Residues in a Class I tRNA Synthetase Which Determine Selectivity of Amino Acid Recognition in the Context of tRNA†

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ABSTRACT: Certain aminoacyl-tRNA synthetases discriminate between closely similar amino acids by hydrolytic editing reactions in the presence of their cognate tRNA. An example is the class I isoleucyl-tRNA synthetase. We recently showed that a mutation which eliminates discrimination between isoleucine (Ile) and valine (Val) in the initial amino acid binding and activation steps had little effect on the hydrolytic editing of activated valine in the presence of isoleucine tRNA ($tRNA^{Ile}$). The result showed that initial amino acid binding and discrimination are functionally independent of tRNA-dependent amino acid discrimination. In this work, we cross-linked (to isoleucyl-tRNA synthetase) a reactive analog of valine misacylated onto $tRNA^{Ile}$. Mutation of specific residues within a peptide segment identified by the cross-linking analysis severely affected discrimination of Val-$tRNA^{Ile}$ versus Ile-$tRNA^{Ile}$. The mutationally sensitive residues are part of an insertion into the catalytic domain and are themselves completely conserved among all known prokaryotic and eukaryotic sequences of the enzyme.

Aminoacyl-tRNA synthetases catalyze the esterification reaction between an amino acid and its cognate tRNAs. In order to maintain fidelity in protein synthesis, each enzyme must specifically recognize one of 20 amino acids and a small subset of the many cellular tRNAs. Recent biochemical data have led to the discovery of "identity elements" in several tRNAs that allow for specific recognition by a tRNA synthetase (Normanly & Abelson, 1989; Schimmel et al., 1993; Giegé et al., 1995; McClain, 1995), and structural studies on synthetase−tRNA cocrystals have identified enzyme residues that mediate specific recognition (Rould et al., 1989; Ruff et al., 1991). There has been considerably less biochemical or structural data directed at understanding specific amino acid discrimination. The small size of amino acids and their frequent structural similarity (isoleucine and valine both having β-branched hydrophobic side chains, for example) create a difficult problem for accurate molecular recognition (Pauling, 1957).

Escherichia coli isoleucyl-tRNA synthetase (IleRS)[1] is a 939 amino acid monomeric enzyme which promotes the attachment of isoleucine to $tRNA^{Ile}$. The reaction proceeds through a two-step process. In the first step (termed amino acid activation), the amino acid is activated through a condensation reaction with ATP to form an enzyme-bound aminoacyl adenylate. In the second step (termed acyl transfer), the activated amino acid is transferred to the 3′-end of $tRNA^{Ile}$ to yield the aminoacyl-tRNA (Schimmel, 1987). The amino acid activation step is error prone due to difficulty in discriminating against valine. The k_{cat}/K_m for activation of isoleucine is only 180 times greater than that for valine activation (Schmidt & Schimmel, 1994). The acyl transfer step provides additional selectivity, which *in vivo* decreases erroneous valine for isoleucine substitutions to 1:3000 (Loftfield, 1963; Loftfield & Vanderjagt, 1972). This additional selectivity (termed hydrolytic editing) can be thought of as tRNA-dependent amino acid recognition (Schimmel & Schmidt, 1995). While $tRNA^{Ile}$ is required for the increased selectivity (Baldwin & Berg, 1966), its exact role in hydrolytic editing is not well understood.

A "double-sieve" model has been developed to explain increased discrimination in the presence of $tRNA^{Ile}$ (Fersht, 1985), and recent work on IleRS has generated support for this kind of model. In particular, a Gly56 → Ala (G56A) mutation was identified that eliminated discrimination between isoleucine and valine in the amino acid activation reaction. However, the mutant enzyme still corrected valine misactivations in a tRNA-dependent manner (Schmidt & Schimmel, 1994). This result showed that the site for tRNA-dependent amino acid recognition is functionally independent of that for amino acid activation.

Hydrolytic editing reactions of IleRS have been postulated to remedy errors in valine activation both before (pretransfer editing) and after (posttransfer editing) the attachment of valine to $tRNA^{Ile}$ (Fersht, 1977). While $tRNA^{Ile}$ is required for both forms of editing, the posttransfer editing assay (which measures the enzymatic deacylation of Val-$tRNA^{Ile}$) unambiguously focuses on the contribution of $tRNA^{Ile}$ to amino acid recognition. Using this assay, the relative deacylation rates of Val-$tRNA^{Ile}$ and Ile-$tRNA^{Ile}$ can be compared in isolation from factors such as ATP, pyrophosphate, and adenylate, which idividually or collectively could affect rates of editing.

