Alternative pathways for editing non-cognate amino acids by aminoacyl-tRNA synthetases

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Received 16 May 1981

ABSTRACT

Evidence is presented that the editing mechanisms of aminoacyl-tRNA synthetases operate by two alternative pathways: pre-transfer, by hydrolysis of the non-cognate aminoacyl adenylate; post-transfer, by hydrolysis of the mischarged tRNA. The methionyl-tRNA synthetases from Escherichia coli and Bacillus stearothermophilus and isoleucyl-tRNA synthetase from E. coli, for example, are shown to reject misactivated homocysteine rapidly by the pre-transfer route. A novel feature of this reaction is that homocysteine thiolactone is formed by the facile cyclisation of the homocysteinyl adenylate. Valyl-tRNA synthetases, on the other hand, reject the more readily activated non-cognate amino acids by primarily the post-transfer route. The features governing the choice of pathway are discussed.

INTRODUCTION

An essential factor in the accuracy of protein synthesis is the involvement of enzymic editing or proofreading mechanisms. Certain amino acids, for example, are so similar in structure that the enzymes responsible for their selection, the aminoacyl-tRNA synthetases, have a special hydrolytic activity to remove the frequent products of misactivation (1,2,3). Since the aminoacylation of tRNA is a two step reaction, consisting of activation of the amino acid followed by transfer to tRNA (eqs. 1,2), editing can occur by

\[ E + AA + ATP \rightarrow E.AA-AMP + PP_i \]  
\[ E.AA-AMP + tRNA \rightarrow E + AA-tRNA + AMP \]  

either the hydrolysis of the misformed aminoacyl-adenylate or the aminoacyl-tRNA (2). The first direct demonstration of a specific reaction pathway came from rapid quenching studies on the rejection of threonine by the valyl-tRNA synthetase from Bacillus stearothermophilus: most of the misactivated threonyl adenylate forms Thr-tRNAVal which is rapidly deacylated before it...
dissociates from the enzyme (4). This pathway of post-transfer editing has
been subsequently rigorously demonstrated for the rejection of α-aminobutyrate
by this enzyme and other valyl-tRNA synthetases (5). It has been claimed that
hydrolysis of the mischarged tRNA is the general mechanism for editing errors
of aminoacylation (6,7). However, the evidence for this stems from experi-
ments on tRNAs which had been modified at the site of acceptance and on yields
rather than rates of formation of incorrectly aminoacylated tRNAs and so is
not conclusive (8).

We have separately gathered evidence that in some cases editing may oc-
cur before the transfer to tRNA, at the level of aminoacyl adenylate: (a) in
two examples, notably the rejection of homocysteine by the methionyl-tRNA
synthetase, no transiently misacylated tRNA could be detected by rapid quench-
ing experiments (9,10); (b) the hydrolysis of enzyme-bound non-cognate amino-
acyl adenylates occurs at a significant rate in the absence of tRNA with a
plant valyl-tRNA synthetase (Lupinus luteus) (11,12). In particular, the
hydrolysis of cysteinyl adenylate occurs at 25% of the rate found in the
presence of tRNA, although the threonyl and α-aminobutyryl adenylates hydrol-
yze at only 2 - 2.5% of the tRNA-stimulated rate. We now wish to present
evidence that the rejection of cysteine and homocysteine by the isoleucyl-
tRNA synthetase, and the rejection of homocysteine by the methionyl-tRNA
synthetase occurs primarily at the level of aminoacyl adenylate as does a
substantial fraction of the rejection of cysteine by the valyl-tRNA synthetase
from E. coli. An unusual feature of the rejection of homocysteine by these
enzymes is that the transiently formed homocysteinyl adenylate undergoes the
facile intramolecular cyclization to yield the thiolactone. Evidence is also
presented that the Lupinus luteus valyl-tRNA synthetase rejects α-aminobu-
tyrate and threonine at the level of mischarged tRNA.

