The proofreading of hydroxy analogues of leucine and isoleucine by leucyl-tRNA synthetases from *E. coli* and yeast

Sabine Englisch, Uwe Englisch, Friedrich von der Haar* and Friedrich Cramer

Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen, FRG

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ABSTRACT

Three analogues each of leucine and isoleucine carrying hydroxy groups in Y- or δ- or γ- and δ-position have been synthesized, and tested in the aminoacylation by leucyl-tRNA synthetases from *E. coli* and yeast. Hydrolytic proofreading, as proposed in the chemical proofreading model, of these analogues and of homocysteine should result in a lactonisation of these compounds and therefore provide information regarding the proofreading mechanism of the two leucyl-tRNA synthetases.

Leucyl-tRNA synthetase from *E. coli* shows a high initial substrate discrimination. Only two analogues, Y-hydroxyleucine and homocysteine are activated and transferred to tRNA where a post-transfer proofreading occurs. Lactonisation of Y-hydroxyleucine and homocysteine could be detected.

Leucyl-tRNA synthetase from yeast has a relatively poor initial discrimination of these substrates, which is compensated by a very effective pre-transfer proofreading on the aminoacyl-adenylate level. No lactonisation nor mischarged tRNA is detectable.

INTRODUCTION

The selection of amino acids during protein biosynthesis has to be extremely accurate in order to maintain the overall accuracy of ~3 mistakes in 10^4 amino acids incorporated (1-4). The selection of the correct amino acid takes place during the aminoacylation of tRNAs by the aminoacyl-tRNA synthetases (5,6). The aminoacylation is a two-step reaction, divided in activation of the amino acid by ATP to form an aminoacyl-adenylate (assayed by the reverse reaction, the ATP/PP_i pyrophosphate exchange) and in transfer of the aminoacyl residue to the corresponding tRNA (assayed by incorporation) of labelled amino acid.

\[ E^{aa} + aa + ATP \rightarrow E^{aa}\cdot aa\cdot AMP \rightarrow E^{aa} \rightarrow aa\cdot tRNA^{aa} + AMP \]

In some cases like histidine (7), tryptophane (8) or cysteine (9) the selection is easy since the amino acid is so different from its competitors that the initial discrimination of substrates is better than ~1 x 10^4. In other cases such as the rejection of valine by the isoleucyl-tRNA synthetase (10-
14) or of tyrosine by the phenylalanyl-tRNA synthetase (15,16) the differences in binding energy between the incorrect and the correct substrate are too low for sufficiently accurate selection. The accuracy of aminoacylation can only be maintained by proofreading mechanisms acting either at the level of the aminoacyl-adenylate (3,4,17,18), called pre-transfer proofreading, or at the level of the misaminoacylated tRNA (3,4,19-21), called post-transfer proofreading. These phenomena are subject of several models (4,18). The "chemical proofreading" model (16,20), based on the misaminoacylation of yeast tRNA\(_{\text{Ile}}\) -C-C-3'dA by valine, postulated that the nonaccepting hydroxy group of the terminal ribose of tRNA serves as a general base for the "activation" of the hydrolysing water molecule (Fig. 1); this hydroxy group, in turn, may be activated by a nucleophilic protein residue similar as in the serine hydrolases. In the case of misaminoacylation of threonine by the valyl-tRNA synthetase from yeast (22) this 3'hydroxy group of tRNA\(_{\text{Val}}\) is not necessary for the hydrolysis (Fig. 1); the water molecule might be activated directly by the hydroxy group of the threonine.

In the present study we examine the activation, transfer and proofreading of six different leucine and isoleucine hydroxy analogues and homocysteine by the leucyl-tRNA synthetases from baker's yeast and E. coli. The approach is based on the idea of an "activated" water molecule in the chemical proofreading model which could be simulated by the hydroxy and thiol groups of the amino acid analogues and their ability to form stable lactones which can easily be detected; lactone formation from homocysteine by two other synthetases has already been reported (23).

**EXPERIMENTAL PROCEDURES**

The preparation of the amino acid analogues is described elsewhere (24).  
\([^{14}\text{C}]\)Leucine of specific activity 336 mCi/mmol, [\(^{14}\text{C}\)]ATP of specific activity 55 mCi/mmol, [\(^{32}\text{P}\)]pyrophosphate of specific activity 11.3 mCi/mmol were purchased from Amersham International (Amersham, England). Homocysteine was purchased from Merck (Darmstadt, FRG). All buffer substances were of ultrapure grade.