The double sieve or two-site model for amino acid selection has also been proposed to explain the discrimination of valine from threonine by valyl-tRNA synthetase (Fersht & Kaethner, 1976), but in neither isoleucyl- nor valyl-tRNA synthetase have any enzyme residues been identified that

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[1] Abbreviations: IleRS, isoleucyl-tRNA synthetase; CP1 and CP2, connective polypeptides 1 and 2; Ile, isoleucine; Val, valine; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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comprise the hydrolytic editing site. Although it is possible that $tRNA^{Ile}$ may itself play a direct role in forming all or part of the posttransfer hydrolytic editing site, we set out to identify those residues in IleRS that might be involved in posttransfer editing. For this purpose, isoleucyl-tRNA synthetase was probed using *N*-bromoacetyl-Val-$tRNA^{Ile}$, a novel misacylated cross-linking reagent. We were particularly interested in whether a peptide on the enzyme could be labeled with this reagent but not with the closely similar *N*-bromoacetyl-Ile-$tRNA^{Ile}$. One such peptide was identified. Subsequent mutational analysis of residues in the uniquely labeled peptide segment of the enzyme showed that these residues contribute specifically to the selectivity of tRNA-dependent discrimination but not to recognition in the amino acid activation step.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Escherichia coli K-12 strain MV1184 (*ara*, Δ[*lac-proAB*],*rspL*, *thi*, [ϕ80 *lacZ*ΔM15], Δ[*srl-recA*], 306::Tn*10*[*tet*^{r}]/F′ [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) was used as a host strain for mutagenesis. The phagemid pKS21 (donated by Dr. Kiyotaka Shiba) encoding wild-type IleRS was used as a template for the introduction of mutations via the Amersham Sculptor mutagenesis system (Amersham). Mutant enzymes were tested for their ability to rescue growth of the Δ*ileS* strain IQ843 (Δ*ileS*, *203::kan*, *recA56*, *araD139*, Δ(*argF-lac*)*U169*, *rpsL150relA1*, *flb-B5301*, *deoC1*, *ptsF25*, *rbsR*) (Shiba & Schimmel, 1992) as described (Schmidt & Schimmel, 1993). This strain is maintained by production of isoleucyl-tRNA synthetase from plasmid pRMS711. This plasmid has a temperature-sensitive replicon so that plasmid replication is defective at the restrictive temperature of 42 °C. For that reason, the resulting strain IQ843/pRMS711 is temperature sensitive.

Mutant enzymes H401Q, Y403F, D461A, and S465A supported cell growth at 42 °C when expressed from phagemid pKS21 in the IQ843 null strain and were purified as described (Shepard et al., 1992) from Δ*ileS* null strain IQ843. Mutant enzymes G56A, W407F, R408Q, R408A, W462F, and R466Q were unable to sustain cell growth. These mutant enzymes were purified from strain MI1 (Iaccarino & Berg, 1971; Treiber & Iaccarino, 1971), which contains a mutant *ileS* allele that allows for cell growth in isoleucine-rich media. Enzymes were plasmid overexpressed (10−20-fold in relation to chromosomally encoded enzyme) and purified. While the activity of contaminating chromosomally encoded IleRS is undetectable in amino acid activation and aminoacylation assays performed *in vitro* (Schmidt & Schimmel, 1993), the chromosomally encoded mutant enzyme does retain deacylase activity toward Val-$tRNA^{Ile}$ (Schmidt & Schimmel, 1994). Therefore, the discrimination factors (Figure 5) for the G56A, W407F, W462F, and R466Q mutant enzymes may include some background level of contaminating activity which we estimate as no more than 10% of the total activity. Deacylation rates reported for the R408Q and R408A mutant enzymes (Table 2 and Figure 5) may derive as much as 15% of their value from contaminating activity, as the overall rate of deacylation was slower for these enzyme preparations.

Production of N-Bromoacetyl[^{3}H]Val-$tRNA^{Ile}$ and Reaction with IleRS. [^{3}H]Val-$tRNA^{Ile}$ (8.5 μM, prepared as described) (Schmidt & Schimmel, 1994) was incubated with bromoacetic acid *N*-hydroxysuccinimide ester (3 mM, Sigma) in 75 mM triethanolamine hydrochloride (pH 7.8) and 25% 1,4-dioxane for 75 min at room temperature in a volume of 195 μL (Santi et al., 1973). *N*-Bromoacetyl[^{3}H]Val-$tRNA^{Ile}$ was recovered after two rounds of ethanol precipitation and judged to be greater than 80% derivatized as measure by resistance to cupric ion hydrolysis (Schofield & Zamecnik, 1968).