EXPERIMENTAL PROCEDURES

Materials. Aminoacyl-tRNA synthetases were obtained as described pre-
viously and were all purified to homogeneity by the accepted criteria (12,13).
Concentrations were determined by active site titration using nitrocellulose
disk filtration (12,13). Unfractionated tRNA from Lupinus luteus was enriched
in tRNAVal to a valine acceptance of 200 pmol/A260 by chromatography on
benzoylated diethylaminoethylcellulose. Unfractionated tRNA from E. coli
and B. stearothermophilus was provided by the Imperial College Pilot Plant.
Radiochemicals were purchased from the Radiochemical Centre, Amersham, U.K.
α-(1-14C)Aminobutyrate was prepared as previously described (5). ATP, yeast
inorganic pyrophosphatase, L-amino acids and other reagents were obtained from Sigma. L-Homocysteine was prepared by the reduction of L-homocystine by a 2-fold molar excess of dithiothreitol for 30 min at 37°C and pH 8.

Methods. All kinetic measurements were performed at 25°C in a standard buffer containing 50 mM Hepes (pH 8.0), 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.2 mM EDTA and 0.2 mg/ml bovine serum albumin unless otherwise stated. ATP-pyrophosphate exchange assays in the presence of 1 mM ATP and 1 mM [³²P]PPi were performed as previously described (12). Amino acid-dependent ATP-pyrophosphatase activities in the presence of 0.1 mM [³H]ATP (216 cpm/pmol), 14 – 33 mM amino acid and 0.12 – 0.25 μM enzyme were assayed by thin layer chromatography on polyethyleneimino-cellulose as described previously (12).

Rapid Quenching Experiments with Lupin Valyl-tRNA Synthetase: Transfer of [¹⁴C]Amino Acid from the Enzyme-Bound [¹⁴C]Aminoacyl Adenylate to tRNA. These experiments were performed at 25°C in a buffer containing 50 mM potassium phosphate buffer (pH 6.8), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 unit/ml inorganic pyrophosphatase using a micro version of the pulsed quenched flow apparatus (14).

(a) Transfer of [¹⁴C]Valine. One syringe of the apparatus contained 0.28 μM enzyme-bound [¹⁴C]Valyl adenylate formed in situ with 0.28 μM lupin valyl-tRNA synthetase, 4.4 μM [¹⁴C]Valine (285 Ci/mol; 1 Ci = 3.7 x 10¹⁰ Becquerels) and 1 mM ATP. The other syringe contained 2.5 μM lupin tRNA Val and 1 mM unlabelled valine.

(b) Transfer of α-[¹⁴C]Aminobutyrate. One syringe contained 0.37 μM of the enzyme-bound α-[¹⁴C]Aminobutyryl adenylate formed in situ with 1.2 μM lupin valyl-tRNA synthetase, 25 μM α-[¹⁴C]Aminobutyrate (57.8 Ci/mol) and 1 mM ATP. The other syringe contained 2.5 μM lupin tRNA Val.

(c) Transfer of [¹⁴C]Threonine. One syringe contained 0.6 μM enzyme-bound [¹⁴C]Threonyl adenylate formed in situ with 1.2 μM lupin valyl-tRNA synthetase, 56 μM [¹⁴C]Threonine (232 Ci/mol) and 1 mM ATP. The other syringe contained 2.5 μM lupin tRNA Val. Aliquots (50 μl) were automatically mixed and quenched with 10% trichloroacetic acid. The concentration of the enzyme-bound adenylate was assayed by nitrocellulose disk filtration throughout the duration of the experiments. The concentration of valyl and α-amino-butyryl adenylates remained constant, while the concentration of threonyl adenylate dropped from 0.6 μM to 0.45 μM during the experiment.

Detection of Homocysteine Thiolactone by Thin Layer Chromatography. The TLC system (cellulose plates from Merck developed in butanol : acetic acid : water 4 : 1 : 1) used for detection of homocysteine thiolactone is the same.
which had been used earlier for determination of aminoacyl adenylates (15).

Reaction mixtures (20 μl) contained 50 mM Hepes (pH 8.0), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1.5 mM ATP, 5 mM homocysteine and 1 - 30 μM aminoacyl-tRNA synthetase. In some experiments, tRNA was added in 1.5 - 20-fold molar excess over enzyme. After 20 - 100 min at 25°, 2-4 μL aliquots were spotted on to the origin line of the cellulose plates. The chromatograms were developed for 1-2 hours at room temperature (16). The spots were visualised both under uv and after spraying with ninhydrin. Homocysteine ($R_f = 0.34$) did not absorb the uv light and stained red with ninhydrin. Homocysteine thiolactone ($R_f = 0.57$) was visible under uv light and gave a yellow colour upon staining with ninhydrin. Similar results were obtained using silica plates.

