butanol/acetic acid/water (12:3:5)). The first two lanes contain complete reaction mixtures incubated with various forms of Ala-His. Unk1 appears in the reaction that uses commercially purchased Ala-His (Lane 1) containing trace amounts of ethanol. The spot is not detected in the reaction using purified Ala-His which does not contain ethanol (Lane 2). Furthermore, the intensity of unk1 increases when 5% ethanol is used instead of Ala-His in the reaction mixture (Lane 3). Lane 4 contains a superficially spotted Leu-OEt standard ($R_f = 0.76$) and Lane 5 contains the free leucine standard ($R_f = 0.54$) and the Leu-Leu dipeptide standard ($R_f = 0.76$). A control reaction containing [^{14}C]-leu-tRNA^{leu} with water instead of Ala-His (Lane 6) shows that unk1 is not generated, ruling out that residual ethanol could be present from insufficient drying during its preparation. Lane 7 contains [^{14}C]-leu-tRNA^{leu} spotted directly onto the TLC plate. **B)** Migration patterns of duplicate reactions were compared after development with Solvent 2 (chloroform/methanol/32% acetic acid (5:3:1)). Although Leu-OEt and Leu-Leu migrate very close to each other with Solvent 1, it is obvious that they are well separated in the Solvent 2 system (Lanes 4 and 5). Obvious differences in the migration patterns showed that unk1 ($R_f = 0.84$) could not be the dipeptide Leu-Leu ($R_f = 0.75$). It is migrating with the Leu-OEt standard as well as with the product of the reaction that contains exogenously added ethanol. Therefore, it appears very likely that the putative Leu-Leu product is actually a product of ethanolysis, specifically Leu-OEt, which is easily distinguished in an alternative solvent system.

(data not shown). In reactions in which the tRNA is internally radiolabeled and the amino acid is not, unk1 was not detected. In summary, it is likely that the species which initially appears to be Leu-Leu dipeptide in our work, is actually a by-product of ethanolysis rather than dipeptide product resulting from Ala-His catalyzed peptidyl transferase. Since Shimizu (1996) did not examine the reaction products with a second mobile phase, the possibility that by-products produced by ethanolysis were misinterpreted as dipeptides can not be ruled out.

3.4. IDENTIFICATION OF UNKNOWN SPOT 2

We hypothesized that unk2 may be a charged nucleotide cleaved from leu-tRNA^{leu} resulting in the formation of adenosine-leucine (Ad-Leu). To test this hypothesis, [^{14}C]-leu-tRNA^{leu} was degraded with RNase T_2 or RNase A in order to generate Ad-Leu that could be compared to the putative Ad-Leu product formed in reactions containing Ala-His and Mg^{2+}. Each reaction was chromatographed in the alternative solvents (Figure 4) and the TLC analysis shows that the Ad-Leu generated by RNase A (Figure 4B, Lane 1) or RNase T_2 (Figure 4A and 4B, Lane 2) degradation migrates to the same position as the putative Ad-Leu formed in the reaction (Figure 4A and 4B, Lane 3). To further investigate this result, *in vitro* transcribed tRNA was synthesized with [8-^{14}C]-ATP in order to internally label the tRNA. The radioactive tRNA molecules were incubated with the Ala-His reaction mixture, as well as with RNase A and RNase T_2. For each reaction, adenosine was in fact detected migrating above the Leu-Leu standard (data not shown). The radioactive tRNA was then aminoacylated and tested under the same reaction conditions. Each reaction yielded another spot migrating to a similar position as the Leu-Leu standard, confirming that this spot could only be Ad-Leu because the leucine molecule did not carry a detectable radioactive component. Furthermore, other peptides or amino acids containing an imidazole ring also generated unk2, including N-α-t-BOC-L-Histidine, N-α-t-BOC-π-Benzyloxymethyl-L-Histidine, His-Ala, and free histidine at pH 7 (data not shown).