Yeast tRNA\(_{\text{Leu}}\) was isolated by the procedure of Schneider et al. (25) from unfractionated baker's yeast tRNA (Boehringer, Mannheim, FRG) to a leucine acceptance of 1420 pmol/A\(_{260}\) unit. E. coli tRNA\(_{\text{Leu}}\) was purified from unfractionated E. coli tRNA (Boehringer, Mannheim, FRG) to a leucine acceptance of 500 pmol/A\(_{260}\) unit by the procedure of Nishimura et al. (26) and then applied to HPLC using derivatized reverse-phase material (27) re-
suiting in a leucine acceptance of 1340 pmol/A_260 unit. Leucyl-tRNA synthetase from baker's yeast was purified to homogeneity according to the procedure of Kern et al. (28) to a specific activity of 1340 units/mg. The enzyme from E. coli was isolated using DEAE Cl-6B-chromatography, salting out elution from Sepharose 4B, and chromatography on hydroxylapatite to a final specific activity of 2580 units/mg.

The radioactivity was counted in a Berthold betaizint BF 8000 liquid scintillation counter, HPLC was carried out with a DuPont 850 liquid chromograph, and UV measurements were done in a Zeiss PMQ3 spectrometer.

**ATP/PP_4 Pyrophosphate Exchange**

In a total volume of 100 µl were incubated at 37°C 2 mM [³²P]pyrophosphate, 0.5 - 20 mM amino acid, 2 mM ATP, 1 mg/ml unfractionated tRNA from yeast or E. coli in 150 mM Tris/HCl pH 8.0, containing 150 mM KCl, 10 mM MgSO_4 and 5 mM β-mercaptoethanol. The reaction was started by addition of 5 µg of leucyl-tRNA synthetase. Aliquots of 10 µl were taken after 2, 4, 6, 8, 10, 20, 30, 40, 60 min, and spotted onto charcoal filter discs. The discs were washed twice 10 min in 1.5% trichloroacetic acid containing 40 mM unlabelled pyrophosphate, once 5 min in water and were then dried (29,30) and the radioactivity was counted.

**AMP formation**

In 100 µl total volume were incubated at 37°C either 2 mM [¹⁴C]ATP, or 2 mM [¹⁴C]ATP plus 0.5 - 5 mM amino acid, or 2 mM [¹⁴C]ATP plus 0.5 - 5 mM amino acid plus 30 µM tRNA^Leu_ in 150 mM Tris/HCl pH 8.0 containing 150 mM KCl, 10 mM MgSO_4 and 5 mM β-mercaptoethanol. The reaction was started by addition of 6 µg enzyme. Within 1 - 120 min 1 µl aliquots were withdrawn, spotted onto PEI Cellulose plates, fluorescing at 254 nm. Ascending chromatography was carried out in 1 M LiCl + 1 mM acetic acid/isopropanol 2:1 (v/v) with ATP, ADP and AMP as reference substances (20,30). The nucleotide spots were identified with uv, cut out and the radioactivity was counted.

**Aminoacylation**

In 100 µl total volume were incubated at 37°C 30 µM [¹⁴C]amino acid, 10 mM ATP, 1 mg/ml unfractionated tRNA from yeast or E. coli in 150 mM Tris/HCl pH 8.0, containing 150 mM KCl, 10 mM MgSO_4, and 5 mM β-mercaptoethanol. The reaction was started by addition of 5 µg leucyl-tRNA synthetase (16,30). After 2, 5, 10 min 10 µl aliquots were withdrawn and spotted onto 3 MM filter discs. The discs were washed three times for 10 min with 5% trichloroacetic acid, then 5 min with ethanol and 2 min with ether, then the radioactivity was counted.
Detection of lactones by thin layer chromatography

From aminoacylation assays (started by addition of enzyme) 2-μl- aliquots were taken after 2, 4, 6, 8, 10, 20, 40, 60, 120, 180 min, and spotted onto silica thin layer chromatography plates. The chromatography was run in butanol:acetic acid:water (4:1:1) for 5 hours, then the plates were dried followed by another 5 h chromatography. The spots were visualized by ninhydrin spray reagent (23,31).

Preparative aminoacylation of γ-Hydroxyleu-tRNA\textsubscript{Leu} and Homocys-tRNA\textsubscript{Leu}

In 1 ml total volume were incubated at 37°C 5 mM amino acid, 10 mM ATP and 1 mg tRNA in 150 mM Tris/HCl pH 8.0 containing 150 mM KCl and 10 mM MgSO\textsubscript{4}. The reaction was started by addition of 25 μg enzyme.

