Aspartyl-tRNA Synthetase Requires a Conserved Proline in the Anticodon-binding Loop for tRNA\textsuperscript{Asn} Recognition \textit{in Vivo}*

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Most prokaryotes require Asp-tRNA\textsuperscript{Asn} for the synthesis of Asn-tRNA\textsuperscript{Asn}. This misacylated tRNA species is synthesized by a non-discriminating aspartyl-tRNA synthetase (AspRS) that acylates both tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Asn} with aspartate. In contrast, a discriminating AspRS forms only Asp-tRNA\textsuperscript{Asp}. Here we show that a conserved proline (position 77) in the L1 loop of the non-discriminating \textit{Deinococcus radiodurans} AspRS2 is required for tRNA\textsuperscript{Asn} recognition \textit{in vivo}. \textit{Escherichia coli trpA34} was transformed with DNA from a library of \textit{D. radiodurans} aspS2 genes with a randomized codon 77 and then subjected to \textit{in vivo} selection for Asp-tRNA\textsuperscript{Asn} formation by growth in minimal medium. Only proline codons were found at position 77 in the aspS2 genes isolated from 21 of the resulting viable colonies. However, when the aspS temperature-sensitive \textit{E. coli} strain CS89 was transformed with the same DNA library and then screened for Asp-tRNA\textsuperscript{Asp} formation \textit{in vivo} by growth at the non-permissive temperature, codons for seven other amino acids besides proline were identified at position 77 in the isolates examined. Thus, replacement of proline 77 by cysteine, isoleucine, leucine, lysine, phenylalanine, serine, or valine resulted in mutant \textit{D. radiodurans} AspRS2 enzymes still capable of forming Asp-tRNA\textsuperscript{Asp} but unable to recognize tRNA\textsuperscript{Asn}. This strongly suggests that proline 77 is responsible for the non-discriminatory tRNA recognition properties of this enzyme.

Accurate aminoacyl-tRNA formation, the basis for error-free protein synthesis, is achieved by the exquisite substrate specificity of an aminoacyl-tRNA synthetase (1). For better quality control, many aminoacyl-tRNA synthetases have also developed editing mechanisms to hydrolyze the potentially harmful misaminoacylated tRNAs (2–5).

One surprising result of the analysis of the many microbial genomes is the discovery of the extensive use of Asp-tRNA\textsuperscript{Asn} and Glu-tRNA\textsuperscript{Cln} for the biosynthesis of Asn-tRNA\textsuperscript{Asn} and Gln-tRNA\textsuperscript{Cln} (6). For example, Asp-tRNA\textsuperscript{Asn} is required for Asn-tRNA\textsuperscript{Asn} formation in most bacteria and archaea because of the lack of asparaginyl-tRNA synthetase in these organisms. This misacylated aminoacyl-tRNA is formed by a synthetase with relaxed tRNA specificity, the non-discriminating aspartyl-tRNA synthetase (ND-AspRS). 1 This enzyme, unlike the discriminating AspRS (D-AspRS) that forms only Asp-tRNA\textsuperscript{Asp}, does not discriminate tRNA\textsuperscript{Asn} from tRNA\textsuperscript{Asp} and therefore synthesizes Asp-tRNA\textsuperscript{Asn} in addition to Asp-tRNA\textsuperscript{Asp} (7, 8). \textit{Deinococcus radiodurans} is among a small number of organisms that have two AspRSs, a discriminating AspRS1 and the non-discriminating AspRS2 (8).

Despite the widespread presence of ND-AspRS enzymes in the microbial world (6), our understanding of tRNA selection by ND-AspRS is still fragmentary. Although the crystal structure of \textit{Thermus thermophilus} ND-AspRS is known (9), the lack of a tRNA-bound AspRS structure limits our ability to understand the tRNA specificity of the enzyme. Moreover, the absence of any tRNA\textsuperscript{Asn} structure, either free or complexed with an aminoacyl-tRNA synthetase, complicates the attempts to model the interaction of tRNA\textsuperscript{Asn} and AspRS.

