Yeast tRNA<sub>Asp</sub> Charging Accuracy Is Threatened by the N-terminal Extension of Aspartyl-tRNA Synthetase*

Received for publication, October 29, 2002, and in revised form, December 2, 2002
Published, JBC Papers in Press, December 13, 2002, DOI 10.1074/jbc.M211035200

Michaël Ryckelynck, Richard Giegé, and Magali Frugier

From the Département "Mécanismes et Macromolécules de la Synthèse Protéique et Cristallogénèse," UPR 9002, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue René Descartes, F-67084 Strasbourg Cedex, France

This study evaluates the role of the N-terminal extension from yeast aspartyl-tRNA synthetase in tRNA aspartylation. The presence of an RNA-binding motif in this extension, conserved in eukaryotic class IIb aminoacyl-tRNA synthetases, provides nonspecific tRNA binding properties to this enzyme. Here, it is assumed that the additional contacts the 70 amino acid-long appendix of aspartyl-tRNA synthetase makes with tRNA could be important in expression of aspartate identity in yeast. Using in vitro transcripts mutated at identity positions, it is demonstrated that the extension grants better aminoacylation efficiency but reduced specificity to the synthetase, increasing considerably the risk of noncognate tRNA mischarging. Yeast tRNA<sub>Asp</sub>(GUC) and tRNA<sub>Asp</sub>(GUG) were identified as the most easily mischarged tRNA species. Both have a G at the discriminator position, and their anticodon differs only by one change from the GUC aspartate anticodon.

Aminoacylation of tRNA is a key step during translation of genetic information. It is performed by aminoacyl-tRNA synthetases (aaRSs) and involves specific recognition of their cognate tRNAs and amino acids (reviewed in Ref. 1). Fidelity of translation depends on accurate tRNA aminoacylation, but unexpectedly, this reaction does not occur with absolute specificity (2). This dilemma is resolved since synthetases developed kinetic artifices ensuring destruction of the mischarged tRNAs (editing), thereby reducing error levels in protein synthesis (3). Furthermore, specialized proteins, by interacting with certain synthetases, increase their affinity for the cognate tRNA and thus ensure increased aminoacylation efficiency. This is the case in the association of Arc1p with MetRS and GluRS in yeast (4) and of p43 with ArgRS in mammals (5).

A similar “helper” role seems to be played by the extra domains within eukaryotic synthetases. Indeed, these enzymes differ usually from their prokaryotic counterparts by appendices located in their N- or C-terminal regions (6), outside the structural core defining the two classes of synthetases (7). In mammals, the extensions are involved in the formation of the multi-synthetase complex (6), but such architecture has not been detected in unicellular eukaryotes until now. In fact, little is known about the functional role of the extensions in unicellular eukaryotic synthetases, except that in two different yeast aminoacylation systems, they were shown to provide nonspecific tRNA binding properties to GluRS (8, 9) and AspRS (10).

Yeast AspRS contains an N-terminal appendix of 70 amino acids that adopts a helical structure (11, 12). It encompasses a RNA-binding motif (XSXXKLKKX) conserved in eukaryotic AspRSs but also in eukaryotic LysRSs and AsnRSs (10), the two other class IIb synthetase families. The appendix, located next to the anticodon-binding module, was shown to considerably increase the stability of the complex between AspRS and cognate tRNA<sup>Asp</sup> as shown by gel-shift binding assays and footprinting experiments. Moreover, the AspRS appendix can be replaced by the appendices of LysRS or AsnRS (10). Likewise, it was recently demonstrated that the extension in human LysRS shares the same properties of those in yeast AspRS (13). However, its presence in AspRS is not mandatory for tRNA aminoaacylation (11, 14). As seen in the crystal structure of yeast AspRS complexed with tRNA<sup>Asp</sup> (15), and in that recently solved of the free enzyme truncated of its first 70 amino acids (16), only the core of the enzyme is required for tRNA aspartylation.

Specificity of tRNA recognition by yeast AspRS is primarily ensured by the tRNA identity determinants. Only six such determinants, located in three distinct regions of tRNA<sup>Asp</sup> (Fig. 1A), are important to confer the aspartate identity. They are the discriminator base G73, four bases in the anticodon loop (G34, U35, C36, and C38), and base pair G10-U25 in the core region of the tRNA (17, 18). Mutation of these elements leads to losses in aspartylation efficiencies (17), the strongest determinants being those leading to the strongest effects upon mutation (e.g. G73 and the aspartate anticodon G34, U35, C36). Except for identity base pair G10-U25, which is involved in the correct folding of the tRNA (19) and has no direct contact with the enzyme, all other identity bases make specific interactions with amino acid residues on AspRS (20). Nonspecific contacts, however, are much more numerous. As shown by biochemical and x-ray data, they extend along the side of the tRNA comprising the variable region (15, 21, 22) (Fig. 1B, left). Additional nonspecific contacts are provided by the N-terminal extension of native AspRS and are found along the opposite side of the tRNA (Fig. 1B, right). They increase the stability of the complex and the global aminoaacylation efficiency (10).