After 20 min the reaction was stopped by addition of 200 μl of 2 M sodium acetate, adjusted to pH 5.0, and applied at 4°C to a DEAE A25 column equilibrated at pH 5 with a buffer of 20 mM sodium acetate pH 5.2, containing 10 mM MgSO\textsubscript{4} and 0.3 M NaCl. After washing with 0.1 and 0.5 M NaCl the tRNA was eluted with 1 M NaCl and desalted over a P-2-column (21,32).

Back titration

Back titration is carried out essentially as aminoacylation. With the beginning of the back titration new enzyme is added and [14C]leucine is added in surplus (16).

Enzymatic and spontaneous hydrolysis of aminoacyl-tRNA\textsubscript{Leu}

The reaction was carried out in the aminoacylation medium without or with (6 μg) enzyme. At 10 min intervals aliquots were withdrawn and the content of tRNA\textsubscript{Leu} estimated by back titration (22).

RESULTS

Activation of the amino acid analogues by the leucyl-tRNA synthetases from E. coli and yeast

Among the tested derivatives (Fig. 1) only the γ,δ-dihydroxy analogues of leucine and isoleucine are not activated by either one of the leucyl-tRNA synthetases, probably because they are too voluminous. The other analogues, however, although they are bulkier than leucine are activated. The γ- and δ-derivatives of leucine and isoleucine exhibit $K_m$ values of ~100fold of leucine; the isoleucine derivatives having higher $K_m$ and lower $k_{cat}$ values. The most surprising result is the activation of the hydroxyisoleucine analogues by the yeast enzyme because, beside the additional OH group, the pattern of methyl groups is changed as well. The substrate properties of the
Fig. 1. Above: Schematic display (left) of valine esterified to tRNA\textsuperscript{Ile} and of the proofreading hydrolysis mechanism proposed by von der Haar and Cramer (20,36), and (middle) of threonine esterified to tRNA\textsuperscript{Val} exhibiting a hydrolytic proofreading without participation of the ribose 3' OH group (22), and (right) of \(\gamma\)-hydroxyleucine esterified to tRNA\textsuperscript{Leu} and of the mechanism possibly explaining lactone formation (\(-\text{BI}\) design basic residue of the enzyme).

Below: Structural formulae of the nine amino acids used in this work.

derivatives listed in Table 1 show that the E. coli enzyme has a considerably higher specificity towards the noncognate analogues than the yeast synthetase, only \(\gamma\)-hydroxyleucine and homocysteine are not discriminated.
Table 1: ATP/PP<sub>i</sub> pyrophosphate exchange and AMP formation by leucyl-tRNA synthetases: Kinetic constants using various amino acids and analogues

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>ATP/PP&lt;sub&gt;i&lt;/sub&gt; pyrophosphate exchange</th>
<th>AMP formation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>yeast enzyme</td>
<td>E. coli enzyme</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (mM)</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (sec&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>γ-Hydroxyleucine</td>
<td>2.5&lt;sup&gt;a,b,c&lt;/sup&gt; 3.4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>3.3  2.8</td>
</tr>
<tr>
<td>δ-Hydroxyleucine</td>
<td>2.6  3.6</td>
<td>-</td>
</tr>
<tr>
<td>γ,δ-Dihydroxyleucine</td>
<td>-  -</td>
<td>-</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.02  52</td>
<td>0.09  31</td>
</tr>
<tr>
<td>γ-Hydroxyisoleucine</td>
<td>4.5  1.6</td>
<td>-</td>
</tr>
<tr>
<td>δ-Hydroxyisoleucine</td>
<td>16.7  2.3</td>
<td>-</td>
</tr>
<tr>
<td>γ,δ-Dihydroxyisoleucine</td>
<td>-  -</td>
<td>-</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>3.4  2.2</td>
<td>2.1  6</td>
</tr>
</tbody>
</table>

*Pyrophosphate exchange and AMP formation (-tRNA) have been carried out with slightly different assay conditions (see Materials and Methods). A substance is called a substrate if a reliable pyrophosphate exchange kinetic is measurable for a substance concentration up to 20 mM what is 10 times of the ATP concentration in the assay. This limit is based on the physiological in vivo concentration of other amino acids. A dash means that the substance was not a substrate. The values given are average values from five independent measurements. The accuracy falls within a factor 1.5 of the data shown.
Fig. 2. Above: Formation of homocysteinyl-tRNA$_{\text{Leu}}$ by leucyl-tRNA synthetase from E. coli, measured as level of remaining tRNA$_{\text{Leu}}$ (estimated by back titration with $^{14}$C]leucine).

