Chapter 7

Puromycin Analogs and the Homochirality of Life

Abstract

How nature evolved to use L-amino acids instead of the D-enantiomers to synthesize proteins remains an unanswered question. The physical basis for evolution of amino acid homochirality is proposed based on the physical properties of puromycin analogs bearing L- and D-amino acid residues. While L- and D-amino acids are enantiomers with similar physical properties, L- and D-puromycin analogs are diastereomers with unique physical properties as assessed by liquid chromatography and solubility determination. These data indicate that selection of L-amino acids could be made based on the different physical properties of aminoacyl-adenosine derivatives.

7.1 Introduction

The origin for homochirality of life continues to be a source of intense debate. Since Louis Pasteur first discovered the chirality of molecules in the 19^{th} century, scientists are divided on where and how enantiomer excess first originated. Enantiomer selection evolving on earth is supported by data that shows the surface of calcite crystals, a

widely present molecule on early earth, separate L- and D-amino acids on different faces of the crystal (1). Others speculate that sunlight, which shows a predominant direction of circular polarized light at dusk, directed enantiomer selection through light-induced reactions on early earth. Other data support models where molecular handedness was directed from events in space. One theory is that Earth formed from accretion of primordial dust containing an excess of left-handed amino acids (2). Further, enantiomer excess is thought to have originated from meteorites, which now have been shown to have L-enantiomeric excesses (3). A significant drawback for this theory is that L-enantiomer excesses were not found to exceed 3% for naturally occuring amino acids (4).

Biochemical experiments have also been carried out showing that while aminoacylation of RNA by amino acids is not stereoselective, RNA that is surface-bound (thought to mimic prebiotic surface monolayers) is aminoacylated specifically with L-residues (5). Another terrestrial process thought to play a role in homochirality is the observation that L- and D-enantiomers do not have exactly the same energy (6). However, this energy difference equates to an enantiomer excess of merely $10^{-13} - 10^{-16}\%$ (7).

How amino acid enantiomer excess evolved remains one of many 'origin of life' questions. Data collection can be gained not only from astrochemisty laboratories but also from chemistry and biochemistry experiments. Evaluating and understanding biological systems, such as ribosome-mediated protein synthesis (eukaryotes) or nonribosomal peptide synthesis (prokaryotes), could provide evidence for why handedness is a fundamental law of biology. Pushing the limits of such systems could yield results that support or refute existing models for enantiomeric selection. Development of reagents and assays to thoroughly elucidate the mechanism of protein synthesis, for example, is the starting point for drawing conclusions about how and why biological systems are stereospecific.

7.2 Results and Discussion

L- and D-amino acid pairs are enantiomers and have similar physical properties. This physical similarity puts into question how the L-enantiomer was selected to be used instead of the D-enantiomer. Current theories about enantiomer selection argue that this event occurred prior to the evolution of biological systems. However, the following data argue that selection of L-amino acids may have been possible based on the distinct physical properties of aminoacyl-nucleotides, such as puromycin. The theory presented assumes that the evolution of homochirality occurred at stages, i.e., D-sugars and then L-amino acids. Several pieces of data should be reviewed first in order to provide a framework for this argument.

A seminal discovery in 1967 showed that peptidyl transferase was carried out on isolated ribosomes in a reaction called the 'fragment reaction' where a peptide bond can be formed on isolated large 50S ribosomal subunits between a P-site held terminal hexanucleotide with a 3'-formylmethionine (CAACCA-Met-f) and puromycin (Figure 7.1) (8). Soon thereafter, peptidyl transferase activity under fragment assay conditions persisted despite the removal of most ribosomal protein (9).