Considering the better affinity of tRNA<sup>Asp</sup> for native AspRS and the increased aspartylation efficiency correlated with the presence of the N-terminal extension in the native enzyme, it can be conjectured that the additional contacts between the two macromolecular partners play a role in the mechanisms ensuring the specificity of genetic code expression at the translational level. By mutational analysis of tRNA<sup>Asp</sup> and AspRS, it is shown that the N-terminal extension is responsible for a
decrease in aminoacylation specificity, thereby favoring aspar-tylation of noncognate tRNAs. The identified mischarged tRNAs are related with tRNA\textsubscript{Asp} by their anticodon sequence, in agreement with the concept of partial conservation of determinants in mischarged tRNAs. Altogether, a mechanistic role of the yeast AspRS N-terminal extension in the aspartylation reaction of tRNA is established. This has functional implications for the specific expression of aspartate identity in vivo.

EXPERIMENTAL PROCEDURES

Preparation of Pure AspRS\textsubscript{s} and of Crude Synthetase Extracts

Native AspRS\textsubscript{1-557} from yeast and truncated AspRS\textsubscript{71-557}, deleted of its N-terminal extension, were expressed and purified as described previously (10). In short, the genes were cloned into the \textit{Escherichia coli} expression vector pQE70 (Qiagen) fused to a C-terminal His\textsubscript{6} tag. AspRS variants were purified according to the Qiagen protocol, quantified by Bradford assay (Bio-Rad), and stored at \(-20\) °C in 50 mM phosphate buffer, pH 7.2, 150 mM KCl, 50% glycerol, and 10 mM \(\beta\)-mercaptoethanol. AspRS preparations yielded only one band on SDS-polyacrylamide gel, even when overloaded, and can be estimated to be 99% pure. Specific activities, expressed as turnover numbers, correspond to 0.20 and 0.19 s\textsuperscript{-1} for AspRS\textsubscript{1-557} and AspRS\textsubscript{71-557}, respectively (assays conducted in the presence of 25 \(\mu\)M aspartate, a subsaturating amino acid concentration, see Ref. 23) or to 3.4 and 3.1 s\textsuperscript{-1} for AspRS\textsubscript{1-557} and AspRS\textsubscript{71-557}, respectively (assays conducted in the presence of 500 \(\mu\)M aspartate, a concentration close to the physiological level of aspartate in yeast cells, see Ref. 24). An enriched protein extract deprived of nucleic acids and ribosomes was made by chromatography on a Mono-Q column (Bio-Rad) and used as a crude enzyme preparation containing the activity of aminoacyl-tRNA synthetases (except for prolylation and glutaminylation).

Preparation of tRNAs

\textit{In Vitro} Transcription of tRNA\textsubscript{Asp} Variants—tRNA transcripts used in this work have been obtained by \textit{in vitro} transcription of synthetic genes cloned in pUC 118 (17). Each of these genes corresponds to the T7 RNA polymerase promoter region directly connected to the downstream tRNA sequence. \textit{In vitro} transcriptions were performed in reaction mixtures containing 40 mM Tris-\(\text{HCl}, \text{pH} 8.1 (37\) °C), 22 mM MgCl\textsubscript{2}, 5 mM dithioerythritol, 0.1 mM spermidine, 4 mM each of nucleoside triphosphate, 5 mM GMP, 50 ng/\(\mu\)l linearized plasmid, and 5 \(\mu\)g/ml T7 RNA polymerase. Transcription mixtures were incubated for 2 h at 37 °C, and reactions were stopped by phenol/chloroform extraction.

![Diagram](image_url)

\textbf{Fig. 1. Structure of yeast tRNA\textsubscript{Asp} with AspRS recognition elements emphasized.} A, cloverleaf structure of tRNA\textsubscript{Asp}. Identity determinants recognized specifically by AspRS are \textit{circled} in red. B, structure of the AspRS/tRNA\textsubscript{Asp} complex as determined in the crystal structure (left) and modelization of the N-terminal appendix (right) (adapted from Refs. 15 and 10, respectively). The N-terminal extension was modeled in \textit{blue}, and the RNA-binding motif is indicated in \textit{pink}.
Full-length transcripts correctly ending with the CCA sequence were purified by preparative electrophoresis on 12% polyacrylamide denaturing gels (8 M urea) followed by electrophoration in Tris-borate-EDTA (Schleicher and Schuell apparatus). The concentration of tRNA transcripts was determined by absorbance at 260 nm.

Purification of Yeast tRNAs—Countercurrent fractions enriched in tRNA\(^{\text{Asp}}\), tRNA\(^{\text{Glu}}\) or tRNA\(^{\text{Gln}}\) were gifts from G. Keith. Pure tRNA\(^{\text{Asp}}\) and tRNA\(^{\text{Glu}}\) were obtained by purification on a 10% polyacrylamide gel (19/1), in semidenaturing conditions (4 M urea). Migration was performed at 300 V for 48 h at 4 °C. Bands containing pure tRNAs were handled as described for tRNA transcripts. Pure tRNA\(^{\text{Asp}}\) was a gift from A. Théobald-Dietrich. The purity of tRNAs (>95%) was established by testing their cognate amino acid acceptance.

