Proofreading in \textit{trans} by an aminoacyl-tRNA synthetase: a model for single site editing by isoleucyl-tRNA synthetase

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**ABSTRACT**

Editing of errors in amino acid selection by an aminoacyl-tRNA synthetase prevents attachment of incorrect amino acids to tRNA, thereby greatly enhancing accuracy of translation of the genetic code. Editing of the non-protein amino acid homocysteine, a frequent type of an error-correcting process, involves reaction of the side chain sulfhydryl group of homocysteine with its activated carboxyl group forming a cyclic thioester, homocysteine thiolactone. Here, it is shown that isoleucyl-tRNA synthetase (IleRS), which occasionally misactivates homocysteine in vitro and \textit{in vivo}, catalyzes reactions of activated isoleucine with organic thiols (analogues of the side chain of homocysteine). That these enzymatic reactions occur under physiologic conditions is suggested by the observation that the two sub-sites are physically close on the surface of IleRS, forming a single synthetic/editing active site of the enzyme. Although IleRS–Val–AMP undergoes thiolysis as efficiently as do IleRS–Ile–AMP (bound in the synthetic sub-site) and a thiol (an analogue of the side chain of homocysteine, bound in the editing sub-site), indicates that the two sub-sites are physically close on the surface of IleRS, forming a single synthetic/editing active site of the enzyme. Although IleRS–Val–AMP undergoes thiolysis as efficiently as do IleRS–Ile–AMP and IleRS+Ile–tRNA\textsuperscript{Ile}, IleRS+Val–tRNA\textsuperscript{Ile} does not react with thiols. These and other data suggest that the mischarged valine residue in IleRS+Val–tRNA\textsuperscript{Ile} is, most likely, positioned off the enzyme.

**INTRODUCTION**

Proofreading or editing mechanisms are an essential part of biological information transfer processes, including translation (reviewed in 1–3). The non-protein amino acid homocysteine (Hcy), an obligatory precursor of methionine in all cells, poses a major accuracy problem for the protein biosynthetic apparatus. Three closely related class I aminoacyl-tRNA synthetases (AARS) (reviewed in 4), MetRS, IleRS and LeuRS, misactivate Hcy \textit{in vitro} (5,6) at a frequency exceeding the frequency of translational errors \textit{in vivo} (2). Two other synthetases, class I ValRS (5,7) and class II LysRS (H. Jakubowski, unpublished), misactivate Hcy less efficiently. These five enzymes possess an efficient editing mechanism which prevents misincorporation of Hcy into tRNA (5,8) by destroying the Hcy–AMP intermediate. The editing pathway involves reaction of the side chain sulfhydryl group of Hcy with its activated carboxyl group yielding a cyclic thioester, Hcy thiolactone (5–7). For at least one synthetase, the editing reaction occurs in the same active site that carries out the synthetic reaction, as demonstrated by structure–function studies of MetRS (9). Editing reactions catalyzed by three class I AARS, MetRS (10–13), IleRS (14) and LeuRS (14), have been shown to occur \textit{in vivo}.

Recently, a class I synthetase, ArgRS, has been shown to catalyze synthesis of dipeptides Arg-Cys and Arg-Hcy from Arg–tRNA\textsuperscript{Arg} and a corresponding thioamino acid (15). The mechanism involves a nucleophilic attack of the thiolate of cysteine (or homocysteine) on the ester bond in Arg–tRNA\textsuperscript{Arg}. The resulting thioesters S-arginyl-cysteine and S-arginyl-homocysteine are not observed as discrete intermediates because of their rapid rearrangement to form a stable peptide bond. The involvement of thioester intermediates in the synthesis of Arg–Cys and Arg–Hcy is supported by the observation that thioesters of arginine do form with cysteine derivatives that do not have a free amino group, such as N-acetylcysteine and 3-mercaptopropionate. Although ArgRS does not appear to have an editing mechanism (15), its ability to catalyze the formation of thioesters of arginine is reminiscent of the formation of the thioester homocysteine thiolactone in proofreading reactions of other AARSs (5–7).

