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Mutational Isolation of a Sieve for Editing in a Transfer RNA Synthetase

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Editing reactions are essential for the high fidelity of information transfer in processes such as replication, RNA splicing, and protein synthesis. The accuracy of interpretation of the genetic code is enhanced by the editing reactions of aminoacyl transfer RNA (tRNA) synthetases, whereby amino acids are prevented from being attached to the wrong tRNAs. Amino acid discrimination is achieved through sieves that may overlap with or coincide with the amino acid binding site. With the class I *Escherichia coli* isoleucine tRNA synthetase, which activates isoleucine and occasionally misactivates valine, as an example, a rationally chosen mutant enzyme was constructed that lacks entirely its normally strong ability to distinguish valine from isoleucine by the initial amino acid recognition sieve. The misactivated valine, however, is still eliminated by hydrolytic editing reactions. These data suggest that there is a distinct sieve for editing that is functionally independent of the amino acid binding site.

Editing reactions in information transfer have long been recognized (*1–5*), and those associated with tRNA synthetases were among the first to be investigated (*3*, 6–8). The aminoacylation reaction occurs in two steps: (i) an amino acid activation reaction in which an amino acid is condensed with adenosine triphosphate (ATP) to form an aminoacyl adenylate and (ii) the transfer reaction in which the aminoacyl moiety is transferred from the adenylate to the tRNA substrate cognate to the amino acid (9). The overall accuracy of aminoacylation depends on the specificity of amino acid activation (first sieve) and on pre- and posttransfer editing events (second sieve), which correct for occasional errors of amino acid activation (*3*, 6, *10*). *Escherichia coli* isoleucine tRNA synthetase (IleRS) is a 939–amino acid monomeric class I tRNA synthetase (*11*) that misactivates the closely related valine at a relative frequency of about 0.5% (*12*). When presented with $tRNA^{Ile}$, the enzyme-bound misactivated valyl–adenosine monophosphate (Val-AMP) is hydrolyzed either directly or by transient formation of Val-$tRNA^{Ile}$ followed by rapid deacylation of the mischarged species (*13–17*). These pre- and posttransfer editing events are manifested by a $tRNA^{Ile}$-dependent hydrolysis of ATP in the presence of valine (*17*).

In spite of considerable progress on the elucidation of structures of class I and class II tRNA synthetases (*18–23*), the structural basis for editing has not been revealed. One difficulty has been the lack of crystal structures for free amino acid or aminoacyl-tRNA complexed with the cognate enzyme. Because both the first sieve and the pre- and posttransfer editing of the second sieve show strong amino acid discrimination, the structural basis for both sieves, although unknown, has been assumed to be connected in some way with an amino acid binding site (*15*).

Although not determined, the three-dimensional structure of IleRS is believed to be most related to that of methionine tRNA synthetase (*11*). On the basis of the close evolutionary relation between these enzymes, a model for the active site region of IleRS has been developed (*24–27*). The nucleotide binding fold of alternating β strands and α helices that characterizes the class I tRNA synthetases incorporates one (the 11–amino acid signature sequence) of the two class-defining sequence motifs into a loop between the first strand (β_A) and helix (α_A), and this segment in turn forms part of the ATP interaction site (*21*). Mutational analysis of *E. coli* methionine tRNA synthetase shows that this region also contributes to amino acid binding (*25*). We confirmed the significance of this part of the structure for interaction with the aminoacyl moiety by determining that it can be cross-linked to the isoleucyl group of bound *N*-bromoacetyl isoleucyl-$tRNA^{Ile}$ (*28*). Within the 20 amino acids that form β_A and the following loop, position 56 is the only one at which IleRS and ValRS have a different and yet strictly conserved amino acid (Gly^{56} and Pro, respectively) (*26*). With this in mind, we introduced substitutions at position 56 to see whether these substitutions affected isoleucine versus valine discrimination and the editing reactions.

A Gly^{56} to Ala substitution (G56A) (*29*) completely eliminated the discrimination between isoleucine and valine in the