Aminoacylation of tRNAs

Aminoacylations of tRNA\(^{\text{Asp}}\) variants have been performed at 37 °C in 100 mM HEPES-KOH, pH 7.4, 15 mM MgCl\(_2\), 5 mM ATP, 30 mM KCl, 25 μM 1\(^{-}\)\[^{3}\]HAspartate (Amersham Biosciences), and appropriate amounts of tRNA transcripts. Before aminoacylation, transcripts were reconstituted in H\(_2\)O by heating at 85 °C for 90 s and slow cooling to room temperature prior to adjunction of MgCl\(_2\). Aminoacylation reactions were initiated by addition of appropriate amounts of enzyme diluted in 100 mM HEPES-KOH, pH 7.4, 10% glycerol, 1 mM dithioerythritol, and 5 mg/ml bovine serum albumin. Aminoacylated tRNA samples were quenched in trichloroacetic acid and treated by the conventional way on Whatman paper 3 MM (25). Kinetic constants (\(k_{\text{cat}}\) and \(K_m\)) were derived from Lineweaver-Burk plots. In general, the concentration of tRNA was changed from 0.1 to 5 \(K_m\), when possible, except for mutants with \(K_m\) over 10 \(K_m\), where the highest concentration was at most 2–3 \(K_m\). Plateaus of tRNA aspartylation were determined by incubating 1 μM pure tRNA (transcript or modified species) or 20 μM total tRNA in the presence of different AspRS (0.05–3 μM) and aspartate (25–500 μM) concentrations and otherwise optimal reaction conditions (as above). Reactions were stopped after 2.5, 5, 10, and 20 min incubation at 37 °C. Displayed kinetics and plateaus represent an average of at least two independent experiments. Values of \(k_{\text{cat}}/K_m\) ratio for replicates varied by at most 15%.

Identification of Mischarged tRNAs

Total Yeast tRNA—The brewers' yeast tRNA was from Roche Molecular Biochemicals. Titration by aminoacylation assays, using crude synthetases extract and 25 μM aspartate, indicated a content of 4.5 ± 0.5% tRNA\(^{\text{Asp}}\) in this total tRNA (deduced from plateau values).

Protection of Thiolated Bases—To avoid modification of thiolated bases during periodate oxidation (26), the total yeast tRNA was DTNB-treated as follows. Total tRNA (20,000 pmol) was incubated for 20 min at room temperature in 25 mM phosphate buffer, pH 6.8, in the presence of 0.5 mM DTNB (Sigma). DTNB was removed by dialyzing 12 h against milliQ water, and tRNA was recovered by ethanol precipitation.

Aspartylation Reactions—Total tRNA (20 μM, containing ~1 μM tRNA\(^{\text{Asp}}\)) was incubated with 1 μM native or truncated AspRS in the presence of 500 μM aspartic acid (ratio [AspRS]/[tRNA\(^{\text{Asp}}\)] ~ 5). Aminoacylation was conducted at 37 °C for 25 min; it was stopped by phenol extraction (pH 5.0), and the tRNA was recovered by ethanol precipitation. Controls without AspRS were run in parallel.

Oxidation of Non-aspartylated tRNAs—The periodate treatment of tRNA was adapted from Hansske and Cramer (27). The pellet containing charged and uncharged tRNAs was dissolved in 50 mM sodium acetate buffer, pH 5.0, to a final concentration of 80 μM. Oxidation was started by addition of 40 mM m-periodate (Sigma) and was run at room temperature in the dark for 25 min. The reaction was stopped by neutralizing the excess periodate with 5% glycerol. Glycerol and remaining periodate were removed by dialysis (2 h against 5 mM sodium acetate at pH 5.0). The mixture of aspartylated and of oxidized tRNA species was recovered by ethanol precipitation.

Decay and Deprotection of tRNAs—The tRNA pellet was dissolved in 10 mM Tris-HCl, pH 8.0, to a final concentration of 80 μM and incubated for 30 min at 37 °C for complete decay of aspartylated tRNA species. After precipitation, tRNAs were first incubated for 3 h on ice in 0.1 M dithioerythritol (reduction of thiol groups) and for 20 min at room temperature in 0.1 mM NaBH\(_4\) (reduction of periodate-generated aldehyde groups on the terminal ribose of non-aspartylated tRNAs). NaBH\(_4\) was removed by an overnight dialysis in milliQ water. tRNAs were recovered by ethanol precipitation and quantified by absorbance at 260 nm.