IleRS, which has an editing mechanism directed against Hcy \textit{in vivo} (14) is also capable of forming Ile-Cys from Ile–tRNA\textsuperscript{Ile} and cysteine (15). However, the mechanism of Ile-Cys formation has not been determined and it is not known whether IleRS can also catalyze the formation of thioesters of isoleucine. If it occurred, the synthesis of thioesters of isoleucine by IleRS would mimic editing reaction, in which misactivated Hcy is cyclized to the thioester homocysteine thiolactone by the enzyme (see Fig. 4). In addition to editing Hcy (5,14), IleRS is also capable of editing \textit{val in vitro} (16,17). Editing of valine was suggested to involve a site separate from the synthetic site (18). It is not known whether noncognate valine and Hcy are edited in the same or separate sites of IleRS. One way to answer this question is to compare reactivities toward thiols of aminocyl ester bonds in cognate IleRS+Ile–tRNA\textsuperscript{Ile} and noncognate IleRS+Val–tRNA\textsuperscript{Val} complexes. Similar reactivities with thiols of both complexes would suggest that the Val and Ile residues (bound to iRNA\textsuperscript{Val}) occupy the same site on the enzyme. If the Val residue in mischarged Val–tRNA\textsuperscript{Val} occupies a different site, the mischarged iRNA should not react with thiols.

Here, it is shown that IleRS catalyses reactions of cognate Ile–tRNA\textsuperscript{Ile} and Ile–AMP, as well as noncognate Val–AMP, but not Val–tRNA\textsuperscript{Val} with organic thiols (analogues of the side chain of Hcy) in reactions mimicking editing of Hcy by the enzyme. The data indicate that two major physiologic functions of IleRS,
formation of Ile-tRNA and editing of inadvertently misactivated Hcy, reside in one active site of the enzyme. A misacylated valine residue in an IleRS•Val-tRNA^Ile complex, which may form transiently during editing (19), appears not to be bound on the enzyme but is still hydrolyzed off the tRNA.

**MATERIALS AND METHODS**

**Plasmids and host strain**

Plasmids containing the genes for *Escherichia coli* IleRS (18,20) and *Bacillus stearothermophilus* ValRS (21) were obtained from P. Schimmel and E. Schmidt. Plasmids were over-expressed in *E.coli* strain JM101 and used as a source of AARS. Cells for enzyme purifications were obtained from overnight cultures (usually 400 ml, yielding ~2 g cells) grown at 37°C in LB medium containing 100 µg/ml ampicillin.

**Aminoacyl-tRNA synthetases**

*Escherichia coli* IleRS (20) and *B.stearothermophilus* ValRS (21,22) were purified to homogeneity from the overproducing strains using standard procedures (20,22).

**Preparation of [14C]Ile-tRNA^Ile and [14C]Val-tRNA^Ile**

[14C]Ile-tRNA^Ile was prepared from aminoacylation mixtures (0.1 ml) containing 50 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.1 mM EDTA, 2.5 mM ATP, 10 µM tRNA^Ile from *E.coli* (1600 pmol/A₂₆₀, Subrinden RNA), 45 µM [14C]isoleucine (306 Ci/nmol) (NEN) and 0.1 µM *E.coli* IleRS. [14C]Val-tRNA^Ile was prepared by mischarging (22) *E.coli* tRNA^Ile with 25 µM [14C]valine (285 Ci/nmol) (Amersham) using *B.stearothermophilus* ValRS (18,21,22). After 15 min at 37°C the charged tRNA was purified by phenol extraction, and recovered by precipitation with ethanol. The precipitate was washed several times with 70% ethanol to remove traces of free [14C]amino acids, dissolved in 0.1 ml glass-distilled water, and stored at –20°C.

**Enzymatic deacylation of [14C]aminoacyl-tRNA^Ile**

The reactions were carried out at 37°C in 0.1 M K–HEPES, pH 7.4, 10 mM MgCl₂, 0.2 mM EDTA. In one set of experiments, the disappearance of [14C]aminoacyl-tRNA^Ile was monitored by trichloroacetic acid precipitation. In another set of experiments in which all forms of [14C]amino acids were followed, the aliquots were analyzed by TLC.