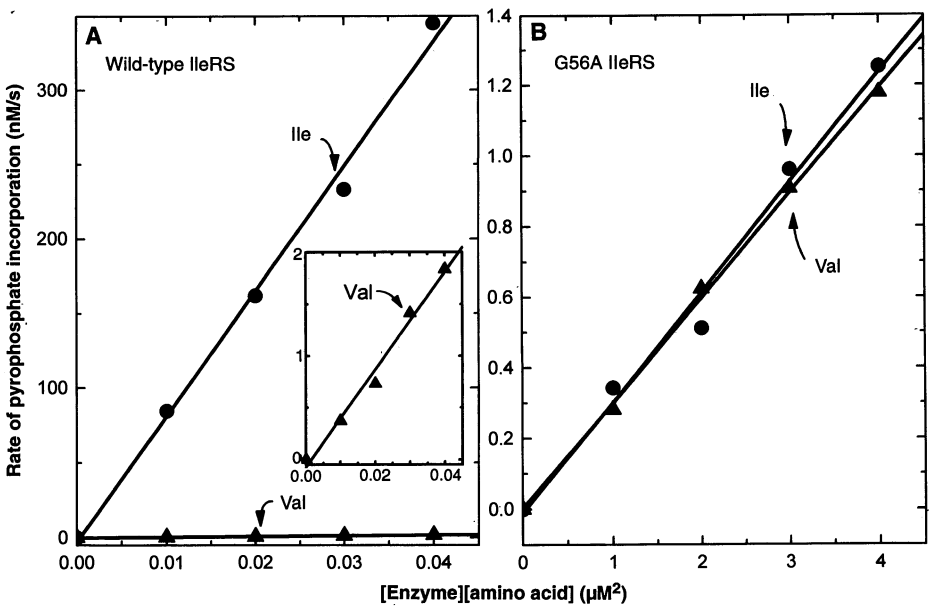

Fig. 1. Amino acid activation activities for wild-type (**A**) and G56A (**B**) IleRS. The inset of (A) gives an expanded scale to show detection of amino acid activation with valine. Initial rates of pyrophosphate incorporation into ATP are plotted versus the product of the enzyme and amino acid concentrations. The slope of the graph is equal to the second-order rate constant k_{cat}/K_m.

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amino acid activation reaction (Fig. 1, A and B) (*30*). This substitution reduced the 180-fold difference in the ratio of the catalytic rate constant and the Michaelis constant (k_{cat}/K_m) for isoleucine versus valine (of the wild-type enzyme) to a value of unity (Table 1). Most of this reduction was caused by a sharp elevation in the value for K_m for isoleucine [from 4 μM (wild type) to 7 mM (mutant)], combined with a more modest change in the value for K_m for valine [from 0.45 mM (wild type) to 7 mM (mutant)]. Thus, the mutation has a major negative effect on the apparent binding of isoleucine to the enzyme; it raises the free energy of binding by 4.5 kcal mol^{-1}, yet affects valine binding only by 1.6 kcal mol^{-1}. The values for k_{cat} are less affected by the substitution, and for the mutant protein are 4.8 s^{-1} (isoleucine) and 4.6 s^{-1} (valine). Consequently, for the G56A mutant enzyme, each of the individual kinetic parameters has the same value regardless of which of the two amino acids is used as substrate, thus demonstrating the elimination by mutation of the first sieve for amino acid discrimination.

The editing activity of the wild-type enzyme was analyzed in two ways. We first investigated the overall pre- and posttransfer ATP hydrolysis induced by addition of $tRNA^{Ile}$ (*31*). We found that the mutant enzyme was as active as the wild-type protein (Fig. 2), with a $tRNA^{Ile}$-dependent rate of hydrolysis of Val-AMP of 2.7 s^{-1} in each case (Table 1). Thus, whereas the first sieve was obliterated by the G56A substitution, the second sieve remained completely active. As an extension of the analysis of editing, we studied independently the IleRS-catalyzed hydrolysis of mischarged Val-$tRNA^{Ile}$ (*16, 32*). There was no diminution of this posttransfer activity caused by the G56A substitution and, if anything, it appeared slightly higher with the mutant enzyme as compared to the wild-type enzyme (Table 1).

For comparison, we investigated two other mutant enzymes. A G56P IleRS (*29*) was constructed first, so that position 56 would have the proline that is conserved among sequenced valine tRNA synthetases. This mutant enzyme was inactive in the activation assay for isoleucine or valine. Because of this, the $tRNA^{Ile}$-induced hydrolysis of Val-AMP could not be investigated. However, we were able to study the IleRS-catalyzed deacylation of Val-$tRNA^{Ile}$ and found that this activity was unimpaired in the G56P enzyme, even though it lacked any activity for amino acid activation. This result further supports the concept that the amino acid activation and editing sites are functionally distinct, even though each provides amino acid discrimination.