Identification of Aspartylated/Protected tRNAs—Three batches of partially aminoacylated tRNAs (a control and two batches that were aspartylated by native AspRS\(^{\text{AspRS1–557}}\) or truncated AspRS\(^{\text{AspRS71–557}}\) were tested for their amino acid acceptance. Aminoacylation activities were measured under the same conditions as those described above, with 25 μM amino acid, 20 μM treated tRNA, and 0.15 μg/μl crude enzymatic extract containing aminoacyl-tRNA synthetase activities. Eighteen amino acids were tested: 1-\[^{14}\]C]-radiolabeled amino acids were alanine, arginine, glutamic acid, glutamine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine; those \([^{3}\]H\)-labeled were asparagine, aspartic acid, glycine, lysine, and threonine (all from Amersham Biosciences). Purity of \([^{3}\]H\)-labeled aspartic acid and asparagine as well as of cold aspartic acid, asparagine, and glutamic acid was checked by TLC for possible contamination with other amino acids. No visible trace of such contamination has been detected.

RESULTS AND DISCUSSION

The N-terminal Extension Confers Increased Aminoacylation Efficiency but Decreased Specificity to AspRS—Aspartylation efficiencies of tRNA\(^{\text{Asp}}\)) transcripts mutated at their major identity positions (the discriminator base G73 and the anticond base G34, U35, C36) were monitored. For each mutant, the kinetic constants (\(k_{\text{cat}}\) and \(K_m\)) were derived from aminoacylation reactions with the truncated and native AspRS (AspRS\(^{\text{AspRS71–557}}\) and AspRS\(^{\text{AspRS1–557}}\) (Table 1). For each AspRS form, aminoacylation efficiencies (\(k_{\text{cat}}/K_m\)) were calculated for mutated tRNAs. For an easier comparison, results will be discussed as losses of aminoacylation efficiency calculated as (\(k_{\text{cat}}/K_m\))-wild-type/(\(k_{\text{cat}}/K_m\))-mutant. The higher the losses of aminoacylation efficiency, the better the discrimination control of the synthetase toward the mutated position in tRNA\(^{\text{Asp}}\).

As shown previously, kinetic constants determined for aspartylation of fully modified tRNA\(^{\text{Asp}}\) are only slightly different from those measured for the corresponding wild-type transcript (25). For truncated AspRS, both \(k_{\text{cat}}\) and \(K_m\) of the aspartylation reaction stay almost the same. With native AspRS, only the \(K_m\) of the modified tRNA\(^{\text{Asp}}\) is lowered ~17 times as compared with the wild-type transcript (Table 1).

With truncated AspRS\(^{\text{AspRS71–557}}\), mutations at single identity positions in tRNA transcripts have strong effects on the global aminoacylation efficiency, which can be reduced up to ~3000-fold. At position 35, replacement of the wild-type U by any other base leads to sharp decreases in aminoacylation efficiency (between 700- and 1400-fold). At the three other major identity positions, 73, 34, and 36, all mutations except one lead to lower decreases of aminoacylation efficiencies with effects fluctuating between 4- and 340-fold depending on the mutation introduced. Noteworthly, the G34 → C exception is the mutation leading to the strongest effect (~3000-fold). In general, comparison of the individual kinetic constants indicates that \(K_m\) is the most affected (exceptions are mutations G73 → A and G34 → C where both \(k_{\text{cat}}\) and \(K_m\) are changed).

The same experiments, done with native AspRS\(^{\text{AspRS1–557}}\) under identical conditions, gave significantly different results. Although as expected, mutations of tRNA\(^{\text{Asp}}\) identity positions still cause decreases in aspartylation efficiency, their effects are quantitatively and qualitatively changed. Indeed, with the native synthetase, the losses of aminoacylation efficiency reach at most ~500-fold (as compared with the ~3000-fold decrease for the strongest loss with AspRS\(^{\text{AspRS71–557}}\)), and the involved positions are different (e.g. strongest effect for U35 → G with native AspRS\(^{\text{AspRS1–557}}\) and for G34 → C with truncated AspRS\(^{\text{AspRS71–557}}\)). For 8 out of 12 mutants, losses in aminoacylation efficiency varies between 1- and 22-fold, and only four tRNA\(^{\text{Asp}}\) mutants are poorly aminoacylated, with losses in aminoacalylation efficiencies above 80. They correspond to transcripts containing mutations G73 → C, G34 → C, or U35 → A or G. Here again, affinity (reflected by \(K_m\)) between native AspRS\(^{\text{AspRS1–557}}\) and the tRNA substrate is mostly affected, whereas \(k_{\text{cat}}\) is only moderately affected.
Extension of Yeast AspRS Favors tRNA Mischarging

Aminoacylation efficiencies \((k_{\text{cat}}/K_m)\) were calculated for each mutated tRNA transcript aminoaacylated with the truncated or the native AspRS. Tabulated \(k_{\text{cat}}\) and \((k_{\text{cat}}/K_m)\) values have to be multiplied by \(10^{-3}\), the effect of the extension corresponds to the gain in aminoaacylation efficiency acquired by native AspRS consequtive to the presence of the N-terminal extension. It is calculated for each tRNA variant as the ratio of the relative aminoaacylation efficiencies determined with native and truncated AspRSs \([k_{\text{cat}}/K_m]^\text{native AspRS}/k_{\text{cat}}/K_m^\text{truncated AspRS}\]. Errors on calculated \((k_{\text{cat}}/K_m)\) values are estimated to be 30%. Numerical values in bold are the controls for the wild-type tRNA transcript; wild-type anticodon residues are in tabulated.