The discriminating and non-discriminating forms of the archaeal-type AspRS (e.g. \textit{Thermococcus kodakaraensis} AspRS and \textit{D. radiodurans} AspRS2) (10) are highly similar and differ systematically only at two amino acid positions (11). Both positions, one in the β strand and one in the L1 loop, are located in the N-terminal domain responsible for tRNA anticodon recognition by the enzyme (Fig. 1). Although histidine (position 28 in \textit{D. radiodurans} AspRS2) and proline (position 77 in \textit{D. radiodurans} AspRS2) are conserved at these positions in ND-AspRSs, glutamine/tryptophan and lysine are found in the corresponding positions in D-AspRS enzymes (Fig. 1). A single amino acid change was sufficient to convert the \textit{T. kodakaraensis} D-AspRS to a non-discriminating enzyme by introducing the W26H and K85P mutations into the discriminating \textit{T. kodakaraensis} AspRS individually or in combination (11). A structure-based model points to a direct interaction between Lys-85 in the L1 loop of the enzyme and C36 of tRNA\textsuperscript{Asp} (11). This interaction is a key identity element that differentiates tRNA\textsuperscript{Asn} from tRNA\textsuperscript{Asp} (12, 13). Mischarging of tRNA\textsuperscript{Asn} was also achieved by replacing the entire L1 loop of the discriminating \textit{T. kodakaraensis} AspRS with that of the non-discriminating \textit{T. thermophilus} enzyme (9). In a similar strategy, substitution of the \textit{Escherichia coli} lysyl-tRNA synthetase L1 loop with that of \textit{E. coli} AspRS enabled the former to mischarge tRNA\textsuperscript{Asp} (14).

The examples above are based on an expansion the tRNA specificity of a discriminating enzyme and tested by \textit{in vitro}...
experiments. We wondered how the tRNA recognition of a non-discriminating AspRS could be limited to tRNA\(^{Asp}\) and whether this could be confirmed in vivo. Therefore we tested the necessity of Pro-77 and His-28 on tRNA\(^{Asp}\) (tRNA\(^{Asp}\)) and tRNA\(^{Asp}\) recognition by D. radiodurans AspRS2 in vivo and in vitro. The importance of the conserved Pro-77 was further analyzed by random mutagenesis of codon 77 of D. radiodurans \(\text{aspS}2\) (gene encoding AspRS2) followed by in vivo selection.

**EXPERIMENTAL PROCEDURES**

**Expression of D. radiodurans AspRSs in E. coli—Oligonucleotide synthesis and DNA sequencing were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. Mutant D. radiodurans \(\text{aspS}2\) genes (8) were generated by overlap extension PCR. For in vivo analyses, each gene was inserted into pCBS200 between NdeI and BglII to yield pCBS\(\text{DRaspS}2\) constructs. The pCBS200 vector was kindly provided by Tong Li (Yale University) and is a derivative of pCBS1 (15). This low copy vector confers ampicillin resistance and allows expression from the constitutive trpS promoter. The vector pCBS200 additionally contains the polylinker NdeI-MscI-NcoI-KpnI-SmaI-XhoI-XbaI-BglII. To obtain purified protein, the mutant genes were also cloned as an intein fusion construct into pTYB11 (New England Biolabs) and in vivo expressed in E. coli, and those with >90% identity are identical. Cloning of the \(\text{aspS}2\) gene cloned in pCBS200. Mutagenic primers contained randomized nucleotides at each of the three positions of the Pro-77 codon.

**Kinetic Analyses**—Random mutagenesis was accomplished by PCR amplification of the region from the unique NdeI and XhoI sites in the \(\text{aspS}2\) gene cloned in pCBS200. Mutagenic primers contained randomized nucleotides at each of the three positions of the Pro-77 codon. The digested PCR product was ligated with the large NdeI/XhoI fragment of pCBS200-\(\text{aspS}2\) vector to generate the P77 variant library. Sixteen individual clones from the library were sequenced to confirm the degeneracy at codon 77 (data not shown). No colonies were obtained in the absence of the insert, indicating that there was no carryover of the original wild type \(\text{aspS}2\) gene. Genes in which mutations were introduced by PCR outside of the randomized position were not considered in this study. Mutations tested for various phenotypes were confirmed upon reisolation of the plasmid, transformation, and sequencing.