RESULTS

Activation of Amino Acids by Valyl-, Isoleucyl- and Methionyl-tRNA Synthetases. The types of misactivation catalysed by the valyl- and methionyl-tRNA synthetases listed in Table I have been documented previously (7,8,10,12). The relatively efficient misactivation of cysteine and homocysteine by the isoleucyl-tRNA synthetase is a novel finding. It is not unexpected, however, since this is predicted by the "double sieve" hypothesis (8): the two amino acids are smaller than the natural substrate and must bind to some extent at the active site of the enzyme. The valyl-tRNA synthetase from \textit{E. coli} has specificities towards the non-cognate amino acids very similar to those found for the lupin enzyme (12) but considerably higher than those reported for the yeast enzyme (7). Whether this reflects an inherently lower specificity of the yeast enzyme or the use of impure preparations of the amino acids (as previously shown for the misactivation of isoleucine (8)) has yet to be determined.

ATP-pyrophosphatase Activity - Rejection of Non-cognate Amino Acids by Editing. Editing of errors of misactivation is characterized by an amino acid-dependent enzyme-catalysed hydrolysis of ATP to AMP as the aminoacyl adenylate is continually formed from ATP and then directly, or indirectly, destroyed. Generally the reaction is strongly dependent on tRNA, being very slow in its absence or in the presence of periodate-oxidised tRNA which cannot accept amino acids (2,4,7,9,12), suggesting that the amino acid is transferred to tRNA prior to hydrolysis. However, as noted previously for the lupin enzyme (12), the valyl-tRNA synthetase from \textit{E. coli} catalyzes an appreciable cysteine-stimulated ATP-pyrophosphatase reaction in the absence of tRNA (Table I). More significantly, the ATP-pyrophosphatase activity of the iso-
Table 1: Activation and Rejection of Amino Acids by Aminoacyl-tRNA Synthetases

<table>
<thead>
<tr>
<th>Aminoacyl-tRNA Synthetase and Source</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Valyl- (Eco)</td>
<td>Val</td>
</tr>
<tr>
<td></td>
<td>Thr</td>
</tr>
<tr>
<td></td>
<td>Cys</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>HCys</td>
</tr>
<tr>
<td>Isoleucyl- (Eco)</td>
<td>Ile</td>
</tr>
<tr>
<td></td>
<td>Val</td>
</tr>
<tr>
<td></td>
<td>Cys</td>
</tr>
<tr>
<td></td>
<td>HCys</td>
</tr>
<tr>
<td>Methionyl- (Eco)</td>
<td>Met</td>
</tr>
<tr>
<td></td>
<td>HCys</td>
</tr>
<tr>
<td>Methionyl- (Bst)</td>
<td>Met</td>
</tr>
<tr>
<td></td>
<td>HCys</td>
</tr>
<tr>
<td>Notes:</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( K_M ) (mM)</th>
<th>Relative specificity</th>
<th>( k_{cat} ) (s(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>Val</td>
<td>46</td>
<td>0.066</td>
<td>1</td>
<td>0.006 (5.4)</td>
</tr>
<tr>
<td>Thr</td>
<td>16</td>
<td>6.2</td>
<td>4 x 10(^{-3})</td>
<td>0.1 (2.9)</td>
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<tr>
<td>Cys</td>
<td>14</td>
<td>20</td>
<td>1 x 10(^{-3})</td>
<td>0.36 (1.5)</td>
</tr>
<tr>
<td>Ala</td>
<td>2.1</td>
<td>27</td>
<td>1 x 10(^{-4})</td>
<td>0.14 (0.8)</td>
</tr>
<tr>
<td>Ser</td>
<td>2.6</td>
<td>25</td>
<td>1.5 x 10(^{-4})</td>
<td>-</td>
</tr>
<tr>
<td>HCys</td>
<td>1.6</td>
<td>10</td>
<td>2 x 10(^{-4})</td>
<td>-</td>
</tr>
<tr>
<td>Ile</td>
<td>18</td>
<td>0.005</td>
<td>1</td>
<td>0.002 (0.6)</td>
</tr>
<tr>
<td>Val</td>
<td>10</td>
<td>0.4</td>
<td>7 x 10(^{-3})</td>
<td>0.05 (0.6)</td>
</tr>
<tr>
<td>Cys</td>
<td>6</td>
<td>6.3</td>
<td>3 x 10(^{-4})</td>
<td>2.0 (1.4)</td>
</tr>
<tr>
<td>HCys</td>
<td>14</td>
<td>1.5</td>
<td>2.5 x 10(^{-3})</td>
<td>1.5 (1.1)</td>
</tr>
<tr>
<td>Met</td>
<td>93</td>
<td>0.03</td>
<td>1</td>
<td>0.003 (3.2)</td>
</tr>
<tr>
<td>HCys</td>
<td>87</td>
<td>5.2</td>
<td>5.4 x 10(^{-3})</td>
<td>1.2 (2.4)</td>
</tr>
<tr>
<td>Met</td>
<td>10</td>
<td>0.05</td>
<td>1</td>
<td>0.03 (1.4)</td>
</tr>
<tr>
<td>HCys</td>
<td>4</td>
<td>1.8</td>
<td>1 x 10(^{-2})</td>
<td>0.53 (0.34)</td>
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</tbody>
</table>