These kinetic differences observed in aspartylation reactions conducted with native or truncated AspRSs have dramatic consequences. To evaluate these consequences, the gain in aminoaacylation efficiency consecutive to the presence of the N-terminal extension in AspRS was calculated for each tRNA variant, comparing their aminoaacylation efficiencies between both native and truncated AspRSs. Whatever the tRNA substrates, wild-type or mutants, the aminoaacylation efficiencies are strongly stimulated when the N-terminal extension is present in AspRS. The strengths of these positive effects, however, are dependent on the mutation introduced in the tRNA. As shown in Table I, these values comprise between 3 and 171. Indeed, the N-terminal appendix triggers only a moderate increase of the aminoaacylation efficiency of tRNA\(^{\text{Asp}}\) transcripts mutated at the discriminator position (at most 11-fold for the G73 \(\rightarrow\) U mutation). In contrast, its effect is increased at least by one order of magnitude when the mutations are introduced in the anticodon (up to \(-170\)-fold for the U35 \(\rightarrow\) C mutation).

Altogether, this means that native AspRS\(^{1-557}\) is less discriminative than truncated AspRS\(^{71-557}\). This property of native AspRS\(^{1-557}\), a priori unexpected, is solely due to the presence of the N-terminal extension. This finding shows that the role of the extension is not marginal and has functional consequences.

**Effects of Degradations in AspRS Extension on tRNA Charging**—Generally, the data in Table I confirm the mutational analysis on tRNA aspartylation identity mutants by truncated AspRS\(^{71-557}\) and native AspRS\(^{1-557}\) and effect of the N-terminal extension on specificity.

### Table I

Comparison of kinetic parameters for aminoaacylation of tRNA\(^{\text{Asp}}\) identity mutants by truncated AspRS\(^{71-557}\) and native AspRS\(^{1-557}\)

| tRNA\(^{\text{Asp}}\) variants | Truncated AspRS\(^{71-557}\) | Native AspRS\(^{1-557}\) |
| --- | --- | --- |
| | \(K_m\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_m\) | Plateaus | \(K_m\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_m\) | Plateaus | \(\%\) Effect of Ext.\(^1-70\) |
| Native tRNA\(^{\text{Asp}}\) | 0.75 | 250 | 330 | 100 | 0.0075 | 250 | 33000 | 100 | 100 |
| tRNA\(^{\text{Asp}}\) transcript | 0.83 | 300 | 571 | 78 | 0.13 | 300 | 208 | 92 | 122 |
| G73 | 2.0 | 300 | 150 | 71 | 0.1 | 148 | 1480 | 79 | 4 |
| U | 2.7 | 72 | 27 | 59 | 0.2 | 50 | 250 | 79 | 9 |
| C | 12.1 | 50 | 4.1 | 33 | 6.2 | 78 | 35 | 35 | 3 |
| G34 | 20.0 | 80 | 4.0 | 2 | 1.1 | 220 | 200 | 83 | 50 |
| A | 21.0 | 56 | 2.7 | 2 | 1.0 | 136 | 136 | 84 | 50 |
| C | 37.0 | 6 | 0.2 | 5 | 3.3 | 38 | 11 | 49 | 55 |
| U35 | 36.5 | 28 | 0.8 | 18 | 1.2 | 164 | 137 | 83 | 171 |
| A | 28.0 | 18 | 0.6 | 6 | 3.4 | 92 | 27 | 50 | 45 |
| C | 32.0 | 14 | 0.4 | 8 | 12.7 | 56 | 4.4 | 33 | 11 |
| C36 | 15.8 | 184 | 12 | 54 | 2.2 | 380 | 172 | 75 | 14 |
| U | 13.4 | 54 | 4.0 | 25 | 1.2 | 126 | 105 | 54 | 26 |
| A | 35.7 | 60 | 1.7 | 30 | 0.7 | 146 | 208 | 92 | 122 |

*Effect of Ext.\(^1-70\)"
aminoacylation assays. However, the raise is much more pronounced in assays conducted with native AspRS\textsubscript{1–557} (Fig. 3). Thus, when present at a 5-fold molar excess as compared with tRNA\textsubscript{Asp}, the amount of aspartylated tRNA catalyzed by native AspRS\textsubscript{1–557} is more than 2-fold of that of tRNA\textsubscript{Asp}, whereas with truncated AspRS\textsubscript{71–557}, it reaches only 1.5-fold. This indicates that AspRS aspartylates noncognate tRNA(s) and that the level of mischarging is significantly higher with native AspRS\textsubscript{1–557}. In control experiments conducted in the presence of limiting aspartate concentration (25 M), the mischarging potential of AspRS, even in its native version, is suppressed (Fig. 3).