**Thiolyis of IleRS•[14C]aminoacyl-AMP**

IleRS•[14C]Ile-AMP was prepared and incubated with thiols in the same mixture. The reactions were carried out at 37°C in mixtures containing 1 µM Ile-AMP, 15 µM [14C]isoleucine, 1 mM ATP, 0.1 M K–HEPES, pH 7.4, 10 mM MgCl₂, 0.2 mM EDTA, 50 mM thiol and 5 U/ml inorganic pyrophosphatase (Sigma). The reactions were monitored by TLC. Under these conditions, reactions with L-cysteine, cysteamine and dithiothreitol (DTT) were completed within 2 min, and with t,L-Hcy in 16 min. With 2-mercaptoethanol (2-ME), reactions were 50% completed in 16 min. Similar reactions were carried out with IleRS•[14C]Val-AMP prepared in reaction mixtures containing 15 or 150 µM [14C]valine instead of [14C]isoleucine.

**RESULTS AND DISCUSSION**

**TLC analysis**

TLC separations were carried out on cellulose plates from Kodak using butanol–acetic acid–water (4:1:1, v/v) as a solvent (5,15,23). Authentic isoleucine and valine (Sigma) standards were co-chromatographed with samples and visualized by staining with ninhydrin. TLC plates were autoradiographed using Reflection™ (NEN) autoradiography film.

**Some thiols accelerate enzymatic deacylation of Ile-tRNA^Ile**

Most AARS (2,3,23), including IleRS (19,24,25), catalyze deacylation reactions which result in discharging of an amino acid from aminoacyl-tRNA in the absence of AMP and PP₃. As shown in Table 1, enzymatic deacylation of Ile-tRNA was accelerated by L-cysteine and DTT but not by D-cysteine, D,L-Hcy and 2-ME, indicating specificity of the reaction with respect to which thiols can participate. This also suggests that stimulation is not merely due to reactivation of essential -SH groups of the enzyme. When reactions were carried out at different thiol concentrations, saturation kinetics were observed (Fig. 1). Control experiments have shown that L-cysteine and DTT did not stimulate nonenzymatic deacylation of Ile-tRNA. The half-life for Ile-tRNA^Ile (pH 7.4, 37°C) in the absence and presence of 0.125 M L-cysteine or DTT was 180 min. Another nucleophile, hydroxylamine, did not accelerate the enzymatic deacylation (Fig. 1), but did stimulate nonenzymatic deacylation of Ile-tRNA. The half-life for Ile-tRNA^Ile (pH 7.4, 37°C) in the absence and presence of 0.125 M L-cysteine or DTT was 180 min. Another nucleophile, hydroxylamine, did not accelerate the enzymatic deacylation (Fig. 1), but did stimulate nonenzymatic deacylation of Ile-tRNA^Ile (the half-life for Ile-tRNA^Ile (pH 7.4, 37°C) in the absence and presence of 0.125 M L-cysteine or DTT was 110 min in the presence of 125 mM hydroxylamine). These results suggest the presence on the enzyme of a specific site (‘-SH subsite’), which affects enzymatic deacylation of Ile-tRNA.