Notes:  

\( ^a \) pH 8.0, 25\(^\circ\), standard buffer.  
\( ^b \) ATP-PP\(_i\) exchange in presence of 1 mM ATP and 1 mM PP\(_i\).  
\( ^c \) ATP-pyrophosphatase activity in the presence of 0.1 mM \(^3\)H\(\text{ATP}, 14-30 \text{ mM amino acid and } 3.3 \mu \text{m inorganic pyrophosphatase.} \)  
\( ^d \) Relative values of \( k_{cat}/K_M \).  
\( ^e \) ATP consumption during the aminoacylation of tRNA.

Leucyl-tRNA synthetase in the presence of cysteine and homocysteine is greater in the absence of tRNA than in its presence. This strongly suggests that the editing of the misactivation of cysteine and homocysteine occurs by hydrolysis of the aminoacyl adenylates. The same appears true for the rejection of homocysteine by the methionyl-tRNA synthetases from \( B. \) stearothermophilus and \( E. \) coli. Subsequent to the completion of this work, Smith and Cohn (17) also reported similar data for this reaction of the \( E. \) coli enzyme.

tRNA which had had its amino acid accepting ability destroyed by periodate oxidation gave little or no stimulation of rate when added to the tRNA-independent reaction mixtures.
The Rapid Hydrolysis of Homocysteine Adenylate Occurs by Cyclization to the Thiolactone. It is expected from our knowledge of intramolecurally-catalysed reactions that homocysteinyl adenylate should rapidly hydrolyze in solution to form the thiolactone (eq. 3), the five membered ring being stable, the thiolate anion a good nucleophile and AMP a good, exocyclic leaving group (18). The thiolactone formed when the valyl- and methionyl-tRNA synthetases from E. coli were incubated with homocysteine and ATP was detected by thin layer chromatography on either cellulose or silica plates (table 2). The thiolactone cochromatographs with authentic samples, gives the same yellow colour with ninhydrin and absorbs uv light in the same manner. It was verified that homocysteine does not cyclise during the developing of the chromatogram. It is seen in Table 2 that the formation of the thiolactone catalysed by the methionyl-tRNA synthetase has the same requirements as the pyrophosphate exchange reaction. Further, the activity is abolished by the presence of 25 mM methionine, the cognate amino acid. tRNA, however, does not suppress the activity. The thiolactone was similarly detected in the reaction of homocysteine with the other two enzymes in Table 1.