**Preparation of tRNA Transcripts**—Three tRNA\(^{Asp}\) genes are encoded by D. radiodurans, and the sequence of tRNA\(^{Asp}\) used in this study and a previous study (17) corresponds to those of tRNA\(^{Asp}\)-2 and tRNA\(^{Asp}\)-3, which are identical. Cloning of D. radiodurans tRNA\(^{Asp}\) has been reported (17). To allow the presence of the natural U1-A72 base pair in the \(\text{aspS}2\) gene, it was cloned into pUC119 between EcoRI and BamHI with a 5' cis promoter of BstNI-digested templates was carried out as described (18). Each transcript was extracted with phenol and chloroform, precipitated with ethanol, and fractionated on a Q-Sepharose column as described (10) or separated by 12% preparative denaturing polyacrylamide gel. RNA was extracted from the crude gel slice containing the appropriate transcript and purified by extractions with phenol and chloroform, ethanol precipitation, and desalting with a NAP-10 gel filtration column (Amersham Biosciences). Purified tRNA transcripts were loaded at \(10 \mu\text{g}\) and in single color. Each transcript was renatured prior to aminoacylation assays as described (10).

**Kinetic Analyses**—Aminoacylation and measurement of kinetic parameters was performed as described previously (11). \(^{[\text{H}]\text{Asp}} (242 \mu\text{M}, 17 \text{ Ci/mmol})\) was from PerkinElmer Life Sciences. Aminoacylation was carried out at \(37^\circ\text{C}\) in \(50 \text{mM Hepes, pH 7.0, 50 mM KCl, 10 mM MgCl}_2, 4 \text{mM ATP}, 5 \mu\text{M dithiothreitol and 10–50 nM AspRS}\). For tRNA\(^{Asp}\), the reaction additionally contained 500 \(\mu\text{M} \) \(^{[\text{H}]\text{Asp}} (0.85 \text{ Ci/mmol})\) and renatured prior to aminoacylation assays as described.
tRNA Recognition by a Non-discriminating AspRS

<table>
<thead>
<tr>
<th>Transformed strain</th>
<th>Codon 77</th>
<th>Amino Acid</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>trpA34</td>
<td>CCC</td>
<td>Proline</td>
<td>19</td>
</tr>
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<td>trpA34</td>
<td>CCA</td>
<td>Proline</td>
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</tr>
<tr>
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<td>CCC</td>
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<tr>
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<td>AAA</td>
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<tr>
<td>CS89</td>
<td>TGT</td>
<td>Cysteine</td>
<td>1</td>
</tr>
<tr>
<td>CS89</td>
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<td>Isoleucine</td>
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</tr>
<tr>
<td>CS89</td>
<td>TTC</td>
<td>Phenylalanine</td>
<td>1</td>
</tr>
</tbody>
</table>

* Individual isolates were obtained following selection for growth of E. coli trpA34 in minimal medium without tryptophan at 37 °C or of E. coli CS89 at the non-permissive temperature of 40 °C.

RESULTS AND DISCUSSION

In Vivo Selection Reveals the Importance of Pro-77 for the Non-discriminating Nature of D. radiodurans AspRS2—Given the strict conservation of Pro-77 in the known archaeal ND-AspRS proteins (Fig. 1), we decided to completely randomize codon 77 in the D. radiodurans aspS2 gene and test the amino acid preference for tRNA<sup>Asp</sup> recognition at this position in vivo. Transformation of the E. coli trpA34 strain, a tryptophan auxotroph containing an Asn-60 (19) instead of the universally conserved active site residue Asp-60 present in all known tryptophan synthetase α-subunits, with the mutant library DNA that could be aminoacylated was determined for each transcript and is around 40% for tRNA<sup>Asp</sup> and 50–60% for tRNA<sup>Asn</sup>. The concentration reported therefore reflects the amount of chargeable tRNA. The values of <i>k</i><sub>cat</sub> and <i>K</i><sub>m</sub> were calculated by non-linear regression fitting of the data to the Michaelis-Menten equation.

0.2-5 μm transcript. For tRNA<sup>Asn</sup>, the reaction contained 750 μm [3H]Asp (0.57 Ci/mol) and 0.5-12 μm transcript. The concentration of aspartate was kept at least 2-fold higher than the <i>K</i><sub>m</sub> for the amino acid. Initial velocities were determined from the average of duplicated sets of data at varying tRNA concentrations. The percentage of renatured tRNA selection of the transformed strains (20). Approximately 200 tryptophan prototrophic colonies were isolated and retested; then the aspS2 genes from 21 isolates were sequenced. All 21 isolates contained Pro-77 (Table I), suggesting that proline is preferred for tRNA<sup>Asn</sup> recognition by D. radiodurans ND-AspRS. In contrast, when the temperature-sensitive aspS E. coli CS89 strain (16) was transformed with the same D. radiodurans aspS2 mutant library DNA, 12 (of 16) aspS2 isolates, which endowed strain CS89 with “wild type-like” growth (Fig. 2) at the non-permissive temperature of 40 °C, contained seven different amino acids at position 77 (4 isolates with Pro-77) (Table I).