**Identification of Aspartylated Noncognate tRNAs**—At first, to prevent false interpretation of results, care was taken that AspRS preparations as well as amino acids were of enough purity to avoid possible misaminoacylation catalyzed by trace contaminations of *E. coli* synthetases (see “Experimental Procedures”). The mischarged tRNA species were identified in a three-step procedure. First, total tRNA was aspartylated by either native AspRS\textsubscript{1–557} or truncated AspRS\textsubscript{71–557} in assays conducted with 500 M aspartate, i.e. under conditions where the specific activity of both AspRSs is the highest. Further, the AspRS concentration was in 5-fold molar excess as compared with the amount of tRNA\textsuperscript{Asp} present in total yeast tRNA. Second, the pool of aspartylated tRNA (comprising tRNA\textsuperscript{Asp} and putative mischarged noncognate tRNAs) was periodate-treated to inactivate all uncharged tRNAs by oxidation of their 3′-terminal ribose. This treatment does not affect the aspartylated species protected by the aspartate residue esterified on their 3′-end. Before treatment, care was taken to protect tRNAs containing thiolated bases against inactivation by periodate (see “Experimental Procedures”), i.e. yeast tRNA\textsubscript{Glu}, a potential candidate for mischarging, which contains a 5-methoxycarbonylmethyl-2-thiouridine (mcm\textsuperscript{s}U) residue in its anticodon (30). Third, the periodate-treated tRNA, still containing the pool of aspartylated tRNA species, was decylated, and formerly mischarged species were identified by their original amino acid acceptance (see “Experimental Procedures”).

Aminoacylation of total yeast tRNA with both truncated AspRS\textsubscript{71–557} or native AspRS\textsubscript{1–557} leads to quantitatively different, although qualitatively comparable, results. As seen in Table II, besides tRNA\textsuperscript{Asp}, three families of noncognate aspartylatable tRNA species can be identified. The first one comprises, as anticipated, tRNA\textsubscript{Asn} and tRNAGlu and corresponds to molecules abundantly aminoacylated. The second family, with tRNA\textsubscript{His}, tRNA\textsubscript{Lys}, and tRNA\textsubscript{Arg}, comprises molecules moderately aminoacylated. The third family encompasses molecules that were not mischarged, or if so, mischarged to trace levels.
With truncated AspRS<sub>71–557</sub>, the amount of aspartylated tRNA was only slightly above the predicted level of tRNA<sup>Asp</sup> charging (6.25% versus 4.5%). As expected, tRNA<sup>Asp</sup> was the major species identified, representing 40% of the charged tRNAs. The 60% tRNAs left were mischarged species. Surprisingly, tRNA<sup>Glu</sup> and tRNA<sup>Asn</sup> were mischarged to comparable levels representing each as much as half of the charged tRNA<sup>Asp</sup>. The minor species correspond to tRNAs mischarged to plateau levels comprising between 1 and 8%.

With native AspRS<sub>1–557</sub>, as a consequence of the presence of the N-terminal extension, ~50% more tRNA was aspartylated (Table II). The same tRNA species were identified in the aspartylated pool, but they were charged to a higher level. Altogether, aspartylation by the synthetase comprising the extension reaches 74% of the amount of tRNA<sup>Asp</sup> present in the bulk yeast tRNA. At a low AspRS<sub>1–557</sub> concentration, only tRNA<sup>Asp</sup> was charged, and excessive mischarging was detected with plateaus reaching 75% of tRNA<sup>Glu</sup> and 30% of tRNA<sup>Asn</sup> aspartylated.

| tRNA species | % Aspartylation of cognate & noncognate tRNAs | % Aspartylation of cognate & noncognate tRNAs |
|--------------|---------------------------------------------|---------------------------------------------|
| tRNA<sup>Asp</sup> | 2.45% | 57.0% |
| tRNA<sup>Glu</sup> | 3.80% | 6.58% |
| tRNA<sup>Asn</sup> | 3.20% | 5.40% |
| Minor mischarging | 0.44% | 0.67% |
| Trace levels of mischarging | 0.16% | 0.51% |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Ala</sup> | 0.1% | ND |
| tRNA<sup>Phe</sup> | 0.1% | ND |
| tRNA<sup>Val</sup> | 0.1% | ND |
| tRNA<sup>Arg</sup> | 0.1% | ND |
| tRNA<sup>Met</sup> | 0.1% | ND |
| tRNA<sup>Ser</sup> | 0.1% | ND |
| tRNA<sup>Lys</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Thr</sup> | 0.1% | ND |
| tRNA<sup>Ile</sup> | 0.1% | ND |
| tRNA<sup>Gln</sup> | 0.1% | ND |
| tRNA<sup>Leu</sup> | 0.1% | ND |
| tRNA<sup>Cys</sup> | 0.1% | ND |
| tRNA<sup>His</sup> | 0.1% | ND |
| tRNA<sup>Trp</sup> | 0.1% | ND |
| tRNA<sup>Trypt</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Lys</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
| tRNA<sup>Asp</sup> | 0.1% | ND |
| tRNA<sup>Tyr</sup> | 0.1% | ND |
| tRNA<sup>Pro</sup> | 0.1% | ND |
| tRNA<sup>Glu</sup> | 0.1% | ND |
| tRNA<sup>Asn</sup> | 0.1% | ND |
tRNA misacylations. Different mechanisms have been considered to overcome these errors. Editing is an option for synthetases having low specificity toward their cognate amino acid, as E. coli IleRS (34) and ValRS (35) and its structural foundation explicitly established for prokaryotic IleRS (36) and ThrRS (37). Correction or prevention of tRNA mischarging due to misrecognition of tRNA is less documented. The presence of antideterminants in tRNAs, responsible for blocking noncognate aminoacylation, has been studied. For example, methylation of G37 in yeast tRNA<sup>Asp</sup> blocks arginylation (38).