Rejection of α-Aminobutyrate and Threonine by Lupin Valyl-tRNA Synthetase Involves Transient Formation of Aminoacyl-tRNA\textsuperscript{Val}. Enzyme-bound aminoacyl adenylate complexes were prepared in situ at pH 6.8 where they are relatively stable (12). It is seen in figure 1 that on mixing the E.\textsuperscript{14}C\textsubscript{Val}-AMP complex with tRNA\textsubscript{Val}, 62% of the radioactivity is transferred to the tRNA with a rate constant of 4.6 s\textsuperscript{-1}. On mixing the E.α-\textsuperscript{14}Caminobutyryl-AMP complex with tRNA\textsubscript{Val}, there is a transient formation of α-aminoacyl-tRNA\textsubscript{Val} reaching a maximum at 50 ms of 25% of the radioactivity transferred to tRNA. A very tentative analysis fits the data to the rate constant of 15 s\textsuperscript{-1} for formation of charged tRNA and 30 s\textsuperscript{-1} for the subsequent hydrolysis. Thr-tRNA\textsubscript{Val} is also transiently formed when the E. Thr-AMP complex is mixed with tRNA, but the maximum level observed is much lower, at about 4%.
Table 2: Requirements for the formation of homocysteine thiolactone catalysed by the methionyl-tRNA synthetase (E. coli)

<table>
<thead>
<tr>
<th>Additions or omissions</th>
<th>Detection of homocysteine thiolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete^a</td>
<td>+</td>
</tr>
<tr>
<td>- HCys</td>
<td>-</td>
</tr>
<tr>
<td>- HCys, + 25 mM Cys^b</td>
<td>-</td>
</tr>
<tr>
<td>- ATP</td>
<td>-</td>
</tr>
<tr>
<td>- MgCl₂</td>
<td>-</td>
</tr>
<tr>
<td>- 2-mercaptoethanol</td>
<td>+</td>
</tr>
<tr>
<td>- enzyme</td>
<td>-</td>
</tr>
<tr>
<td>+ 7 μM tRNA^Met</td>
<td>+</td>
</tr>
<tr>
<td>+ 25 mM Met^c</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: ^a pH 8.0, 25°C, 30 min, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.2 mM EDTA, 1.25 mM ATP, 20 mM homocysteine (HCys), 1 μM methionyl-tRNA synthetase from E. coli. ^b R_f = 0.22, separates satisfactorily from the homocysteine (R_f = 0.34) and the thiolactone (R_f = 0.57) when run simultaneously. ^c R_f = 0.45, no other ninhydrin staining spot was detected.

The amount of mischarged tRNA present at the maximum depends on the ratio of rate constants for its formation and hydrolysis, and on the fraction that passes through this pathway. It may be calculated for α-aminobutyrate, for example, that a transfer of 100% combined with the rate constants of 15 s⁻¹ and 30 s⁻¹ should give 25% at the maximum, the observed value. Clearly, the major fraction of the α-aminobutyrate is edited by deacylation of the mischarged tRNA. The lower level of Thr-tRNA^Val detected may just reflect a higher ratio of rate constants for hydrolysis and formation rather than a lower fraction edited by the deacylation route.

These data were obtained from only 350 μg enzyme using the micro pulsed-quenched flow apparatus.

DISCUSSION

The hydrolytic editing of errors of misactivation of amino acids can
Figure 1. Transfer of valine (○), α-aminobutyrate (●) and threonine (△) from their aminoacyl adenylate-valyl-tRNA synthetase complexes (Lupinus luteus) to tRNA\(^{Val}\) at 25\(^0\)C and pH 6.8 (see text).

They take place by essentially the three routes of Scheme I: (a) via \(k_1\), the dissociation of the complex to give the free aminoacyl adenylate complex which hydrolyses in solution ("kinetic proofreading" (19)); (b) via \(k_2\), the hydrolysis of the aminoacyl adenylate when bound to the enzyme; (c) via \(k_3\), the enzyme-catalysed deacylation of the mischarged tRNA (20,21). We have evidence from this study that all three pathways are used to some extent. Their relative contributions to the overall editing depends on the ratios of
all the rate constants in Scheme I. The fraction of errors edited by, say, the k₁ pathway of Scheme I is given by \( \frac{k_1}{(k_1 + k_2)} \). Thus for k₁ to make a significant contribution to error correction, k₁ must be significantly greater than k₂. Similarly for k₃, the fraction edited is \( \frac{k_3}{(k_3 + k_0)} \) and so k₃ must be greater than k₀ if deacylation of the mischarged tRNA is to be of importance.