**Thiols react enzymatically with Ile-tRNA^Ile**

To analyze products of enzymatic deacylation of Ile-tRNA, aliquots of reaction mixtures containing IleRS, [14C]Ile-tRNA^Ile and various thiols were subjected to TLC. In the absence of thiols,
isoleucine was a major product (lane 1, Fig. 2A, B and C). In the presence of L-cysteine (lane 4, Fig. 2A; lane 2, Fig. 2B), DTT (lane 3, Fig. 2A), cysteamine (lane 5, Fig. 2A; lane 3, Fig. 2B), 3-mercaptopropionate, N-acetyl-L-cysteine, L-cysteine methyl ester (lanes 4, 7 and 8 respectively, Fig. 2B), and D-cysteine (lane 7, Fig. 2A), new products formed, suggesting that these thiols bind to the -SH subsite and react with Ile-tRNA. Deacylations in the presence of L-cysteine (lane 4, Fig. 2A; lane 2, Fig. 2B) and D,L-Hcy (lane 6, Fig. 2A) also yielded isoleucine. These observations indicate that, in addition to binding to the -SH subsite in the editing mode common for all thiols (see Fig. 4B.iv), cysteamine, in addition to Hcy, is a noncognate substrate which co-chromatographed with an authentic Ile-Ala standard (15). These properties suggest that products of cysteamine-, D-cysteine-, L-cysteine- and L-cysteine methyl ester-dependent enzymatic deacylation of Ile-tRNA\textsubscript{Ile} are the corresponding isoleucyl-dipeptides.

**Identification of products of thiol-dependent deacylation of Ile-tRNA\textsubscript{Ile}**

To determine identities of these new products, several chemical tests were carried out followed by TLC analysis of treated and untreated samples. For example, products of enzymatic reactions of Ile-tRNA with N-acetyl-L-cysteine, 3-mercaptopropionate and DTT were hydrolyzed to isoleucine upon NaOH treatment and, with the exception of the DTT-dependent product, were not sensitive to thiol reagents such as iodoacetate and DTNB. These properties suggest that products formed in the presence of N-acetyl-cysteine, 3-mercaptopropionate and DTT were the corresponding thioesters of isoleucine.

In contrast, products of enzymatic reaction of Ile-tRNA with cysteamine, D-cysteine, L-cysteine and L-cysteine methyl ester were not sensitive to NaOH but reacted with iodoacetate and DTNB. Upon Raney nickel treatment (which desulfurizes cysteine into alanine), a product of the IleRS-dependent reaction of Ile-tRNA with cysteine was transformed into a new product which co-chromatographed with an authentic Ile-Ala standard (15). These properties suggest that products of cysteamine-, D-cysteine-, L-cysteine- and L-cysteine methyl ester-dependent enzymatic deacylation of Ile-tRNA\textsubscript{Ile} are the corresponding isoleucyl-dipeptides.

**Table 1.** First order rate constants, \( k \), for enzymatic deacylation of Ile-tRNA\textsubscript{Ile} and Val-tRNA\textsubscript{Ile} catalyzed by IleRS

<table>
<thead>
<tr>
<th>Additions</th>
<th>( k (s^{-1}) )</th>
<th>( k ) (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Val-tRNA\textsubscript{Ile}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.04</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>N.D.</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>D,L-Hcy</td>
<td>0.04</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0.08</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Isoleucine (2.5 mM)</td>
<td>0.04</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Cysteine + isoleucine (2.5 mM)</td>
<td>0.04</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>0.08</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>DTT + isoleucine (2.5 mM)</td>
<td>0.04</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2-ME</td>
<td>0.04</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>D-Cysteine</td>
<td>0.04</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Decaylative reactions were carried out at 37°C in reaction mixtures containing 100 mM HEPES–KOH (pH 7.4), 10 mM MgCl\(_2\), 0.1 mM EDTA, 1.4 \( \mu \)M [\(^{14}\)C]Ile-tRNA\textsubscript{Ile} (1 pmol = 400 c.p.m.) or 1.1 \( \mu \)M [\(^{14}\)C]Val-tRNA\textsubscript{Ile} (1 pmol = 350 c.p.m.), 0.2 or 0.025 \( \mu \)M IleRS and indicated additions at 50 mM unless indicated otherwise. The \( k \) values (calculated as in ref. 15) are means of two to three independent determinations with errors of 20%. N.D., not determined.