N-Bromoacetyl[^{3}H]Val-$tRNA^{Ile}$ (6 μM) was incubated with IleRS (6 μM) in 20 mM potassium phosphate buffer (pH 7.2) and 5 mM $MgCl_2$ for 0−2 h at 37 °C. Aliquots were removed at times 0, 10, 30, 60, and 120 min. At these times an equal volume of 4 mM crude tRNA (Boehringer Mannheim) was added to displace any noncovalent *N*-bromoacetyl-[^{3}H]Val-$tRNA^{Ile}$−IleRS complexes. The quenched reaction was applied to a nitrocellulose filter (Schleicher and Schuell) which had been prewashed with 20 mM Tris-HCl (pH 7.5) and 150 mM KCl. Four successive washes with the same buffer were applied to remove free *N*-bromoacetyl[^{3}H]Val-$tRNA^{Ile}$ (Yarus & Berg, 1969, 1970). The nitrocellulose filters were air-dried, and the level of remaining radioactivity was determined by scintillation counting in Betaflour (National Diagnostics). The yield of covalent complex formation was calculated from the filter-bound radioactivity and the specific activity of [^{3}H]valine in *N*-bromoacetyl[^{3}H]Val-$tRNA^{Ile}$.

In preparative-scale reactions of *N*-bromoacetyl[^{3}H]Val-$tRNA^{Ile}$ with IleRS, reaction conditions were identical to those above, except that the reaction was allowed to proceed for 1 h at 37 °C. After this time, RNase-free trypsin [1:20 (w/w) ratio to IleRS)], urea (to 3 M), and $CaCl_2$ (to 100 μM) were added. Proteolytic digestion was carried out for 3 h at 37 °C. The products of the digest were applied onto a G-100 Sephadex (Pharmacia) column (0.5 cm × 40 cm) equilibrated in 50 mM ammonium acetate (pH 6.6). Elution was performed using the same buffer, and fractions of 500 μL were collected. Those fractions corresponding to an early peak of radioactivity (Santi & Cunnion, 1974) were pooled and further purified by two rounds of ethanol precipitation (Hill & Schimmel, 1989). The resulting peptide-acetyl[^{3}H]-Val-$tRNA^{Ile}$ was submitted for seven cycles of peptide sequence analysis (MIT Biopolymers Laboratory) on an Applied Biosystems Model 477A protein sequencer with on-line Model 120 PTH amino acid analyzer.

Enzyme Assays. For deacylation assays [^{3}H]Val-$tRNA^{Ile}$ was prepared as described from $tRNA^{Ile}$ (Subriden RNA) (Schmidt & Schimmel, 1994). [^{3}H]-Ile-$tRNA^{Ile}$ was prepared by phenol/chloroform extraction and multiple ethanol precipitations of an aminoacylation reaction containing 20 mM 4-(2-hydroxy)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 0.1 mM EDTA, 0.15 M NH_4Cl, 100 μg/mL bovine serum albumin, 2 mM ATP, 6 mM $MgCl_2$, 20 μM [4,5-^{3}H]-L-isoleucine (specific activity of 1200 counts/min per pmol; Amersham, UK), 5 μM $tRNA^{Ile}$ (Subriden RNA), and 200 nM pure IleRS. Deacylation reactions were performed at ambient temperature (22−23 °C) in 150 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 3.25 μM aminoacyl-tRNA, pyrophosphatase (4 units/mL, Boehringer Mannheim), and 5.8−230 nM IleRS. Aliquots of the reaction mixture were quenched on Whatman 3MM filter pads soaked in 5% 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 both [^{3}H]Val-$tRNA^{Ile}$ and [^{3}H]Ile-$tRNA^{Ile}$

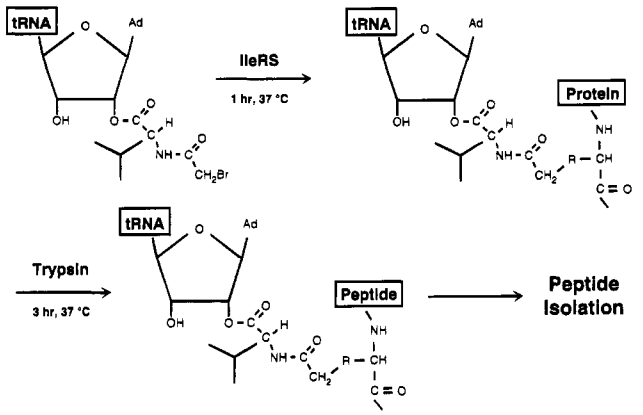

FIGURE 1: Reaction of *N*-bromoacetyl-Val-tRNA^{Ile} with IleRS. Schematic illustration of the cross-linking strategy employed in this work. Shown is *N*-bromoacetyl-valine attached to the 3′-end of tRNA^{Ile}. The *N*-bromoacetyl-Val moiety could be positioned at either the 2′- or 3′-hydroxyl of the terminal adenosine due to rapid acyl transfer. After incubation with IleRS, the bromine is displaced by a nucleophilic group on the enzyme leading to formation of a covalent complex. Trypsin cleavage reduced the complex to a covalent RNA−peptide complex, which was then isolated as described in Materials and Methods.