This discovery led Francis Crick and Leslie Orgel to argue that the primitive ribosome may have consisted entirely of RNA (11). In their discussion, Crick and Orgel suggested that while the primordial ribosome may have been entirely RNA, present day ribosomes evolved to contain protein to 'do the job with greater precision' (12). Then tandem discoveries of catalytic RNA in 1980 (13) and experiments showing that stripping protein from 23S rRNA in 50S ribosomal subunits showed continued peptidyl transferase activity (14) further bolstered the theory of a primitive RNA only ribosome. Finally, in August 2000 Tom Steitz and co-workers published an atomic resolution crystal structure of the large ribosomal subunit with a transition state analog (10). This work unambiguously assigned the peptidyl transferase center (PTC) to be completely within a region of RNA (Figure 7.1). In fact, the closest amino acid side chain is more than 18 $\AA$ removed from the PTC (10). This confirmed the

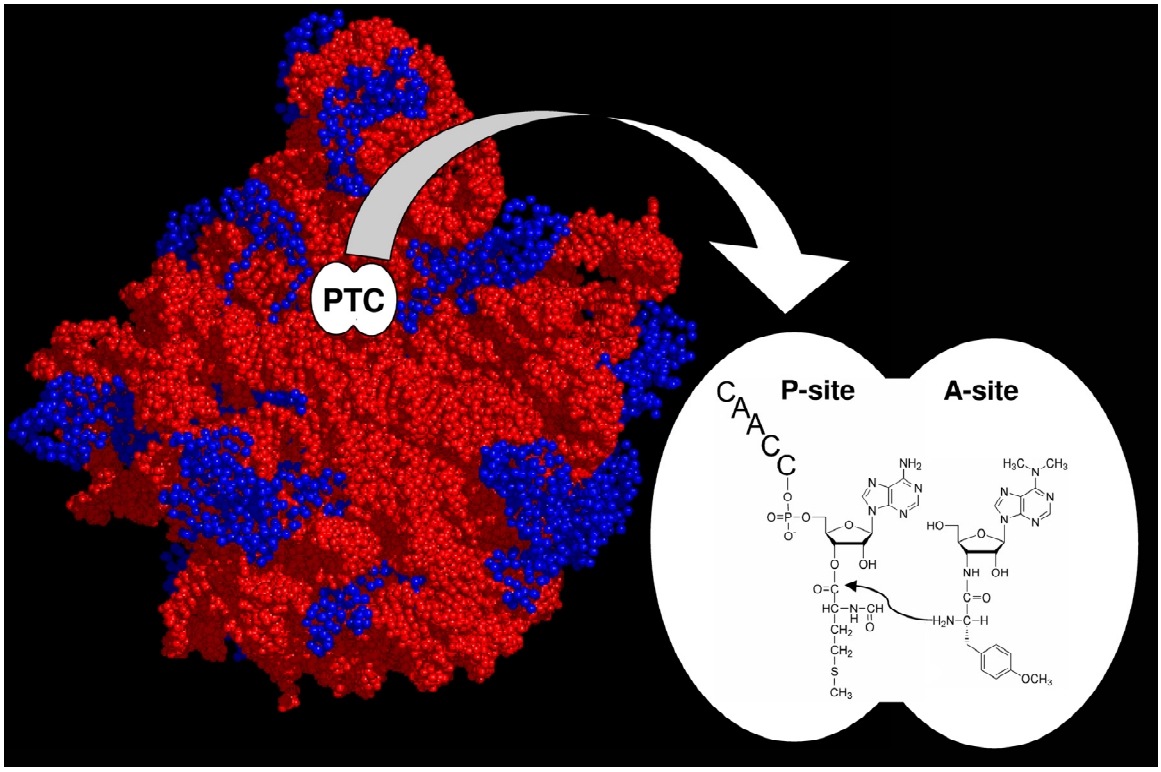

Figure 7.1: The fragment reaction between a P-site held CAACCA-Met-f and puromycin (RNA = red and protein = blue). Cystral structure of the 50S subunit from *H. marismortui* adapted from (10).

theory that the ribosome is a ribozyme (10). Taken together, these data are evidence that a primordial system for protein synthesis may have evolved from an RNA world where an RNA-only ribosome performed dipeptide bond formation between aa-tRNA fragments (15, 16).

At one point in evolution, prebiotic life had selected D-sugars, mastered RNA polymer synthesis, and the identification of the optimal RNA sequence and structure for basic ligation and cleavage reactions. However, the presence of amino acids in the prebiotic environment and pressure to search for alternative polymers prompted RNA to react with amino acids. In a scenario where the 'fragment reaction' was the progenitor of current day protein synthesis, the presence of puromycin-like molecules (e.g., 3'-*N*- and *O*-aminoacyl adenosine derivatives) would be expected. In fact, RNA catalysts have been selected that perform aminoacyl group transfer (17, 18). Therefore, primitive aminoacylation reactions may have yielded a variety of amino acid-nucleoside molecules. The current existence of puromycin, modified nucleosides, and co-factors such as S-adenosyl methionine (Figure 7.2) may be primordial remnants of the first amino acid-ribonucleoside compounds (19, 20, 21).