Above: Hydrolysis by leucyl-tRNA synthetase from E. coli of homocysteinyl$_{\text{Leu}}$ tRNA$_{\text{Leu}}$ (preparatively aminoacylated), measured as level of emerging tRNA$_{\text{Leu}}$ (estimated by back titration with $^{14}$C]leucine).

Below: Decrease of homocysteine (left) and increase of its thiolactone (right) upon aminoacylation of tRNA$_{\text{Leu}}$ with homocysteine by leucyl-tRNA synthetase from E. coli as monitored by thin layer chromatography.

These findings support earlier reports of a lower initial specificity of yeast aminoacyl-tRNA synthetases in comparison to their E. coli counterparts (16,31,33).

AMP formation in absence of tRNA and in aminoacylation of tRNA$_{\text{Leu}}$

In cognate aminoacylation the formation of one aminoacyl-tRNA needs formation of one aminoacyl-adenylate and consumes one ATP. After a misactivation the aminoacyl-adenylate formed (or later on the aminoacyl-tRNA) is destroyed directly (34) or indirectly (17) and the ATP is wasted. Therefore, the hydrolysis of ATP to AMP during enzyme catalysed formation of the aminoacyl-adenylate from ATP and amino acid is characteristic for proofreading events in aminoacylation of tRNAs. There are many examples in the literature where the AMP formation is strongly dependent on tRNA (16,19,21) suggesting that the aminoacyl residue is transferred to tRNA prior to hydrolysis. However,
as noted for the valyl-tRNA synthetases from lupin seeds and E. coli the enzymes catalyse an appreciable AMP formation in the absence of tRNA (31,35). The AMP formation by isoleucyl-tRNA synthetase from E. coli in the presence of cysteine or homocysteine is even greater in the absence of tRNA (31). The influence of tRNA on the AMP formation by leucyl-tRNA synthetase from yeast and E. coli during the activation of the analogues is summarised in Table 1. In both enzyme systems occurs an AMP formation in presence of tRNA, whereas in absence of tRNA only the yeast leucyl-tRNA synthetase hydrolyzes misformed aminoacyl-adenylates. The rate of hydrolysis is not significantly increased by adding the cognate tRNA. This strongly indicates that the proofreading of the yeast enzyme occurs on the aminoacyl-adenylate level independently of tRNA presence, whereas the E. coli enzyme needs tRNA for the hydrolysis of these leucine analogues.

Aminoacylation of tRNA\textsuperscript{Leu} with leucine analogues

The amount of aminoacylation of tRNA\textsuperscript{Leu} with nonradioactive amino acid analogues is followed by back titration of tRNA\textsuperscript{Leu} with \[^{14}\text{C}]\text{leucine. According to the AMP formation experiments no transfer of the analogues to yeast tRNA\textsuperscript{Leu} was detectable using yeast leucyl-tRNA synthetase. In contrary to this with the E. coli system the aminoacylation of only two analogues, homocysteine (Fig. 2) and \(\gamma\)-hydroxyleucine (data not shown) could be detected by back titration. The hydrolysis of the other analogues is either too fast or they are, despite the exclusive AMP formation in the presence of tRNA, proofread on the pretransfer level, however in tRNA-dependent reaction.}

Lactonisation of homocysteinyll-tRNA\textsuperscript{Leu} and \(\gamma\)-hydroxyleucyl-tRNA\textsuperscript{Leu}

Whilst no lactonisation at all could be detected with the yeast leucyl-tRNA synthetase, with the E. coli system in two cases lactone formation occurs: When E. coli leucyl-tRNA synthetase is incubated with homocysteine or \(\gamma\)-hydroxyleucine, ATP and tRNA\textsuperscript{Leu}, the thin layer chromatography clearly shows the increase of thiolactone (Fig. 2) or lactone (not shown) with time until the spot has the intensity as the amino acid. This lactonisation is strongly dependent on the presence of tRNA. Thin layer chromatography of aliquots from the pyrophosphate exchange assays did not show any lactonisation of the analogues.