Formation of isoleucyl-dipeptides is consistent with the following mechanism. The ester bond in Ile-tRNA\textsubscript{Ile} undergoes thiolation by cysteine (or similar thiol amino acid). The resulting thioester is not observed as an intermediate because of the rapid...
rearrangement to form a stable peptide bond. Facile intramolecular reaction results from the favorable geometric arrangement of the α-amino group of cysteine with respect to the thioester bond (26). Support for this mechanism comes from the observations that
the SH group is required for the reaction (alanine, serine and S-methyl-cysteine do not react with Ile-tRNA^Ile^, Fig. 2B) and that
isoleucine thiosters do indeed form with cysteine derivatives that
do not have a free amino group, such as 3-mercaptopropionate
and N-acetyl-L-cysteine. A similar mechanistic analysis for
synthesis of the dipeptide Arg-Cys by Arg-RS (15).

Products of nonenzymatic deacylation of Ile-tRNA in the
presence of cysteine and DTT were also analyzed by TLC. The
half life of Ile-tRNA^Ile^ (180 min) was not affected by thiols as
stated above. More than 95% isoleucine and <5% Ile-Cys or
Ile-DTT formed during these nonenzymatic reactions. These
results further indicate that reactions of thiols with Ile-tRNA^Ile^ are
catalyzed by IeRS.

Thiols bind at a distinct site of IleRS

To test whether binding sites for isoleucine and thiols interact,
thiol-dependent deacylation reactions were carried out in the
absence and presence of exogenous isoleucine. The cysteine- and
DTT-dependent deacylations of Ile-tRNA^Ile^ were 2-fold slower
in the presence of isoleucine than in its absence (Table 1). As
shown in Figure 2C, exogenous isoleucine prevented formation
of [^14C]Ile-DTT (thio)ester (compare lanes 6 and 5) and
[^14C]Ile-Cys dipeptide (compare lanes 4 and 3) from
[^14C]Ile-tRNA^Ile^. This suggests that free isoleucine displaces the
[^14C]Ile residue in [^14C]Ile-tRNA^Ile^ from the thiol-reactive site
on IleRS either to another site on the enzyme (perhaps the editing
site for valine) or off the enzyme. Because [^14C]Ile-tRNA^Ile^ is
decacylated by IeRS even in the presence of free isoleucine or
valine (24; see also Table 1), it follows that under these conditions
the[^14C]Ile residue in an[^14C]Ile-tRNA^Ile•IleRS complex is
positioned off the enzyme: excess of free isoleucine or valine is
expected to prevent binding of the[^14C]Ile residue in
[^14C]Ile-tRNA^Ile•IleRS to the enzyme. Alternatively, free isoleucine
might bind to a second Ile-binding site which in turn would
either displace a thiol or affect reactivity of the ester bond in
Ile-tRNA^Ile•IleRS, respectively. However, IleRS, a monomeric enzyme
(20), is very unlikely to possess two Ile-binding sites. That thiols
do not bind at the isoaceuline binding site of IleRS is indicated by
the inability of thiols to inhibit the aminocacylation reaction
catalyzed by IleRS (see below).

Thiols and valine do not affect deacylation of
Val-tRNA^Ile^ by IleRS

The rate of IleRS-dependent deacylation of[^14C]Val-tRNA^Ile^ in
contrast with the rate of deacylation of[^14C]Ile-tRNA^Ile^, was not
accelerated by DTT or L-cysteine (Table 1). Saturating
concentrations of Hey and valine, noncognate substrates which are
missilicated and edited by IleRS (5, 16), did not prevent enzymatic
decacylation of[^14C]Val-tRNA^Ile^ (Table 1). TLC analyses
confirmed that[^14C]valine was the only product of the
decacylation reaction in the presence or absence of 100 mM
L-cysteine, N-Cys and DTT (not shown). The sensitivity of these
analyses was such that < 5% of Val-Cys or Val-DTT would have
been detected if present. These results suggest that the charged
valine and isoleucine residues in Val-tRNA^Ile•IleRS and Ile-
Ile-tRNA^Ile•IleRS complexes respectively, are positioned differently.
The[^14C]sulfocysteine residue in[^14C]Ile-tRNA^Ile•IleRS is positioned
on the enzyme and therefore able to react with thiols; excess of
free isoleucine or valine displaces the[^14C]cysteine to the off
enzyme position thereby preventing reaction with thiols.