It is tempting to attribute any observed hydrolytic activity to the presence of an editing mechanism. The above reasoning, however, may be applied to determine whether the activity is significant or just adventitious. For example, it is clear the the pre-transfer pathway makes an insignificant contribution to the reduction of errors in the activation by the valyl-tRNA synthetase of threonine, α-aminobutyrate, cysteine, alanine and threonine (Table 1 and ref. (12) Table 3). In all cases, the value of k₇ for the ATP-pyrophosphatase activity in the absence of tRNA is far lower than in its presence. Thus the sum of (k₁ + k₂) is less than k₁ so that there is only a fractional reduction in the error rate. Further, we have been able to detect the transient formation of mischarged tRNA Val in this and earlier studies (4,5) and find k₇ to be very fast compared with the rate constants for the hydrolysis of enzyme-bound aminoacyl adenylate complexes (e.g. k₇ in the range 10-40 s⁻¹ as in figure 1). Thus, even though the tRNA-independent hydrolysis in the presence of cysteine is 24% of the tRNA-dependent rate, the pre-transfer pathway cannot improve the accuracy by more than a factor of 1.24.

The rejection of homocysteine by the methionyl- and isoleucyl-tRNA synthetases does appear to be by the pre-transfer route: the tRNA-independent ATP-pyrophosphatase activity is in all cases significantly high and generally greater than the tRNA-dependent activity (Table 1 and ref. (17)). Further, a previous extensive search using rapid quenching methods (10) found no evidence for the accumulation of homocysteinyl-tRNA Met in the reaction of the enzyme from B. stearothermophilus. As described earlier (eq. 3), homocysteinyl adenylate does have the propensity to cyclise rapidly and so there is a specific chemical process for its removal. This type of cyclisation should readily occur with certain other aminoacyl adenylates that may also form a five-membered ring such as homoserine. Cysteine is also rejected at a high tRNA-independent rate by the isoleucyl-tRNA synthetase. Whether this involves the transient formation of cysteine thiolactone is not known. Although four-membered ring lactones are very unstable, it is possible that the larger size of sulphur and the different bond angles could render the form-
ation of the four-membered thiolactone ring more energetically favourable. Cysteinyl esters are, in any case, more reactive towards hydrolysis because of the inductive effect of the sulphur atom (e.g. hydrolysis of Cys-tRNA\textsuperscript{Cys} (22)).

Qualitative predictions may be made about the relevance of each of the three editing pathways of Scheme I.

(a) Pre-dissociation of aminoacyl adenylate ($k_1$). It has been pointed out previously (23) that, because the rate constant for the dissociation of the cognate aminoacyl adenylate from its enzyme is in general so low, only very dissimilar non-cognate complexes should dissociate sufficiently rapidly to compete significantly with transfer. Further, the rejection of amino acids similar in structure to the cognate causes considerable wastage of ATP because a high proportion of the cognate aminoacyl adenylate will also be destroyed by this route for it to be effective (23-25). This mechanism becomes of more importance for the non-cognate substrates of very dissimilar structure that dissociate rapidly. But, these are the very substrates which are only weakly activated in the first place so that their editing is not crucial to obtain the desired accuracy of protein synthesis (about 1 mistake per 3000 residues incorporated (26,27)). The rejection of cysteine by the isoleucyl-tRNA synthetase could well be an example of this route: the amino acid is activated slowly by the enzyme; the ATP-pyrophosphatase activity is independent of tRNA and occurs at about the rate estimated for the dissociation of the complex (1 s\textsuperscript{-1}, calculated from the measured dissociation rate constant of 0.04 s\textsuperscript{-1} for valyl adenylate (9) and the ratios of their relative values of $k_{cat}/K_M$ for activation listed in Table 1). The initial discrimination by the isoleucyl-tRNA synthetase against cysteine, $3 \times 10^{-4}$ (Table 1), is, however, just adequate to maintain the desired overall accuracy of protein synthesis without editing. The rejection of cysteine by the valyl-tRNA synthetase occurs mainly by the post-transfer pathway. This route is favoured by the valyl-tRNA synthetase compared with the isoleucyl-tRNA synthetase for two reasons: the rate constant for transfer, $k_T$, is generally higher than with the isoleucyl-tRNA synthetase; cysteine is relatively more similar to valine than to the larger isoleucine - reflected in the poorer relative specificity of activation by the valyl-tRNA synthetase (Table 1) - and so dissociates relatively more slowly from the valyl-tRNA synthetase. This illustrates the balance between the dissociation and post-transfer pathways. Glycine, for example, which is activated at a negligible rate by the three enzymes in Table 1 will almost certainly be rejected by the $k_1$ route,
its binding being so poor.