A primordial peptidyl transferase system may not have been stereospecific admist a racemic mixture of L- and D-amino acids. This phenomena is still apparent in current day aminoacylation reactions (for review see (22)). In this theory, both L- and D-amino acids became covalently linked to 2'-*N*- and 2'(3')-*O*-adenosine ribonucleosides by an RNA-based system yielding a series of 3'-*N*- and 2'(3')-*O*-L- and D-aminoacylnucleosides. These compounds recapitulate the 3'-end of every aa-tRNA molecule where the terminal nucleoside is an adenosine. Now, a series of dipeptides may have been coupled by the peptidyl transferase reaction of a primordial RNA-only ribosomal subunit. Since aminoacylation is not stereochemically controlled in this theory, the primitive ribosome would equally incorporate 3'-*N*- and -*O*-L- and D-aminoacylnucleosides (Figure 7.3).

As such, the homochirality of proteins may have resulted from a primordial RNA-

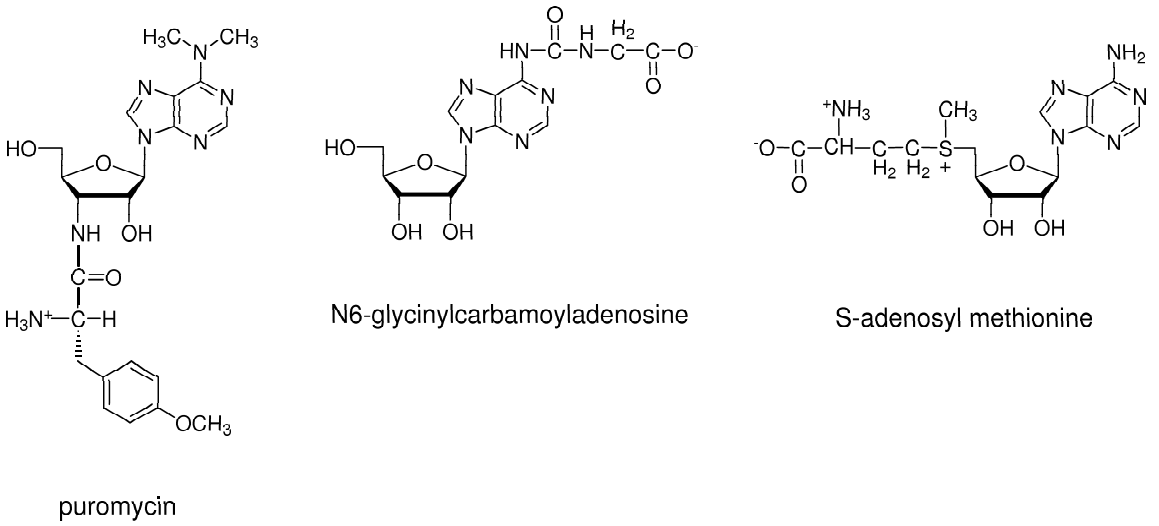

Figure 7.2: Current day ribonucleoside adenosine compounds with amino acid moieties.

only ribosome evolving to preferentially select L-amino acids. The data presented herein suggest that based on chemical differences between 3'-*N*- and L- and D-aminoacylnucleosides the ribosome was able to differentiate the L-isomer from the D-isomer. Two pieces of data are presented: 1) HPLC analysis of L- and D-puromycin analogs and 2) Solubility data for L- and D-analogs.