The valine residue in Val-tRNA^Ile•IleRS is, most likely,
positioned off the enzyme and is therefore unreactive towards
thiols. Because neither exogenous valine nor isoleucine significantly
inhibits deacylation of Val-tRNA^Ile^ by IleRS (Table 1), it is
unlikely that the valine residue in Val-tRNA^Ile•IleRS is positioned
on the enzyme. A proposed second site for editing of mischarged
valine by IleRS (18) would be functionally equivalent to the
off enzyme position.

Reactions of IleRS•Ile-AMP and IleRS•Val-AMP with thiols

Thiols reacted also with IleRS-bound Ile-AMP or Val-AMP,
yielding thiosters and peptides. For example, IleRS•Ile-AMP
reacted with DTT and 2-ME to yield (thio)esters Ile-DTT and
Ile-(2-ME). Similarly, IleRS•Val-AMP reacted with DTT to yield
(thio)ester Val-DTT. Peptide bond formation occurred with
cysteine to yield either Ile-Cys or Val-Cys. The reactions with
adenylates proceeded at about the same rates as enzymatic
reactions with Ile-tRNA^Ile^ and exhibited similar thiol specificity:
cysteine and DTT reacted faster than 2-ME (not shown). Similar
reactivities of IleRS-bound aminoacyl-adenylates and Ile-tRNA^Ile^
towards thiols indicate that the amino acid residues of Ile-AMP,
Val-AMP and Ile-tRNA^Ile^ occupy the same sub-site in the active
site of the enzyme.

The (thio)esters[^14C]Ile-DTT and[^14C]Val-DTT can be easily
purified by extraction of reaction mixtures with toluene. TLC
analysis showed that mild alkaline hydrolysis of[^14C]Ile-DTT
yields[^14C]cysteine, whose identity was confirmed by showing
that it can re-charge tRNA^Ile^ in the presence of ATP and IleRS.
Similarly, mild alkaline hydrolysis of[^14C]Val-DTT yielded
[^14C]valine, whose identity was confirmed by showing that it can
re-charge tRNA^Val^ in the presence of ATP and ValRS. The
half-life of the spontaneous hydrolysis of both[^14C]Ile-DTT and
[^14C]Val-DTT at pH 7.4, 37 °C was 120 min, somewhat less than
the half-life of Ile-tRNA^Ile^ (180 min), Val-tRNA^Val^ (160 min) or
Val-tRNA^Ile^ (140 min).

Thiols react with isoleucine in complete aminocacylation
mixtures

Reactions of thiols with isoleucine occurred also in complete
aminocacylation mixtures (Fig. 3C). This led to transient
formation of Ile-tRNA followed by its enzymatic thiolysis in reaction
mixtures containing 20 mM L-cysteine or DTT (Fig. 3A) or 200 mM
D-cysteine (Fig. 3B). Although 2-ME reacted with isoleucine in
complete aminocacylation mixtures to some extent (compare lanes
2 and 6 in Fig. 3C), the reaction was very inefficient and did not
lead to transient aminocacylation kinetics (Fig. 3A and B).
Importantly, the rate of the aminocacylation reaction was not
inhibited by thiols, when present at 0.2 M (Fig. 3A and B),
indicating that thiols and isoleucine bind to different (sub)sites on
the enzyme.

A model for single site editing of Hey by IleRS

The ability of IleRS to catalyze synthesis of thiosters of isoleucine
is a consequence of the editing function of the enzyme and can be
Figure 3. Effects of thiols on aminoacylation of tRNA\textsubscript{Ile}. Reactions were carried out at 37°C in mixtures containing 0.1 M HEPES–KOH (pH 7.4) buffer, 15 µM tRNA\textsubscript{Ile}, 16 µM \textsuperscript{14}C\textsuperscript{Ile} (306 Ci/mol), 2.5 mM ATP, 10 mM KF, 10 mM MgCl\textsubscript{2} and 30 nM IleRS. Time courses of aminoacylation in the absence and presence of 20 mM \textsuperscript{A} and 200 mM \textsuperscript{B} indicated thiol are shown. \textsuperscript{C} TLC analysis of aminoacylation mixtures containing 200 mM (lanes 1–3) or 20 mM (lanes 5–9) thiol: lanes 1 and 5, DTT; lanes 2 and 6, 2-ME; lanes 3 and 9, D-cysteine; lane 4, no additions; lane 7, D,L-Hcy; lane 8, L-cysteine. Spots migrating close to Ile-tRNA\textsubscript{Ile} in lanes 3, 8 and 9 are disulfide forms of Ile-Cys dipeptide.