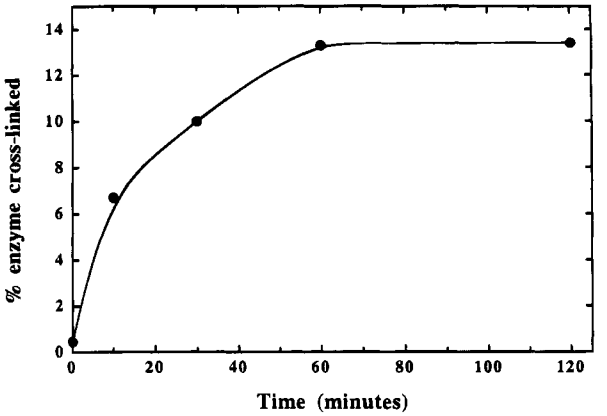

FIGURE 2: Adduct formation in the reaction of *N*-bromoacetyl-Val-tRNA^{Ile} with IleRS. A time course for reaction of *N*-bromoacetyl-Val-tRNA^{Ile} with IleRS, using a 1:1 ratio of reactant and enzyme. The product of the reaction was isolated by a nitrocellulose filter binding assay (see Materials and Methods). In other trials, we determined that less than 0.1% of IleRS could be cross-linked to *N*-bromoacetyl-Met-tRNA^{Met}.

hydrolysis was equal to 0.0006 s^{-1}, and this value was subtracted from all measurements of rates of enzymatic deacylation.

Aminoacylation reactions were performed according to Shepard et al. (1992) but at ambient temperature and with crude *E. coli* tRNAs (120 μM, Boehringer Mannheim). Amino acid activation reactions were performed as previously described (Schmidt & Schimmel, 1994).

RESULTS

Cross-Linking of N-Bromoacetyl[^{3}H]Val-tRNA^{Ile} to IleRS. Posttransfer editing in isoleucyl-tRNA synthetase is characterized by a rapid enzymatic deacylation of mischarged Val-tRNA^{Ile} (0.073 s^{-1}) compared to a slower enzymatic deacylation of Ile-tRNA^{Ile} (0.0023 s^{-1}). This 32-fold rate enhancement in noncognate deacylation improves the accuracy of amino acid selection at a site distinct from that where amino acid activation occurs (Schmidt & Schimmel, 1994). As a probe for identifying residues comprising this particular site, we used a chemically reactive *N*-bromoacetyl[^{3}H]Val-tRNA^{Ile}, which is a version of the N-bromoacetylated aminoacyl-tRNA affinity reagents first used by Santi (Santi et al., 1973; Santi & Cunnion, 1974) (Figure 1). This affinity reagent incorporates both a mischarged tRNA species designed to interact specifically with the hydrolytic editing site and a small bromoacetyl group which is generally reactive toward nucleophillic amino acid side chains (Wilchek & Givol, 1977). The use of tritiated valine provided a radiolabel for the monitoring of cross-linking efficiency.

We found that *N*-bromoacetyl[^{3}H]Val-tRNA^{Ile} is a substrate for enzymatic deacylation and, in addition, was also reactive toward IleRS. A single-species complex was formed between IleRS and *N*-bromoacetyl[^{3}H]Val-tRNA^{Ile} as detected by a PAGE "band-shift" assay (data not shown). Using a nitrocellulose filter binding assay, we determined that 10−15% of the enzyme was routinely derivatized (Figure 2). The yield may have been limited by enzymatic deacylation of the cross-linking reagent.

Enzyme−Val-tRNA^{Ile} complex was digested with trypsin and run over a G-100 Sephadex gel filtration column to separate free peptides from the high molecular weight peptide-tRNA complex (Santi & Cunnion, 1974). Gel filtration was followed by two successive rounds of ethanol precipitation to ensure that only peptides covalently bound to tRNA were recovered (Hill & Schimmel, 1989). The resulting peptide-Val-tRNA^{Ile} complex was subjected to seven cycles of amino acid sequencing, with each cycle yielding two amino acid residues (Table 1). The sequenced residues align unambiguously to peptide 1 (minor) encompassing IleRS residues 399−408 and peptide 2 (major) encompassing IleRS residues 452−466.

Table 1: Amino Terminal Sequence of Reactive Peptides[a]

	cycle number						
	1	2	3	4	5	6	7
amino acids detected	M/I	Q/E	H/S	−/M	Y/V	P/A	−/N
yield (pmol)	1.4/4.9	1.5/2.6	0.5/0.8	−/1.8	1.0/2.6	0.8/2.1	−/2.0
known IleRS sequences							
peptide 1	M^{399}	Q	H	S	Y	P	C
peptide 2	I^{452}	E	S	M	V	A	N

[a] Cross-linked peptides were prepared and submitted for sequence analysis as described in Materials and Methods. Amino acid residues detected in each cycle are reported along with their yields and the known sequence of two regions of IleRS. A dash indicates only one residue detected for this cycle.