HPLC analysis shows the retention times for L- and D-puromycin analogs are different for each diastereomer (Figure 7.4A). This pattern is also observed for L- and D-Phe-PANS, *N*-methyl-L- and D-Phe-PANS, and L- and D-biocytin-PANS (Figure 7.4B-D). One interesting result is that D-analogs consistently elute before their L-counterparts (Figure 7.4). L- and D-amino acid pairs are expected to have identical chromatographic behavior, but when linked to puromycin aminonucleoside (PANS) the molecules are diastereomers with different physical properties. Overall, the presence of a D-side chain changes the solubility of the molecule, independent of the side chain identity (e.g., 4-*O*-methyl-tyrosine or biocytin side chains) or amine substitution (e.g., *N*-methyl-Phe-PANS) (Figure 7.4). The solubility of several L- and

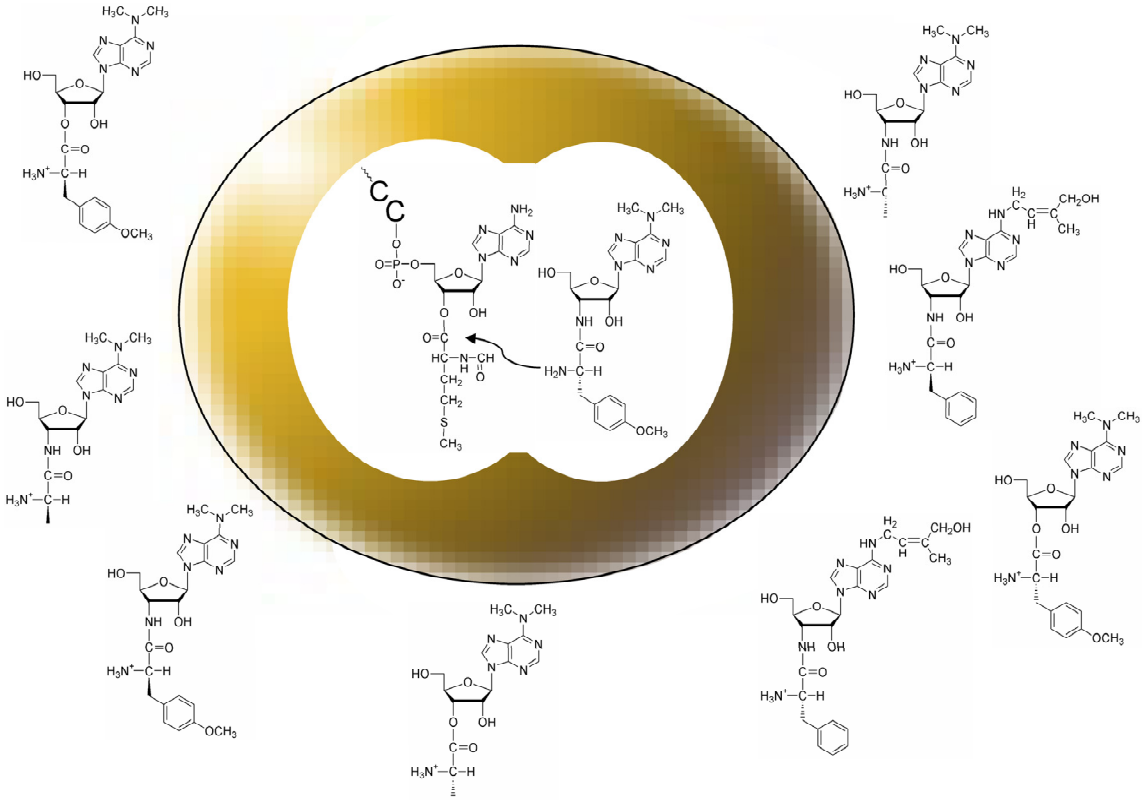

Figure 7.3: A primordial RNA-only ribosome may have generated dipeptides by coupling CAACCA-Met-f and 3'-*N*- and 2'-(3')-*O*-L- and D-aminoacylnucleosides.