Figure 4. A model for single site editing by IleRS. The active site is proposed to have two partially overlapping sub-sites, synthetic and editing. Thet-carbon, carboxyl and amino groups, common to all amino acids, bind to the overlapping region common to the synthetic and editing sub-sites. The side chain of an amino acid can bind to nonoverlapping portions of either synthetic or editing sub-site. Thus, an amino acid binds either in the synthetic or editing mode. Amino acid substrates of IleRS bind initially in the synthetic mode. Binding of a noncognate amino acid in the editing mode is induced at some point of the synthetic pathway. (A) (i) Initial binding of the noncognate Hcy is in the synthetic sub-site of IleRS. (ii) The side chain of Hcy moves to the editing sub-site after formation of Hcy-AMP. Nucleophilic attack of the side chain thiol on activated carboxyl carbon yields Hcy thiolactone (iii). (B) With the synthetic sub-site occupied by the cognate isoleucine, the editing sub-site can be filled with an analogue of the side chain of Hcy, R-\textsubscript{CH}_2-SH (an organic thiol). (iv) This leads to formation of a thioester of isoleucine in a reaction mimicking editing of Hcy. X denotes tRNA\textsubscript{Ile} or AMP.

rationalized by the following model (Fig. 4). When misactivated Hcy is in the active site of IleRS, its side chain -SH group occupies a sub-site (an -SH sub-site) next to its carboxyl carbon (Fig. 4A.ii). This sub-site is presumed to be vacant when a cognate amino acid is in the active site. Filling the -SH sub-site by providing the -SH function in \textit{trans}, i.e., on another molecule (Fig. 4B.iv), leads to the formation of a thioester of a cognate amino acid (Fig. 4B.v). That this reaction occurs between Ile-tRNA\textsubscript{Ile} or Ile-AMP (bound in the synthetic sub-site) and an organic thiol, an analogue of the side chain of Hcy (bound in the editing sub-site), indicates that the two sub-sites are intimately close on the surface of the enzyme, forming a single synthetic/editing active site. Editing of valine can also be accommodated in the same site; however, while a mischarged valine residue (attached to tRNA\textsubscript{Ile}) that undergoes editing appears to be positioned off the surface of the enzyme, the aminoacyl bond is still accessible to hydrolysis by the enzyme. Thus, editing occurs when an activated amino acid is no longer bound in the synthetic sub-site or when the editing sub-site is occupied by a thiol.

The model shown in Figure 4 is also consistent with the data obtained by others (18). Implication of this model is that any mutation that affects binding of the side chain of the amino acid
substrate during synthetic reaction will not affect editing reaction: the side chain of an amino acid that undergoes editing is not bound in the synthetic sub-site. This explains the behavior of a G56A mutant of IleRS that has lost the ability to discriminate between isoleucine and valine in the synthetic reaction (mostly due to the loss of efficient binding of the side chain of the substrate isoleucine) but was completely active in editing of valine. A related mutant, G56P, inactive in the synthetic reaction with either isoleucine or valine, was active in editing of valine (18). The lack of synthetic activity of the G56P IleRS mutant is, most likely, due to a total loss of the ability to bind amino acid substrates; this, according to the model shown in Figure 4, will not affect editing activity of the enzyme. Another mutant IleRS, F570S, has lost the ability to efficiently bind isoleucine and valine during the synthetic reaction, but was still unimpaired in the editing reaction (18), again consistent with the model shown in Figure 4.

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