The amino acid variations observed at position 77 in the CS89 isolates also demonstrated the efficiency of the randomization procedure. However, none of these non-wild type Pro-77 isolates of D. radiodurans AspRS2 supported the growth of trpA34 in the absence of tryptophan (Fig. 3), consistent with the dominant isolation of proline in our randomized mutagenesis experiment (Table I).

P77K Reduced Only the Misacylation Activity of D. radiodurans AspRS2 in Vitro—To examine the effect of Pro-77 on tRNA<sup>Asn</sup> recognition by D. radiodurans AspRS2 in vitro, we investigated the ability of purified wild type and P77K D. radiodurans AspRS2 enzymes to charge homologous tRNA transcripts (Table II). The P77K variant was tested because of the strict conservation of Lys-77 in the archaeal D-AspRSs, which showed a slight preference for tRNA<sup>Asn</sup> over tRNA<sup>Asp</sup> (Fig. 1), but at a cost of aminoacylation efficiency (Table II).
However, the simultaneous introduction of H28Q and P77K did not result in a further gain of tRNA<sub>Asp</sub> recognition in <i>D. radiodurans</i> AspRS2 (Table II), suggesting a negative interaction between these two positions. The smaller changes observed in the <i>D. radiodurans</i> AspRS2 mutant enzymes compared with those found with the reciprocal mutations in the <i>T. kodakaraensis</i> AspRS (11) could be a result of context-dependent recognition of tRNA<sub>Asp</sub> by these AspRSs. Based on their ability to support the growth on LB agar plates of <i>T. kodakaraensis</i> and <i>D. radiodurans</i> and their ability to support the growth on three mischarged aminoacylated tRNAs (21), we have used various selection as a powerful tool to reveal that the conserved Pro-77 in the L1 loop (Fig. 1) could charge tRNA<sub>Asp</sub> but not tRNA<sub>Asn</sub> in vivo. Therefore, a small in vivo effect could lead to a significant response in vivo. Similar results have been reported with other mischarged aminoacylated tRNAs (21).

**Conclusions**—We have used in vivo selection as a powerful tool to reveal that the conserved Pro-77 in the L1 loop (Fig. 1) of archaeal-type ND-AspRS enzymes is critical for tRNA<sub>Asn</sub> recognition. Replacement of this proline in <i>D. radiodurans</i> AspRS2 with seven other amino acids (lysine, serine, cysteine, isoleucine, leucine, valine, and phenylalanine) did not affect tRNA<sub>Asp</sub> recognition in vivo (Fig. 2) but reduced Asp-tRNA<sub>Asn</sub> formation to undetectable levels in vivo. The importance of proline in tRNA<sub>Asn</sub> recognition was further illustrated by the universal appearance of this amino acid in our in vivo missense suppression selection (Table I). The intolerance of other amino acids at position 77 in <i>D. radiodurans</i> AspRS2 when selecting in vivo for Asp-tRNA<sub>Asp</sub> formation (Fig. 3 and Table I) explains the strict conservation of this proline in all the archaeal-type ND-AspRS enzymes (Fig. 1). Because of its unique backbone structure, proline at this position may avoid a potential clash between the protein backbone and the U36 base of the tRNA<sub>Asn</sub> anticodon (11). Interestingly, a conserved proline is also found at the equivalent position in a subgroup of asparaginyl-tRNA synthetases (14, 22) that are closely related to the archaeal-type ND-AspRSs (11). On the other hand, the semi-conserved histidine in the 81 strand appears to be important for both tRNA<sub>Asp</sub> and tRNA<sub>Asn</sub> recognition (Table II), but the molecular basis for this is unclear (11). It is interesting to note that the corresponding L1 loop in the bacterial-type AspRSs has diverged in sequence and length and does not show the strict conservation of proline in the ND-AspRSs and lysine in the D-AspRSs described here. Therefore, a different mechanism might be used by the bacterial-type AspRSs to achieve the non-discriminating tRNA<sub>Asn</sub> recognition.

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