Positions of Peptides 1 and 2 within IleRS. Isoleucyl-tRNA synthetase is a class I tRNA synthetase (Cusack et al., 1990; Eriani et al., 1990). The 10 class I tRNA synthetases have a conserved N-terminal Rossman nucleotide binding fold which supplies the scaffolding for ATP binding (Brunie et al., 1990; Burbaum & Schimmel, 1991). Inserted into the Rossman fold of each tRNA synthetase are two connective polypeptide chains (CP1 and CP2), which are characterized by lower levels of sequence conservation. In the cocrystal of the class I glutaminyl-tRNA synthetase−tRNA^{Gln}−ATP complex, residues within CP1 are involved in making contacts to the acceptor stem of tRNA^{Gln} (Rould et al., 1989).

Peptide 1 consists of 10 residues which map to the large connective polypeptide (CP1) that interrupts the Rossman fold of IleRS between the third and fourth β-strands (Figure 3) (Starzyk et al., 1987). The 276 amino acid CP1 domain (enzyme residues 157−433) has been studied by deletion

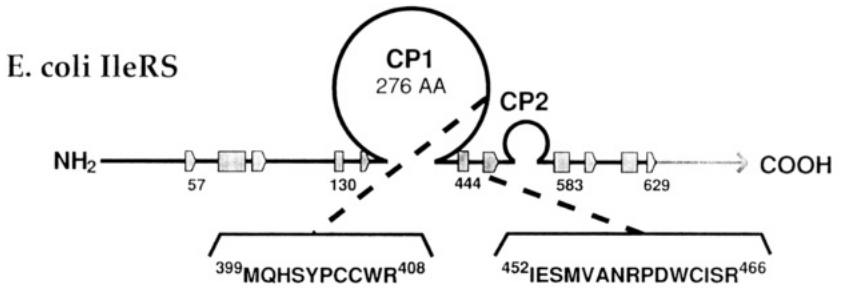

FIGURE 3: Locations of cross-linked peptides within isoleucyl-tRNA synthetase. The locations and sequences of cross-linked peptide 1 (residues 399−408) and peptide 2 (residues 452−466) are shown with reference to the predicted secondary structure of the N-terminal domain of IleRS. The secondary structure of IleRS is based on alignments to the sequence and known three-dimensional structure of *E. coli* methionyl-tRNA synthetase (Starzyk et al., 1987; Brunie et al., 1990). CP1 and CP2 refer to connective polypeptide insertions 1 and 2 in the Rossman nucleotide binding fold (Starzyk et al., 1987). The rectangles indicate α-helices, and the pentagons indicate β-strands.

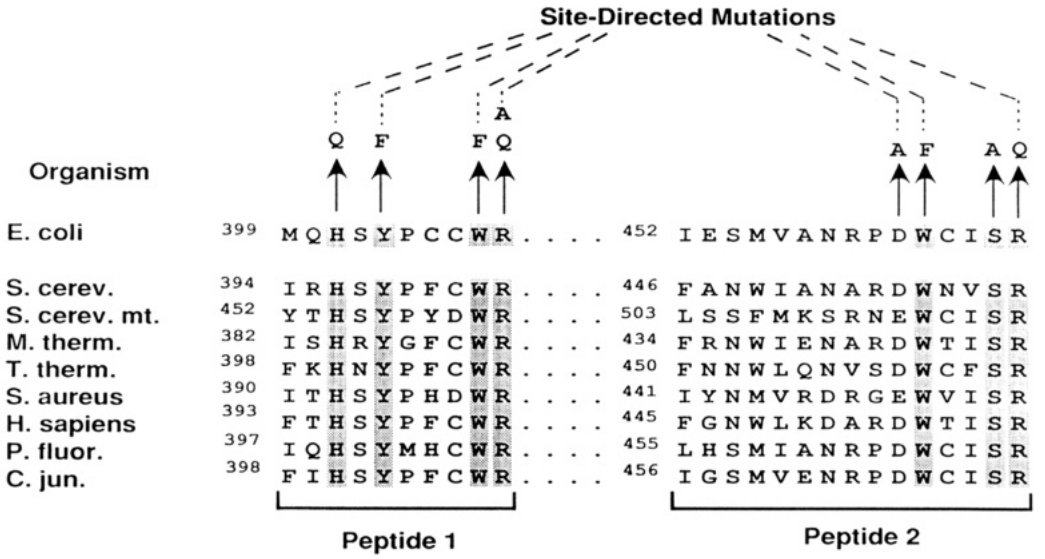

FIGURE 4: Alignment of peptide 1 and peptide 2 sequences with known sequences of other isoleucyl-tRNA synthetases. The sequence of peptides 1 and 2 of *E. coli* IleRS is aligned with the corresponding sequence in eight other sequences of IleRS. Shaded residues are strictly conserved. Amino acid substitutions reported in this work are indicated with arrows. The sequences are from *E. coli* (Webster et al., 1984), *Saccharomyces cerevisiae* (Englisch et al., 1987), *Saccharomyces cerevisiae* mitochondria (Genbank Accession no. l38957), *Methanobacterium thermoautrophicum* (Jenal et al., 1991), *Tetrahymena thermophila* (Csank & Martindale, 1992), *Staphylococcus aureus* (Chalker et al., 1994), *Homo sapiens* (Shiba et al., 1994), *Pseudomonas fluorescens* (Isaki et al., 1990), and *Campylobacter jejuni* (Genbank Accession No. u15295).