We further investigated the identity of unk2 as Ad-Leu by incubating moderately purified [^{14}C]-leu-tRNA^{leu} with Ala-His in a range of acidic (pH 3) to slightly basic (pH 8) conditions, yielding unk1 and unk2. Unk2 disappeared under basic conditions suggesting hydrolysis of the labile ester bond of charged RNA. Figure 5 shows duplicate reactions adjusted to the appropriate pH before incubation, loaded onto a TLC plate, and chromatographed in butanol/acetic acid/water. Lane 1 contains a reaction mixture incubated at pH 4 and Lane 4 contains the same reaction mixture at pH 7. KOH was added to the reactions to increase the pH to 5 (Lane 2) or pH 8 (Lane 5) prior to spotting an aliquot directly onto the TLC plate. After incubation of the remaining reaction mixture for 2 hr at 37 °C as described in the Shimizu protocol (Lanes 3 and 6, respectively), unk2 in Lane 6 disappears. In addition, there is a dramatic decrease in the radiolabeled tRNA spot typically observed at the origin of the TLC plate that also occurs most likely

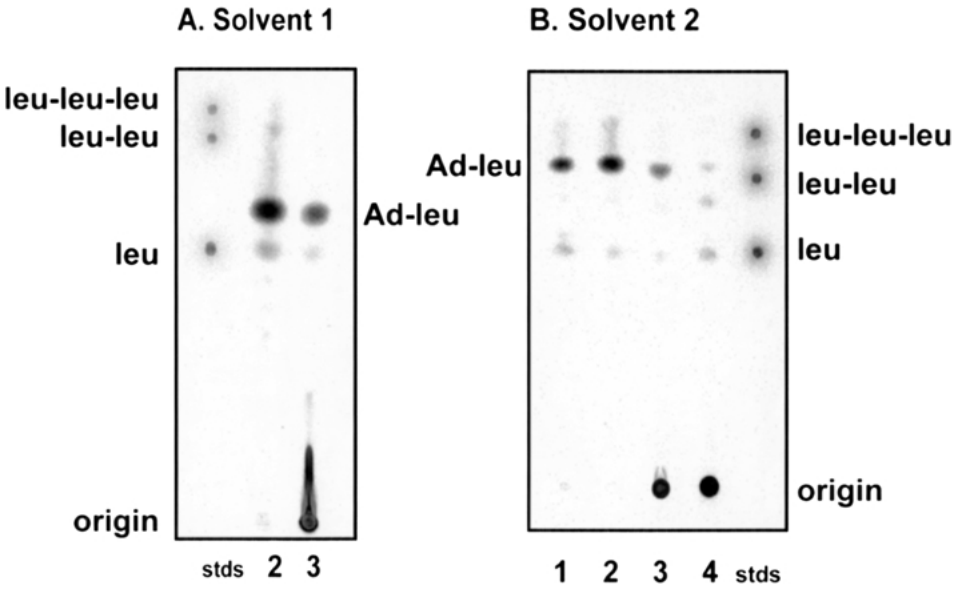

Figure 4. RNase degradation of aminoacylated tRNA confirms the identity of unk2 as adenosine-leu (Ad-Leu). **A)** Lane 1 contains superficially spotted free leucine, Leu-Leu dipeptide, and Leu-Leu-Leu tripeptide standards. Lanes 2 and 3 contain [^{14}C]-leu-tRNA^{leu} that was incubated with RNase T_2 at 37 °C for 3 hr (Lane 2) or with 500 mM Ala-His and 100 mM $MgCl_2$ at 37 °C for 1 hr (Lane 3). The reaction mixtures were spotted onto a TLC plate and developed in Solvent 1 (butanol/acetic acid/water (12:3:5)). Both products migrated to the same distance slightly above the Leu standard and corresponded to unk2. **B)** A separate reaction under the same conditions with RNase A (Lane 1), RNase T_2 (Lane 2), or the reaction mixture (Lane 3) was developed in Solvent 2 (chloroform/methanol/32% acetic acid (5:3:1)). Lane 4 contains [^{14}C]-leu-tRNA^{leu} only. Lane 5 contains superficially spotted free leucine, Leu-Leu dipeptide, and Leu-Leu-Leu tripeptide standards. Each product migrated the same distance on the TLC plate near the Leu-Leu standard. The RNase digestions confirm that the identity of the species generated in the presence of Ala-His and Mg^{2+} is Ad-Leu.

from hydrolysis. In conclusion, we have identified unk2 as a 2′(3′)-aminoacyl-ester (adenosine-leucine), generated from Ala-His interactions with the phosphodiester backbone of the charged tRNA molecule.