To see whether other mutations at the amino acid binding site had a similar effect, we investigated the MI1 enzyme first described by Iaccarino and Berg (*33*). This enzyme has a large value for K_m for isoleucine, and for that reason, a cell harboring this mutation is an isoleucine auxotroph (*33, 34*). Cloning of the mutant gene by polymerase chain reaction (PCR) methods (*35*) and subsequent sequencing established that the phenotype of the MI1 enzyme was due to a single F570S substitution (*29*). [Phe^{570} is within the nucleotide binding fold at the beginning of the fourth (or D) helix and, on the basis of the alignment of this region with methionine tRNA synthetase (*26*), also contributes to the formation of the amino acid binding site (*36*).] We confirmed that the mutant protein had a large value for K_m for isoleucine (8 mM), but we also found that it still discriminated against valine (K_m for valine was greater than 200 mM) and estimated that k_{cat}/K_m for isoleucine versus valine was at least as great as the 180-fold difference observed with wild-type enzyme (Table 1). Thus, unlike the case for the G56A mutant protein, the structural basis for the discrimination of isoleucine and valine is preserved in the F570S enzyme. This suggests that position 570 may be needed for recognition of parts of the amino acid that are common to isoleucine and valine, whereas position 56 is sensitive to the fine structure of the side chain. Similar to the situation with the G56A enzyme, however, the posttransfer editing activity of the F570S enzyme was unimpaired by the mutation (Table 1). This result shows that mutations at distinct positions that affect the amino acid binding site differently have little or no effect on editing reactions.

On the basis of these experiments, we suggest that the second sieve for amino acid discrimination evolved as a distinct functional unit as a result of the need to enhance the accuracy of decoding genetic information. The results with the G56A and F570S mutations demonstrate that the second sieve is sufficiently independent to withstand mutations in the first sieve. These mutations are within the conserved nucleotide binding fold and are believed to alter an integral part of the amino acid binding site of the catalytic region. Residues needed for the amino acid editing reactions may, therefore, be located in other structural units that have been incorporated in close proximity to the active site, such as residues within one or both of the two variable size segments that are inserted into the nucleotide binding folds of class I tRNA synthetases (*24, 37, 38*).

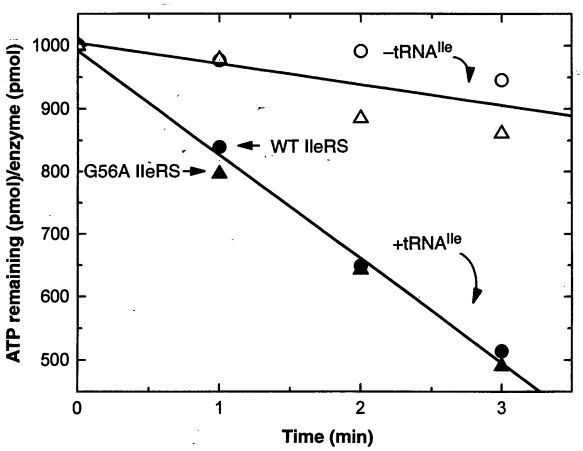

Fig. 2. Adenosine triphosphate consumption of wild-type (closed circles) and G56A (closed triangles) IleRS in the presence of valine and $tRNA^{Ile}$. Also shown is the rate of ATP hydrolysis in the absence of $tRNA^{Ile}$ for wild-type (open circles) and G56A (open triangles) IleRS. The G56A mutant showed more ATP hydrolysis in the absence of $tRNA^{Ile}$ than did the wild-type enzyme when the assay was carried out for longer time periods, but this hydrolysis was 15% or less of the $tRNA^{Ile}$-dependent hydrolysis.

Table 1. Comparison of kinetic data for wild-type and mutant IleRS. Amino acid activation, adenosine triphosphatase, and deacylation assays were performed as described (*30–32*). Values for k_{cat}/K_m are calculated from the individually measured kinetic parameters k_{cat} and K_m. These values are twofold or less of the values for k_{cat}/K_m measured from the plots in Fig. 1, A and B. ND, K_{cat} values for these mutants were not measurable because of low efficiencies of Val-AMP formation.

Enzyme	Amino acid activation			k_{cat} (s^{-1})	
	k_{cat}/K_m (Ile) (s^{-1} M^{-1})	k_{cat}/K_m (Val) (s^{-1} M^{-1})	$\frac{k_{cat}/K_m \text{ (Ile)}}{k_{cat}/K_m \text{ (Val)}}$	Hydrolysis of Val-AMP	Deacylation of Val-$tRNA^{Ile}$
Wild type	6.9×10^6	3.8×10^4	180	2.7	0.07
G56A	690	660	1.0	2.7	0.13
F570S	410	1.8	230	ND	0.17
G56P	<25	<1	–	ND	0.17