(b) Direct destruction of enzyme-bound aminoacyl adenylate ($k_2$). The first-order rate constants for the hydrolysis of aminoacyl adenylates in solution under the present reaction conditions are low compared with the turnover numbers for editing (e.g. $k = 3 \times 10^{-3}$, $4 \times 10^{-3}$ and $8 \times 10^{-3}$ s$^{-1}$ for the hydrolysis of isoleucyl, valyl and tyrosyl adenylates respectively (13)). Therefore, for the hydrolysis of the enzyme-bound aminoacyl adenylate to occur at a sufficient rate to be significant, there must be either a specific enzyme-catalysed reaction or a special chemical mechanism, such as the cyclisation of the homocysteinyl adenylate. It is not clear whether or not the cyclisation of the homocysteinyl adenylate takes place before dissociation from the methionyl-tRNA synthetase. The rate constant for the dissociation of methionyl adenylate from the enzymes from both E. coli and B. stearothermophilus is equal to or less than about $3 \times 10^{-3}$ s$^{-1}$. Coupling this rate constant with the relative values of $k_{cat}/K_M$ for the activation of methionine and cysteine by the enzymes gives values of 0.6 and 0.3 s$^{-1}$ for $k_1$ for the two enzymes respectively. These values are sufficiently close to the observed turnover numbers in Table 1 to render an unambiguous distinction between the $k_1$ and $k_2$ pathway difficult. On balance, however, given that the value of the dissociation rate constant is an upper limit, we favour the $k_2$ pathway as being dominant.

The cyclisation reaction is not just restricted to the homocysteinyl adenylate. Intramolecular cyclisation will occur very readily with the adenylates of the naturally occurring amino acids, aspartate, glutamate and lysine (18). This could thus provide a (further) route for the rejection of these amino acids when misactivated. The geometry of their cognate aminoacyl-tRNA synthetases, however, must presumably be such that the cyclisation is avoided in the correct activations.

(c) Post-transfer pathway ($k_3$). This is the one pathway that can be established unambiguously because an intermediate is formed, the mischarged tRNA, which may be directly detected and trapped. Previous studies have shown its importance with the valyl-tRNA synthetases from B. Stearothermophilus, E. coli and yeast (4,5), and this study extends the mechanism to the lupin enzyme. The type of selection which is used for editing, the "double-sieve" (8), is particularly efficient because it uses different features of the non-cognate and cognate substrates for selection in activation and editing. The principles of double-sieve editing may also be applied to the hydrolysis of the aminoacyl adenylate in the $k_2$ reaction as well as the hydrolysis of the
In conclusion, the post-transfer pathway of editing by deacylation of the mischarged tRNA has been established to be of major importance. The pathway involving hydrolysis of the aminoacyl adenylate by a specific chemical route, such as cyclisation, is also efficient. The pre-dissociation pathway whereby the aminoacyl adenylate hydrolyses in solution is generally of minor importance, being efficient for editing only those amino acids that are activated so weakly as not to require editing.

(Subsequent to the submission of this manuscript, a report appeared suggesting that the phenylalanyl-tRNA synthetases from yeast, \textit{E. coli} and \textit{Neurospora crassa} reject tyrosine, methionine and leucine by the post-transfer route but that the turkey liver enzyme uses the pre-transfer pathway (28)).

**ACKNOWLEDGEMENTS**

This work was supported by an EMBO Short-Term Fellowship tenable at Imperial College (H.J.), MRC Programme Grant G977/985 and Polish Academy of Science Project 09.7.1.

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