Here, in both tRNA<sup>Glu</sup> and tRNA<sup>Asn</sup>, base modifications could influence negatively their recognition by AspRS, but they are not sufficient to repress mischarging with aspartate in vitro. In fact, the biological consequences of this kind of mischarging was considered as marginal because it is further repressed in the presence of the cognate tRNA (reviewed in Ref. 39), and this view is strengthened by the observation that in vivo competitions between different synthetases lower the risk of mischarging (40). However, this study demonstrates that yeast AspRS is able to aspartylate in vitro noncognate tRNAs to unexpectedly high levels, even in total tRNA samples containing cognate tRNA<sup>Asp</sup>. This property is considerably enhanced when the N-terminal extension of the enzyme is present. High mischarging relies on the inherent low specificity of yeast AspRS for its tRNA<sup>Asp</sup> substrate, as shown by the weak catalytic discrimination toward tRNA<sup>Asp</sup> molecules mutated at identity positions. This low discrimination is mainly due to the rather high affinities the mutated or noncognate tRNAs retain for the native AspRS. From these considerations, it can be expected that tRNAs favorably mischarged are those having sequence elements closely related to the tRNA<sup>Asp</sup> identity set.

This is actually the case since the aspartate identity set is undetectable if these concentrations are low enough.

In this work, aspartylation of total yeast tRNA and of pure noncognate tRNA species were conducted under an aspartate difference found in one anticodon position.

This latent mischarging of AspRS, enhanced by the nonspecific tRNA binding properties of its N-terminal extension, represents a vital threat for the yeast cell. Thus, one can question whether elongator factor would discriminate efficiently enough the mischarged tRNAs (45, 46) and protect the cell against their toxicity. Another way to lower the risk of errors during translation would be to reduce the intracellular concentrations of AspRS, thereby decreasing mischarging possibilities. It is remarkable that traces of this potential mischarging ability remain present in organisms other than yeast. Indeed, an evolutionary proximity between tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> has been explicitly demonstrated in some microorganisms, with AspRS mischarging tRNA<sup>Glu</sup> and Asp-tRNA<sup>Glu</sup> being the precursor for the synthesis of Asn-tRNA<sup>Glu</sup> (47, 48). It is also interesting to note that maybe evolution answered to the menace of tRNA<sup>Glu</sup> mischarging by replacing the nature of its discriminator base in higher eukaryotes (G73 in prokaryotes and unicellular eukaryotes changed to A in higher eukaryotes, see Ref. 49).

**Acknowledgments**—We thank Gilbert Eriani, Catherine Florentz, and Daniel Kern for advice and stimulating discussions and Gérard Keith, Caroline Paulus, and Anne Théobald-Dietrich for preparation of biological material.