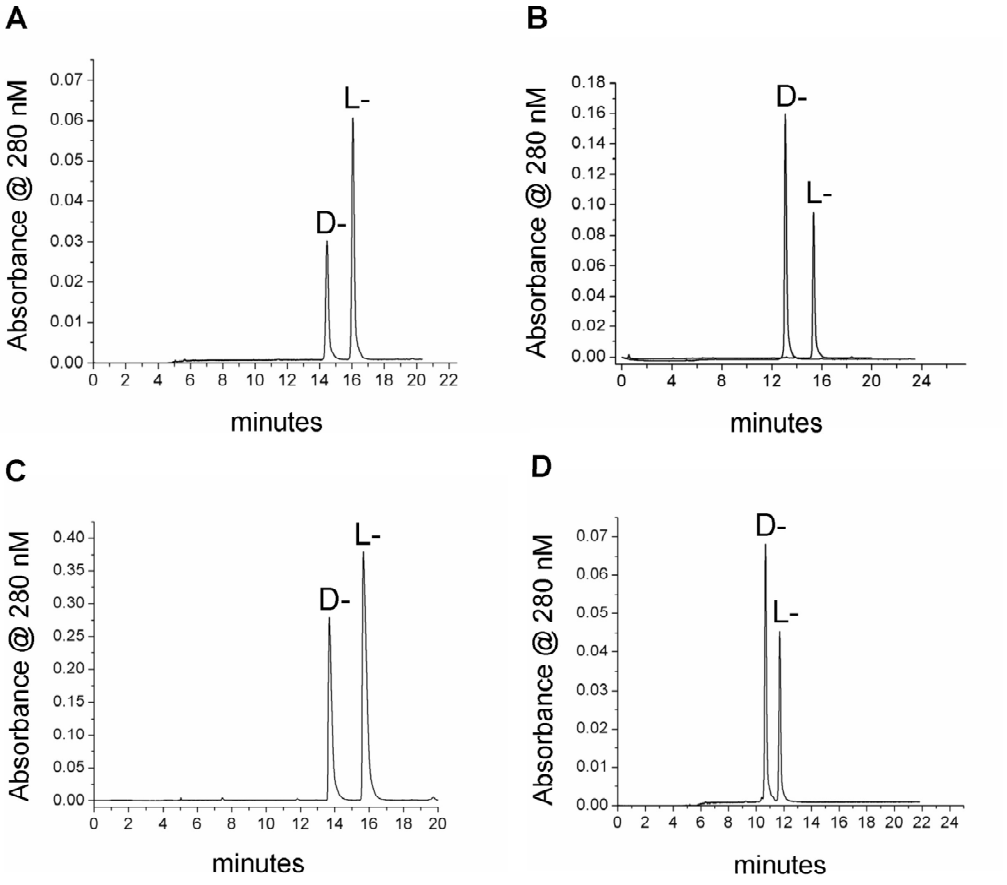

Figure 7.4: HPLC analysis of L- and D-amino acid analogs: (A) L- and D-puromycin; (B) L- and D-Phe-PANS; (C) *N*-methyl-L- and D-Phe-PANS; and (D) L- and D-biocytin-PANS.

Analog	Solubility (K_{sp}, μM)	ΔK_{sp}
L-puromycin	48.0	
D-puromycin	0.45	~100-fold
L-Phe-PANS	23.2	
D-Phe-PANS	4.44	~5-fold
L-Ala-PANS	1120	
D-Ala-PANS	10.4	~100-fold
PANS	147	

Table 7.1: Solubility determination for puromycin analogs.

D-puromycin analog pairs was also determined (Table 7.1).

The solubility of the L-analogs is consistently greater than their D-counterparts. In fact, the D-variants were all less soluble than any one L-variant (Table 7.1). Once the amino acid is coupled to PANS (K_{sp} = 147 mM) the analog solubility decreases for each molecule except L-Ala-PANS which becomes more soluble (Table 7.1). This observation may be related to the high solubility (g/100g, 25 °C) of free alanine which is 16.65 (6th most soluble amino acid) while free phenylalanine is 2.965 (23, 24). Perhaps, the presence of an L-alanine moiety increased the solubility of the L-Ala-PANS derivative relative to PANS alone (Table 7.1). The solubility data shows a consistent pattern for the D-analogs tested since they were all were less soluble than their L-counterparts. This pattern is comparable to that seen from the HPLC data where D-analogs consistently eluted before their L-counterparts (Figure 7.4). However, there does not appear to be a direct relationship between the potency of the analogs and their solubility. A problem with attempting to make this correlation stems from the effect of the amino acid side chain for affinity within the A-site of the ribosome. This variation was first noted by Nathans and Neidle which showed that hydrophobic, aromatic side chains produce the most potent puromycin derivatives

while smaller side chains lead to a less active analog (25).