analysis, which showed that residues 231−377 are dispensable, while residues 378−444 are needed for function (Starzyk et al., 1987). Four of the residues in peptide 1 are strictly conserved among the nine known isoleucyl-tRNA synthetase sequences (Figure 4). Although the biochemical function for residues in peptide 1 or the surrounding region of IleRS has not previously been established, we imagine by analogy to GlnRS that parts of CP1 may make contact with the acceptor helix of $tRNA^{Ile}$ to promote specific tRNA recognition [*cf.* Rould et al. (1989), Burbaum and Schimmel (1991), and Perona et al. (1991)].

Peptide 2 is a 15 amino acid peptide which aligns to the fourth β-strand of the Rossman fold and the surrounding amino acids (Figure 3). This pentadecamer ends in the pentapeptide sequence WCISR, which is conserved among a subgroup of class I tRNA synthetases (Härtlein & Madern, 1987; Heck & Hatfield, 1988) and has been proposed to contribute to editing function (Carter, 1993). Three of its residues are strictly conserved among known sequences of IleRS (Figure 4). Additionally, peptide 2 was also shown to be reactive toward *N*-bromoacetyl[^{3}H]Ile-$tRNA^{Ile}$ (data not shown) and by others to be reactive toward L-isoleucyl-bromomethyl ketone (Rainey et al., 1976), indicating that peptide 2 comprises part of the initial amino acid binding pocket of IleRS. Consistent with this interpretation, Schulman and co-workers have shown that analogous mutations in the region corresponding to peptide 2 of the closely related *E. coli* methionyl-tRNA synthetase disrupt methionine binding and activation (Ghosh et al., 1991).

Mutagenesis of Residues in Peptide 1. In order to decipher the function of the four invariant residues in peptide 1, conservative replacements were made as follows: His401 → Gln (H401Q), Tyr430 → Phe (Y403F), Trp407 → Phe (W407F), and Arg408 → Gln (R408Q) (Figure 4). In addition, Arg408 was also changed to Ala. All mutant enzymes were overexpressed from a multicopy plasmid and subsequently isolated as greater than 95% pure. To measure editing activity *in vitro*, enzyme variants were assayed for their ability to deacylate both Val-$tRNA^{Ile}$ and Ile-$tRNA^{Ile}$. The difference in deacylation rates toward cognate and noncognate substrate (termed discrimination factor) was taken as a measure of tRNA-dependent amino acid discrimination and was compared to the 32-fold discrimination factor for the wild-type IleRS enzyme.

When enzymes with mutations in peptide 1 were tested for their relative ability to deacylate Val-$tRNA^{Ile}$ and Ile-

Table 2: Deacylation Rates for IleRS with Point Mutations in Peptide 1[a]

IleRS mutation	deacylation rate $(10^3 \text{ sec})^{-1}$ Val-tRNA^{Ile} (Ile-tRNA^{Ile})	discrimination factor
wild-type	73 (2.3)	32
H401Q	120 (0)[b]	>1200[c]
W403F	110 (0.1)	1100
R408Q[d]	53 (6.9)	7.7
R408A[d]	51 (11)	4.6

[a] Values are corrected for the nonenzymatic rate of 0.0006 s^{-1} for both Val-tRNA^{Ile} and Ile-tRNA^{Ile} hydrolysis. [b] No increase in deacylation rate was detected for the enzymatic reaction. [c] This value is the low estimate for the discrimination factor. [d] Deacylation rates for these enzymes may include low levels of activity from an enzyme contaminant (see Materials and Methods). Therefore, the reported discrimination factors represent an upper limit to actual discrimination factors.