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29. Mutations are given by the single-letter code for amino acids. Thus, $Gly^{56} \rightarrow$ Ala is indicated by G56A. Abbreviations for the amino acid residues are: A, Ala; F, Phe; G, Gly; P, Pro; and S, Ser.
30. Mutant or wild-type enzyme was isolated as described (*27, 39*) from cells [strain MI1 (for G56A) or MV1184 (for wild type)] with a multicopy plasmid expressing the desired protein. The reaction mixture contained 100 mM tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, bovine serum albumin (0.1 mg/ml), 0.1 mM EDTA, 10 nM KF, 5 mM $MgCl_2$, 2 mM ATP, 2 mM [^{32}P]sodium pyrophosphate (5.35 to 11.1 cpm/pmol), 0.1 to 40 nM enzyme, and 1 to 1000 μM amino acid (but always under conditions where the amino acid concentration was less than its K_m). Reactions were incubated at 37°C for up to 2 hours. The reaction was quenched with 0.7 volumes of 15% $HClO_4$ and 400 mM sodium pyrophosphate. Activated charcoal was added, and ATP was separated by filtration with 2.4-cm glass fiber pads (Schleicher and Schuell) (*17*).
31. Reactions contained 150 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM valine, 3 mM [γ-^{32}P]ATP (10 to 20 cpm/pmol), pyrophosphatase (2 U/ml), 14 μM $tRNA^{Ile}$, and 2.8 μM enzyme. Reactions were assayed at 25°C for up to 20 min and were quenched with four volumes of 7% $HClO_4$. Activated charcoal containing 10 mM sodium pyrophosphate was added, and ATP was separated by filtration through glass fiber pads (Schleicher and Schuell).
32. *Bacillus stearothermophilus* valine tRNA synthetase was purified from *E. coli* strain MV1184 harboring plasmid pTB8, which encodes the *B. stearothermophilus* enzyme (*40*). The *B. stearothermophilus* enzyme was used to charge *E. coli* $tRNA^{Ile}$ with [^{3}H]valine (*41*). The [^{3}H]valine-$tRNA^{Ile}$ was purified through a series of phenol-chloroform extractions and ethanol precipitations. Deacylation reactions, performed at 25°C, contained 150 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 3.25 μM [^{3}H]valine-$tRNA^{Ile}$ (2170 cpm/pmol), pyrophosphatase (4 U/ml), and 5.2 nM enzyme. Aliquots of the reaction mixture were quenched on Whatman 3MM filter pads soaked in 5% trichloroacetic acid (TCA) and then washed repeatedly in 5% TCA and then 100% ethanol to remove free [^{3}H]valine. Under these conditions, the spontaneous rate of valine-$tRNA^{Ile}$ hydrolysis was less than 0.0002 s^{-1}.
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Stimulation of Human γδ T Cells by Nonpeptidic Mycobacterial Ligands

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Most human peripheral blood γδ T lymphocytes respond to hitherto unidentified mycobacterial antigens. Four ligands from *Mycobacterium tuberculosis* strain H37Rv that stimulated proliferation of a major human γδ T cell subset were isolated and partially characterized. One of these ligands, TUBag4, is a 5′ triphosphorylated thymidine-containing compound, to which the three other stimulatory molecules are structurally related. These findings support the hypothesis that some γδ T cells recognize nonpeptidic ligands.

Since the discovery of the γδ T cell receptor (TCR), various studies have demonstrated the involvement of γδ T lymphocytes in immune responses to mycobacteria (*1*). The nature of antigens recognized by γδ T cells has remained elusive, as has the physiological function of these cells. In mice, γδ T cells derived from lung and newborn thymus respond to tuberculin and to hsp65-derived peptides, suggesting a specialized role in stress immunity (*2*). In humans, though some γδ T lymphocytes reactive to mycobacteria recognize hsp65 (*3*), most of them appear to have different unidentified ligands (*4*). Such ligands recovered in hydrosoluble extracts from *Mycobacterium tuberculosis* have small molecular masses (1 to 3 kD) and, on the basis of several lines of indirect evidence, were assumed to be carbohydrate in nature (*5*). Here, we show that these ligands are phosphorylated molecules related to a thymidine 5′-triphosphoryl-X nucleotide conjugate.

Isolation of the stimulating ligands in hydrosoluble extracts from *M. tuberculosis* strain H37Rv cultures [mycobacterial extracts (ME)] was monitored by the proliferative response of a mycobacteria-reactive human γδ T cell clone, G115 (*6*). To determine the chemical nature of the ligands, we submitted ME to various treatments. The ligands were resistant to proteases as described (*5*) but sensitive to periodic acid oxidation and to alkaline phosphatase (*7*). Fractions from gel permeation chromatography of ME were analyzed for carbohydrate content and biological activity (Fig. 1A). The stimulating fractions appeared in two broad overlapping peaks containing carbohydrate, with an estimated mass range of 500 to 600 daltons. Taken together, these preliminary findings suggested that the stimulating ligands for the γδ T cell clone G115 were small phosphorylated glycoconjugate molecules.

These ligands were purified from ME by anion exchange chromatography, silicic acid chromatography, and C18 ion-pair reversed-phase high-pressure liquid chromatography (rpHPLC). With this procedure, four biologically active components, named TUBag1 to TUBag4 according to

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