Preparative aminoacylation of E. coli tRNA\textsuperscript{Leu} with homocysteine and enzymatic hydrolysis of homocysteinyll-tRNA\textsuperscript{Leu}

Misaminoacylated tRNAs slowly hydrolyze spontaneously but can also be hydrolyzed enzymatically in a proofreading event. To trace both processes, preparatively aminoacylated tRNAs have to be subjected to these processes.
Homocysteinyl-tRNA\textsuperscript{Leu} can be prepared enzymatically since the proofreading capacity of E. coli leucyl-tRNA synthetase is not high enough to prevent the release of homocysteinyl-tRNA\textsuperscript{Leu} from the tRNA-enzyme complex. Homocysteinyl-tRNA\textsuperscript{Leu} was isolated by anionic exchange chromatography and HPLC on derivatized reversed phase material. The isolated homocysteinyl-tRNA\textsuperscript{Leu} is enzymatically hydrolysed with a $t_{1/2}$ of 14 minutes whereas spontaneous hydrolysis needs more than 4 hours to reach the same extent (data not shown). This is a strong evidence for the action of a post-transfer enzyme-catalysed proof-reading (20,36).

DISCUSSION

The accuracy of aminoacylation of many tRNAs is only achieved by enzyme catalysed proofreading mechanisms effecting hydrolysis of noncognate products. These mechanisms can take place either on the level of the aminoacyl-adenylate, pre-transfer proofreading (17,18) or on the level of the misaminoacylated tRNA, post-transfer proofreading (19,20). Our examinations show that the leucyl-tRNA synthetases from E. coli and yeast use different ways to maintain the high accuracy of aminoacylation.

The yeast enzyme has a relatively poor initial substrate discrimination, all hydroxy analogues with the exception of $\gamma$,$\delta$-dihydroxyleucine are activated although they are bulkier than the cognate substrate. The AMP formation in the absence of tRNA strongly suggests that pre-transfer hydrolysis is the main proofreading event although the stimulation by tRNA\textsuperscript{Leu} (up to 5-fold) might indicate a sequential combination of pre- and post-transfer proofreading as mentioned by Tsui and Fersht (37) for the rejection of serine by alanyl-tRNA synthetase from E. coli. In this case the post-transfer hydrolysis must be very fast because no misaminoacylated tRNA can be detected using backtitration. The proofreading is effective enough to destroy all activated and transferred analogues before misaminoacylated tRNA is released from the tRNA-enzyme complex.

The E. coli leucyl-tRNA synthetase exhibits a much better initial substrate discrimination regarding the analogues. Only two analogues are activated; AMP formation occurs only in the presence of tRNA favouring the idea of transfer of the aminoacyl residues to tRNA prior to hydrolysis. The fact that aminoacylation, using back titration, is detectable for $\gamma$-hydroxyleucine and homocysteine is evidence for action of a post-transfer mechanism. The post-transfer proofreading capacity of the E. coli enzyme is however not effective enough to destroy all misaminoacylated tRNA molecules, some
are released from the tRNA–enzyme complex.

Similar results are reported for the phenylalanyl-tRNA synthetases from different organisms, where better initial substrate recognition and less elaborate proofreading for the E. coli enzyme were compared to the less specific substrate discrimination and the more efficient proofreading of eukaryotic synthetases (33).

The chemical proofreading model — as established from the yeast isoleucine system — postulates participation of the 3' OH group and an "activated" water molecule for the mechanism of post-transfer hydrolysis (compare Fig. 1 left). The fast enzymatic proofreading of threonine by valyl-tRNA synthetase from yeast, however, is dependent on the 8-hydroxy group of threonine and independent of the terminal 3' hydroxy group of the tRNA (22) whereas threonine analogues like O-methylthreonine are very slowly hydrolysed by the enzyme and only in presence of the intact tRNA terminus (22). The hydroxy analogues used in this study are able to form 5- or 6-membered lactone rings. However, there is no lactone formation by the yeast enzyme detectable whereas the E. coli enzyme exhibits post-transfer hydrolysis of homocysteine and γ-hydroxyeucine and in both cases the corresponding lactones could be detected. The lactonisation is absolutely enzyme dependent and occurs on the level of the misaminoacylated tRNA, different to the pre-transfer hydrolysis of homocysteine by valyl- and methionyl-tRNA synthetases from E. coli (23).

In conclusion, the leucyl-tRNA synthetases from E. coli and yeast act by different ways to achieve the necessary accuracy of protein biosynthesis. The yeast enzyme uses pre-transfer hydrolysis while the E. coli enzyme hydrolyses misaminoacylated tRNA. Homocysteine and γ-hydroxyeucine are hydrolysed to the corresponding lactones. These findings favour the basic idea of the chemical proofreading model as an important part of the overall proofreading process.

*Present address: Braun Melsungen AG, Geschäftsbereich Medizin und Labortechnik, Postfach 110/120, D-3508 Melsungen, FRG

References