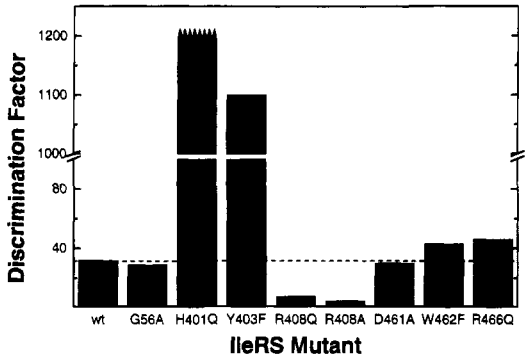

FIGURE 5: Discrimination between Val-tRNA^{Ile} and Ile-tRNA^{Ile} in deacylation assays. Wild-type and point mutant IleRS enzymes were tested for their ability to deacylate Val-tRNA^{Ile} and Ile-tRNA^{Ile}. The ratio of rates of Val-tRNA^{Ile} deacylation to Ile-tRNA^{Ile} deacylation (termed discrimination factor) is shown graphically for enzymes containing peptide 1 substitutions H401Q, Y403F, R408Q, and R408A, as well as for several enzymes containing substitutions outside of peptide 1. For the H401Q substitution, enzyme-catalyzed Ile-tRNA^{Ile} deacylation was not detected. Therefore, the discrimination factor of 1200 for this enzyme represents an estimate of the lower bound to the actual discrimination factor and this is indicated by a jagged top on the bar.

tRNA^{Ile}, large differences from the 32-fold wild-type discrimination rate were obtained (Table 2 and Figure 5). In particular, H401Q and Y403F mutant enzymes showed remarkably slower deacylation rates toward Ile-tRNA^{Ile} along with a slightly faster (than wild-type enzyme) deacylation of Val-tRNA^{Ile}. Taken together, the two changes result in a discrimination factor of over 1000 for the mutant enzymes, which is more than 30-fold higher than that of wild-type enzyme (Table 2).

The effects of H401Q and Y403F mutations on enzyme activity were also tested *in vivo* and *in vitro*. For the *in vivo* analysis we used tester strain IQ843/pRMS711, which contains a Δ*ileS* null allele and is maintained by plasmid pRMS711. Plasmid pRMS711 is a multicopy plasmid with a temperature-sensitive replicon that encodes wild-type isoleucyl-tRNA synthetase (see Materials and Methods). Genes encoding the mutant enzymes were introduced on a compatible plasmid (pKS21) and tested for their ability to rescue the temperature-sensitive phenotype. Both H401Q and Y403F enzymes restored growth at 42 °C to strain IQ843, demonstrating that these mutant proteins are active *in vivo*. In addition to this analysis, we showed that the H401Q and Y403F enzymes had activities for activation of isoleucine and valine and aminoacylation (with isoleucine) that were indistinguishable from the corresponding activities of the wild-type enzyme (data not shown). Thus, H401 and Y403 may be part of a site specifically designed for tRNA-dependent discrimination of isoleucine vs valine.

Consistent with the idea that this region of the protein is important for tRNA-dependent amino acid discrimination, R408Q and R408A substitutions also affected the relative selection of these two amino acids. However, in the case of these substitutions, both showed increased rates of deacylation toward Ile-tRNA^{Ile} coupled with slightly decreased rates of Val-tRNA^{Ile} deacylation (Table 2). Overall discrimination factors for these mutant enzymes were about 4−7-fold lower than that for the wild-type enzyme. These data are consistent with a more easily accessible or expanded hydrolytic site in these mutants which can better accommodate the larger amino acid side chain of Ile-tRNA^{Ile}. Both the R408Q and R408A mutations also destroyed activity for amino acid activation *in vitro* and, as expected, resulted in proteins that could not sustain growth of the IQ843 Δ*ileS* null strain. We conclude that R408 encompasses both the amino acid activation site and the hydrolytic editing site.

Finally, the W407F mutation did not affect Val-tRNA^{Ile} vs Ile-tRNA^{Ile} deacylation rates relative to the rates of the wild-type enzyme.

Mutagenesis of Other IleRS Residues. In order to determine whether the observed changes in posttransfer editing selectivity were specific to mutations targeted in peptide 1, we placed amino acid substitutions at each of the positions of three conserved residues of peptide 2 and also analyzed a fourth previously constructed G56A mutation (Schmidt & Schimmel, 1994). Specifically, individual Asp461 → Ala (D461A), Trp462 → Phe (W462F), and Arg466 → Gln (R466Q) mutations were made (Figure 4), and the mutant enzymes were purified.

The G56A substitution was previously shown to disrupt the initial amino acid binding of IleRS and to create a mutant enzyme that was unable to discriminate valine from isoleucine in the amino acid activation reaction. We tested the G56A enzyme's ability to discriminate between these two amino acids in the posttransfer deacylation assay and found no significant difference from wild-type discrimination levels (Figure 5). This finding is consistent with the earlier hypothesis that the G56A mutation only disrupts initial amino acid recognition.

The D461A, W462F, and R466Q mutant enzymes were also tested for their ability to discriminate Ile-tRNA^{Ile} from Val-tRNA^{Ile} in the deacylation reaction. In each case, only slight differences in discrimination levels (Figure 5) relative to the wild-type enzyme were observed. In addition to the analysis of these three mutant enzymes, the Ser465 → Ala (S465A) variant was also created. This variant showed no apparent defect in rapid deacylation of Val-tRNA^{Ile} and was not pursued further. Collectively, these results show that the WCISR pentapeptide is not involved in editing.