3.5. Analysis of the Mg^{2+} contribution

Because of the important role Mg^{2+} plays in RNA folding (Correll *et al.*, 1997), structural stability, and catalytic activity (Steitz and Steitz, 1993), the effect of Mg^{2+} on the reaction was also scrutinized. Generally, magnesium ions are introduced during the aminoacylation reaction and typically associate with the tRNA molecule during moderate purification. Subsequently, they are present in unknown concentrations for each reaction. Specifically, the charged tRNA used in all of the reactions shown in Figure 1 likely contained residual magnesium ions associated

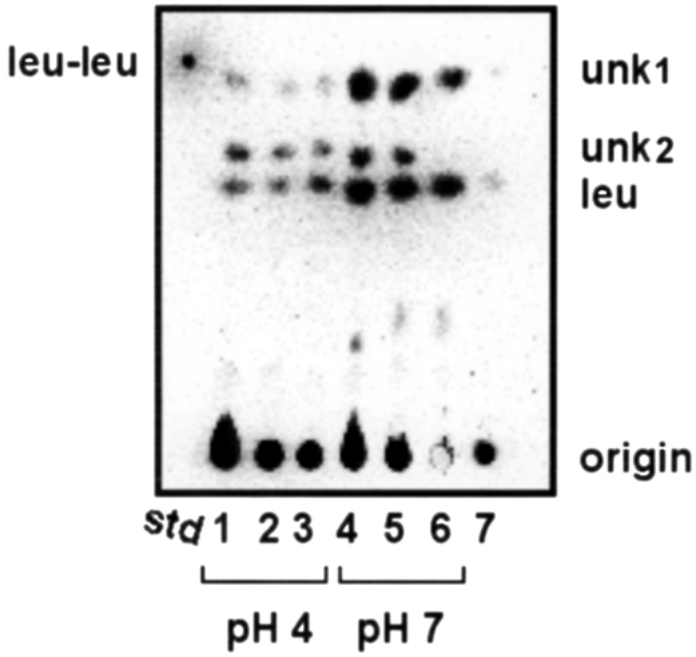

Figure 5. Unk2 is hydrolyzed at pH 8. In the absence of mRNA template, [^{14}C]-leu-tRNA^{leu} was incubated with 500 mM Ala-His at pH 4 (Lane 1) or pH 7 (Lane 4) at 37 °C for 1 hr. Several microliters of the reaction mixture were spotted onto the TLC plate. KOH was added to the remaining mixture to alter the acidity of the environment from pH 4 to pH 5 (Lane 2) and pH 7 to pH 8 (Lane 5). An aliquot of the reaction mixture was spotted directly onto the TLC plate (Lanes 2 and 5). The remaining reaction mixture was incubated at 37 °C for 2 hr and then spotted onto the TLC plate (Lanes 3 and 6, respectively). Lane 7 contains untreated [^{14}C]-leu-tRNA^{leu} spotted directly onto the TLC plate. The samples were chromatographed in Solvent 1 (butanol/acetic acid/water (12:3:5)). Unk2 (Ad-Leu) was detected for each initial reaction, however, treating the sample with KOH for 2 hr at pH 8 caused its disappearance and also that of [^{14}C]-leu-tRNA^{leu} usually detected at the origin (Lane 6). This occurs most likely from hydrolysis of the labile ester bond of charged tRNA in a basic environment. Unk1 (Leu-OEt) is also visible in all the reactions and it appears that ethanolysis occurs more readily at neutral pH.

with its purification. In order to remove excess Mg^{2+} ions, [^{14}C]-leu-tRNA^{leu} was passed thru size exclusion spin columns (equilibrated to pH 5 with 50 mM KH_2PO_4 buffer). The charged tRNA was then exhaustively washed in potassium phosphate buffer using Centricon centrifugal filter devices. Although it is possible that tightly associated Mg^{2+} ions may still be present, excess ions should be removed during the desalting washes. Unk2 does not form in the presence of exhaustively washed [^{14}C]-leu-tRNA^{leu} and Ala-His (data not shown). Furthermore, the reaction yielding unk2 (Ad-Leu) does not occur with 100 mM Mg^{2+} only (Figure 1, Lane 3). Likewise, incubation of [^{14}C]-leu-tRNA^{leu} in the presence of varying concentrations of 0-500 mM $MgCl_2$ did not result in unk2 formation (data not shown). Unk2 does, however, form in the presence of charged tRNA supplemented with 100 mM

$MgCl_2$ in Ala-His mediated reactions. Thus, it appears that residual or exogenously added magnesium ions are important for chemical interactions of Ala-His with the RNA to generate cleaved Ad-Leu.