**REFERENCES**

1. Ibba, M., and Soll, D. (2000) *Annu. Rev. Biochem.* 69, 617–650
2. Ebel, J.-P., Giegé, R., Bonnet, J., Kern, D., Befort, N., Bollack, C., Fasiolo, F., Gangloff, J., and Dirheimer, G. (1973) *Biochimie* (Paris) 55, 547–557
3. Ibba, M., and Soll, D. (1999) *Science* 286, 1883–1887
4. Deinert, K., Fasiolo, F., Hurt, E. C., and Simon, G. (2001) *J. Biol. Chem.* 276, 6000–6008
5. Park, S. G., Jung, K. H., Lee, J. S., Jo, Y. J., Motegi, H., Kim, S., and Shiba, K. (1996) *J. Biol. Chem.* 271, 16773–16776
6. Mirande, M. (1991) *Prog. Nucleic Acid Res. Mol. Biol.* 40, 95–142
7. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1989) *Nature* 347, 203–206
8. Whelihan, E. F., and Schimmel, P. (1997) *EMBO J.* 16, 2968–2974
9. Wang, C.-C., Morales, A. J., and Schimmel, P. (2000) *J. Biol. Chem.* 275, 17180–17186
10. Frugier, M., Moulinier, L., and Giegé, R. (2000) *EMBO J.* 19, 2371–2380
11. Lorber, B., Mejdoub, H., Reinholt, J., Boulanger, Y., and Giegé, R. (1988) *Eur. J. Biochem.* 174, 155–161
12. Agou, F., Yang, Y., Gesquière, J.-C., Waller, J.-P., and Guittet, E. (1995) *Biochemistry* 34, 569–576
13. Franccin, M., Kaminosa, M., Kerjan, P., and Mirande, M. (2002) *J. Biol. Chem.* 277, 1762–1769
14. Eriani, G., Prevost, G., Kern, D., Vincendon, P., Dirheimer, G., and Gangloff, J. (1991) *Eur. J. Biochem.* 200, 337–343
15. Boff, M., Krishnaswamy, S., Jessel, M., Petersson, A., Mitechler, A., Podjarny, A., Rees, B., Thierry, J. C., and Moras, D. (1991) *Science* 252, 1682–1689
16. Sluster, C., Lorber, B., Cavarelli, J., Moras, D., and Giegé, R. (2000) *J. Mol. Biol.* 299, 1313–1324
17. Pütz, J., Puglisi, J. D., Florentz, C., and Giegé, R. (1991) *Science* 252, 1698–1699
18. Pikiet, M., Soll, D., Giegé, R., and Florentz, C. (1994) *Biochemistry* 33, 9912–9921
19. Westhof, E., Dumas, P., and Moras, D. (1985) *J. Mol. Biol.* 184, 119–145
20. Cavarelli, J., Rees, B., Ruff, M., Thierry, J.-C., and Moras, D. (1995) *Nature* 372, 181–184
21. Romby, P., Moras, D., Bergdoll, M., Dumas, P., Vlaisov, V. V., Westhof, E., and Moras, D. (1985) *J. Mol. Biol.* 184, 455–471
22. Rudinger, J., Puglisi, J. D., Pütz, J., Schatz, D., Eckstein, F., Florentz, C., and Giegé, R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 1882–1886
23. Lorber, B., Kern, D., Dietrich, A., Gangloff, J., Jelb, J.-P., and Giegé, R. (1983) *Biochem. Biophys. Res. Comm.* 117, 259–267
24. Messenguy, F., Colin, D., and Ten Have, J.-P. (1980) *Eur. J. Biochem.* 108, 439–447
25. Perret, V., García, A., Puglisi, J. D., Grosjean, H., Ebel, J.-P., Florentz, C., and Giegé, R. (1990) *Biochimie* (Paris) 72, 735–744
26. Houssier, C., Depe, P., Nicoghosian, K., and Grosjean, H. (1988) *J. Biol. Chem.* 263, 1259–1268
27. Hansske, F., and Cramer, F. (1979) *Eur. J. Biochem.* 96, 595–600
28. Giege, R., Sissler, M., and Florentz, C. (1998) *FEBS Lett.* 437, 447–451
29. Kobayashi, T., Irie, T., Yoshida, M., Takeuchi, K., and Ukiti, T. (1974) *Biochem. Biophys. Acta* 317, 265–279
30. Kern, D., and Lapointe, J. (1979) *Biochemistry* 18, 9847–9851
31. Kern, D., Roy, H., and Becker, H. D. (2003) *Biochemistry* 42, 9851–9855
32. Lwoff, R. B., and Vanderjagt, M. A. (1972) *Biochem. J.* 128, 1353–1356
33. Eldred, E. W., and Schimmel, P. (1972) *Biochemistry* 11, 17–23
35. Fersht, A. R., and Kaethner, M. M. (1976) *Biochemistry* **15**, 3342–3346
36. Nureki, O., Vassylyev, D. G., Tateno, M., Shimida, A., Nakama, T., Fukai, S., Kono, M., Hendrickson, T., Schimmel, P., and Yokoyama, S. (1998) *Science* **280**, 578–582
37. Sankaranarayanan, R., Dock-Bregeon, A.-C., Rees, B., Bovee, M., Caillet, J., Romby, P., Francklyn, C. S., and Moras, D. (2000) *Nat. Struct. Biol.* **7**, 461–465
38. Putz, J., Florentz, C., Benseler, F., and Giege, R. (1994) *Nat. Struct. Biol.* **1**, 580–582
39. Giege, R., Puglisi, J. D., and Florentz, C. (1993) *Prog. Nucleic Acid Res. Mol. Biol.* **45**, 129–206
40. Sherman, J. M., Rogers, M. J., and Soll, D. (1992) *Nucleic Acids Res.* **20**, 2847–2852
41. Eriani, G., and Gangloff, J. (1999) *J. Mol. Biol.* **291**, 761–773
42. Bonnet, J., and Ebel, J.-P. (1972) *Eur. J. Biochem.* **31**, 335–344
43. Dietrich, A., Kern, D., Bonnet, J., Giege, R., and Ebel, J.-P. (1976) *Eur. J. Biochem.* **70**, 147–158
44. Smith, W. E., and McNamara, A. L. (1972) *Biochim. Biophys. Acta* **269**, 67–77
45. LaRiviere, P. J., Wolfson, A. D., and Uhlenbeck, O. C. (2001) *Science* **294**, 165–168
46. Asahara, H., and Uhlenbeck, O. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3499–3504
47. Becker, H. D., and Kern, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12832–12837
48. Raccah, G., Becker, H. D., Min, B., and Soll, D. (2001) *J. Biol. Chem.* **276**, 45862–45867
49. Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A., and Steinberg, S. (1998) *Nucleic Acids Res.* **26**, 148–151