Analysis of enzymatic activity *in vitro* showed that, for the W462F and R466Q mutations, amino acid activation was severely impaired. These data are consistent with the earlier prediction that this region of IleRS forms part of the amino acid binding pocket (Rainey et al., 1976; Ghosh et al., 1991).

DISCUSSION

Single binding pocket discrimination between similar amino acid pairs has been recognized as a difficult problem

(Pauling, 1957). Physical chemical calculations predicted that enzymes such as isoleucyl-tRNA synthetase may require two sequentially acting binding sites in order to achieve sufficient amino acid selection (Crick, 1975), with the overall selection being equal to the product of that achieved at each step (Freist, 1989). While the double sieve model has survived biochemical tests, the second tRNA-dependent site has yet to be identified on a molecular level. Although none of the mutations studied here were successful in destroying catalytic deacylation of Val-tRNA^{Ile}, our results clearly show that specific mutations lead to altered tRNA-dependent discrimination, thus implying the existence of a defined site whose character can be changed. In particular, the conservative H401Q and Y403F substitutions altered tRNA-dependent valine vs isoleucine selectivity by 30-fold or more. It remains to be seen whether nonconservative substitutions of H401 or Y403, or mutation of residues in the surrounding region, will lead to the production of mutant tRNA synthetases with even greater defects.

Kinetic studies on isoleucyl-tRNA synthetase suggested that the enzyme also has a pretransfer editing activity whereby, in the presence of tRNA^{Ile}, noncognate valyl adenylate is destroyed before transfer to tRNA^{Ile} (Fersht, 1977). It would be of interest to determine whether tRNA-dependent pretransfer editing has importance in overall amino acid selection, and whether it occurs at the same hydrolytic site as posttransfer editing. To investigate this question, mutant enzymes with decreased deacylation toward Val-tRNA^{Ile} and that retain the ability to form valyl adenylate would be required.

The tRNA synthetases specific for arginine, glutamate, and glutamine are different from all other class I synthetases in that they require bound cognate tRNA in order to achieve amino acid activation (Ravel et al., 1965; Mehler & Mitra, 1967; LaPointe & Söll, 1972; English-Peters et al., 1991). While it is unclear how related the tRNA-dependent editing reaction of IleRS is to the tRNA-dependent amino acid activation reaction of argininyl-, glutamyl-, and glutaminyl-tRNA synthetases, each case may reflect an ancestral condition in which cognate tRNA had a role in specific amino acid recognition. There appears to be no direct contact between amino acid and tRNA^{Gln} in the cocrystal structure of glutaminyl-tRNA synthetase with tRNA^{Gln} (Rould et al., 1989), suggesting an indirect role for tRNA in amino acid binding in this system. The phenotypes of our mutations within the peptide 1 segment of CP1 indicate that some CP1 residues mediate tRNA-dependent amino acid selectivity in IleRS. Possibly, this discrimination is coupled with acceptor helix interactions.

IleRS has been placed in a subgroup of five class I enzymes which are more related in sequence to each other than to the other five class I enzymes (Eriani et al., 1991; Hou et al., 1991). This subgroup includes synthetases specific for valine, isoleucine, leucine, methionine, and cysteine. Interestingly, three of these enzymes have well-characterized editing activities. Valyl-tRNA synthetase primarily uses a fast posttransfer editing reaction to reject the noncognate threonine (Fersht & Kaethner, 1976). As with IleRS, editing is believed to occur via a separate hydrolytic site. Conversely, methionyl-tRNA synthetase uses a single active site to eliminate homocysteine misactivation in a manner that does not require tRNA^{Met} (Jakubowski & Fersht, 1981). Additionally the posttransfer editing activity of methionyl-tRNA synthetase is relatively slow (Jakubowski, 1993), indicating that the editing mechanism of this synthetase may be different from that of isoleucyl- and valyl-tRNA synthetases.

In light of these facts, it is interesting to note that, in published sequence alignments between isoleucyl-, valyl- and methionyl-tRNA synthetases (Shiba & Schimmel, 1992), the region corresponding to peptide 1 (and the surrounding region) of isoleucyl-tRNA synthetase is present in valyl- but not methionyl-tRNA synthetase. We suggest that this part of CP1 plays a general role in tRNA-dependent amino acid recognition. Possibly, the large CP1 insertions of the valyl- and isoleucyl-tRNA synthetases arose later in tRNA synthetase evolution when a need for higher accuracy in amino acid selection developed. Methionyl-tRNA synthetase, due to its ability to reject homocysteine using a single active site, may not have required this addition and has a much smaller (186 fewer residues than IleRS) CP1 insertion (Burbaum et al., 1990).

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