4. Discussion

The TLC studies described herein do not support the notion that Ala-His acts as a catalyst to mediate dipeptide formation in a leucine based system and contrasts the results previously described by Shimizu (1996). Although Leu-Leu dipeptide was not detected, two reaction products were observed. The first product, unk1, was convincingly shown to result from the presence of low levels of ethanol in the Ala-His dipeptide added to reaction mixes, and identified as Leu-OEt. This ethanolysis reaction was demonstrated to produce aminoacyl-ethyl-esters which can be easily mistaken for dipeptide product when the TLC plates are developed with a butanol/acetic acid/water mobile phase. This result has drastic implications with respect to Shimizu's original studies. Since commercially produced Ala-His dipeptides were used by Shimizu, it is likely that trace amounts of ethanol were present in his reactions. This must explain why the products observed by Shimizu (1996) were specific to reactions with Ala-His and that other combinations of potential dipeptide catalysts containing histidine did not form observable products in his work. Shimizu's reaction products were not confirmed by mass spectroscopy or separated with a different mobile phase, therefore this alternative explanation cannot be ruled out. In conclusion, we suggest that the putative dipeptide products reported may have been produced by ethanolysis. Thus, one should not infer from the published work that the Ala-His dipeptide actually catalyzes dipeptide formation to a significant extent in the presence of aminoacylated RNA.

We identified unk2 as Ad-Leu, which originates from the terminal adenosine residue of the tRNA that is cleaved while still covalently linked to leucine. Substantial evidence in the literature demonstrates that histidine alone, histidine containing peptides and imidazole all degrade RNA (Roth and Breaker, 1998; Breslow and Xu, 1993). This species would not have been detected using Shimizu's procedures because treatment with KOH hydrolyzes the labile ester bond releasing the radiolabeled amino acid from the cleaved nucleotide.

Not withstanding these conclusions, we did unequivocally detect small amounts of Leu-Leu dipeptide formation in large-scale reaction mixes containing Ala-His and leu-$tRNA^{leu}$. It is likely that stoichiometrically significant amounts of Ad-Leu were generated in the presence of Ala-His using the increased amounts of tRNA necessary for HPLC procedures as opposed to micromolar concentrations used for TLC analysis. It is well known that 5′-aminoacyl adenylates (aa-AMP), formed in the first step of a tRNA charging reaction, are very reactive and can interact to produce peptide products (Gillet *et al*., 1997; Larkin *et al*., 1999; Nakajima *et al*., 1986). The 2′(3′)-aminoacyl-esters that are likely produced by the degradation of

leu-tRNA^{leu} are significantly less reactive but may condense into peptides at high concentrations. Thus, it is likely that the small amount of Leu-Leu product seen in large-scale reactions is a by-product of tRNA cleavage by Ala-His. Notably, equivalent large-scale experiments in the absence of Ala-His did not produce this small amount of dipeptide, therefore eliminating the possibility that leu-tRNA^{leu} alone is interacting to form the Leu-Leu dipeptide.

The actual synthesis of peptides or nucleotides in the primordial world is not difficult to envision. There are in fact several plausible ways in which it might have occurred (Shen *et al*., 1990; Oró and Stephen-Sherwood, 1974; Weber and Miller, 1981). However, when one views the problem from the perspective of the transition from the late prebiotic world to true organisms, what is at issue is how the protein synthesis machinery that actually is used in modern organisms came to be. Since RNAs carrying the incoming amino acid or growing peptide are at the very heart of the complex modern machinery, one attractive hypothesis is to assume that an aminoacylated RNA was also used in the earliest renditions of the eventual translation apparatus. In fact, it has been shown that aminoacylated RNAs might be generated in an early RNA World in which aminoacyl adenylates are present (Illangasekare *et al*., 1995; Illangasekare *et al*., 1997). It is thus not unreasonable to suppose that at the very earliest stages these aminoacylated RNAs may have been essentially all there was to the machinery. In the absence of a ribosome how then would peptide bond formation have been catalyzed? One logical hypothesis is that this was facilitated by a second RNA that subsequently evolved to become the 23S rRNA. Based on the proposal made by Shimizu (1996), one could not previously take lightly the notion that the original catalyst was a peptide only later surpassed by a catalytic RNA. Although the present studies do not resolve the issue, they do level the playing field in that there is no compelling reason to believe that the original catalyst was more likely to be a peptide rather than RNA.

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