The HPLC and water solubility data identify physical differences between L- and D-puromycin analogs. While this property can not be reconciled with the absolute potency of the analogs, the consistently low solubility of the D-variants is a property that primitive ribosomes may have been able to discern. Previous observations that D-puromycin analogs are incorporated into protein (26) may represent a residual activity that has not yet been lost through evolution. It should be noted that the significant physical difference between L- and D-analogs may not have required such an evolutionarly mature selective step, such as peptidyl transferase. Simply, the solubilty differences between 3'-*N*- and 2'(3')*O*-L- and D-aminoacylnucleosides dictated the concentrations available for subsequent reactions.

7.3 Conclusions

The RNA world hypothesis argues that an RNA-only ribosome (the progenitor of the current day large 50S or 60S subunits) may have carried out reactions analogous to the 'fragment reaction' to yield dipeptides. Assuming that 3'-*N*- and 2'(3')*O*-L- and D-aminoacylnucleosides were present in this primordial reaction, the primitive ribosome would be poised to discriminate between L- and D-molecules based on their distinct physical differences. The solubility data presented here reflect the unique properties of L- and D-puromycin analogs. The primordial ribosome may have initially been able to accommodate L- and D-variants equally and evolved to use only L-variants. This theory is specifically supported by the identification of mutations in RNA of the peptidyl transferase center of *E. coli.* 23S RNA that allow for enhanced incorporation of D-amino acids into protein (27). The confirmation that a primitive ribosome evolved to preferentially select L-residues instead of D-residues would stand as the first terrestrial process known to break the parity of racemic mixtures.

7.4 Experimental Procedures

7.4.1 General Information

Low-resolution mass spectra were recorded on a PE SCIEX API 365 triple quadruple electrospray mass spectrometer at the Beckman Institute Mass Spectrometry Laboratory, California Institute of Technology. High-resolution mass spectra (FAB) were recorded on a JMS-600H double-focusing, high-resolution, magnetic sector mass spectrometer at the Mass Spectrometry Laboratory, Division of Chemistry and Chemical Engineering, California Institute of Technology. All reagents were of highest available commercial quality and were used without further purification. L-puromycin and puromycin aminonucleoside (3′—amino-3′—deoxy-*N*,*N*'-dimethyl-adenosine) (PANS) were purchased from Sigma Chemical Co. D-puromycin and L- and D-alanine-PANS were prepared as described in (26). L- and D-phenylalanine-PANS and *N*-methyl-L- and D-phenylalanine-PANS were prepared as described in Chapter 5. L- and D-biocytin-PANS were prepared as described in Chapter 6. Puromycin analog concentrations were determined with the following extinction coefficients ($M^{-1}cm^{-1}$) at 260 nm: Aromatic puromycin analogs such as L- and D-puromycin, L- and D-phenylalanine, and *N*-methyl-L- and D-phenylalanine-PANS [$\epsilon = 10,500$] in H_2O. Non-aromatic puromycin analogs, such as L-and D-biocytin-PANS and L- and D-Ala-PANS [$\epsilon = 11,000$] in phosphate buffered saline (pH 7.3). Rabbit reticulocyte lysate was purchased from Novagen. Rabbit globin mRNA was obtained from Life Technologies Gibco BRL.

7.4.2 HPLC Analysis

Column chromatography was carried out on silica gel (40 – 63 μm, EM Science). Analytical HPLC was performed using a Vydac C18 column (5 mm, 4.5 x 250 mm) with buffer A (5 mM NH4OAc, pH 5.5 with 10% acetonitrile) and buffer B (5 mM NH4OAc, pH 5.5 with 90% acetonitrile); a linear gradient of 100% buffer B in 50 min

was used with a flow rate of 1 mL/min.

7.4.3 Solubility (K_{sp}) Determination

Saturated solutions were prepared by dissolving the analytes in 0.75 mL of water in 1.5-mL eppendorf tubes. The samples were shaken for 3 days at 25 °C and then filtered. The resulting saturated solution was quantified as described in General Information and K_{sp} = [analyte].

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