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Structural Diversity and Protein Engineering of the Aminoacyl-tRNA Synthetases

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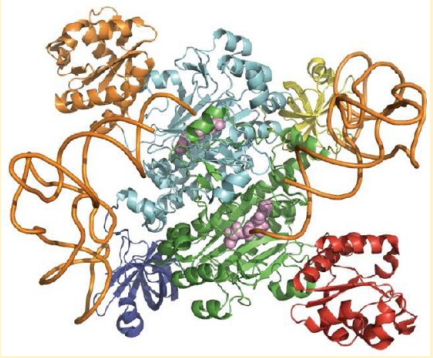
ABSTRACT: Aminoacyl-tRNA synthetases (aaRS) are the enzymes that ensure faithful transmission of genetic information in all living cells, and are central to the developing technologies for expanding the capacity of the translation apparatus to incorporate nonstandard amino acids into proteins in vivo. The 24 known aaRS families are divided into two classes that exhibit functional evolutionary convergence. Each class features an active site domain with a common fold that binds ATP, the amino acid, and the 3′-terminus of tRNA, embellished by idiosyncratic further domains that bind distal portions of the tRNA and enhance specificity. Fidelity in the expression of the genetic code requires that the aaRS be selective for both amino acids and tRNAs, a substantial challenge given the presence of structurally very similar noncognate substrates of both types. Here we comprehensively review central themes concerning the architectures of the protein structures and the remarkable dual-substrate selectivities, with a view toward discerning the most important issues that still substantially limit our capacity for rational protein engineering. A suggested general approach to rational design is presented, which should yield insight into the identities of the protein−RNA motifs at the heart of the genetic code, while also offering a basis for improving the catalytic properties of engineered tRNA synthetases emerging from genetic selections.



The enduring fascination of the aminoacyl-tRNA synthetases (aaRS) arises from their central role in mediating information transfer in all cells.[1,2] The synthesis of aminoacyl-tRNA by the aaRS, not the subsequent use of this product by the ribosome, is the true step in translation at which the chemical language of nucleic acids is converted into that of proteins. Embedded in the highly differentiated architectures of the aaRS, in ways still poorly deciphered, are the motifs and stereochemical determinants that enable the specific couplings of amino acids and tRNAs. We recognize, then, that these enzymes "know" the genetic code. To thoroughly understand aaRS function, it is necessary to identify the key structural elements that operate to exclude all possible pairings except for the correct ones. This ambitious goal has not been accomplished for any of the enzymes.

The nearly universal nature of the genetic code suggests that the aaRS are of ancient origin, possibly replacing RNA-based enzymes that may have existed before the last universal common ancestor (LUCA).[3,4] However, contemporary aaRS do not exhibit vertical inheritance from a common ancestral enzyme but are instead divided into two structurally disparate classes (class I and class II) containing 11 and 13 aaRS families, respectively (Table 1).[5−8] All aaRS of both classes bind and juxtapose the reactive moieties of ATP, the amino acid, and tRNA to catalyze the same general two-step reaction; thus, the two classes exhibit functional evolutionary convergence.[4] Within each class, the enzymes possess some overall architectural similarity, but only the topologies of the catalytic domains and a few sequence motifs in the active sites are strictly conserved. Within each family distinguished by its amino acid type, extents of sequence similarities are generally high only for enzymes from closely related organisms, while detailed domain arrangements and even quaternary structures may differ over greater evolutionary distances (Table 1).[1]

Minimal protein architectures for the aaRS are generally found among the Bacteria and Archaea, while eukaryotic aaRS frequently possess additional domains.[9] For some of these enzymes, the added domains are required for association with a large multi-tRNA synthetase complex (MARS) found in organisms ranging from *Drosophila* to humans, which may enhance the efficiency of protein synthesis by a channeling mechanism.[9−12] Nine of the cytoplasmic aaRS are known to be part of the MARS, which also contains several accessory RNA-binding proteins. Many of the added domains also impart novel functions.[13,14] For example, human cytoplasmic glutaminyl- and leucyl-tRNA synthetases (GlnRS and LeuRS, respectively) function as amino acid binding sensors to trigger glutamine-dependent apoptosis and leucine-dependent cellular proliferation, respectively.[15,16] In some cases, these new functions also implicate the aaRS in human disease, providing further impetus for study.[17,18] Advances in understanding fundamental amino-

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Table 1. Classification and Status of Structure Determinations of Aminoacyl-tRNA Synthetases

					catalysis[b]			
		quaternary structure	subunit size[a]	tRNA coxtl	step 1	step 2	editing	published X-ray structures
IA[c]								
	Met	α,α_2	677 (α_2)	yes[d]	yes	no	yes[e]	51, 211−217
	Leu	α	860	yes	yes	yes[34]	yes	34, 36, 140, 218−224
	Ile	α	939	yes[f]	yes	no	yes	35, 225−227
	Val	α	951	yes[f]	yes	no	yes	37, 228, 229
IB								
	Cys	α,α_2	461 (α)	yes[g]	no	no	no	50, 230
	Gln	α	553	yes	yes	yes[120]	no	31, 113, 120, 132, 135, 136, 201, 231−235
	Glu	α	471	yes	yes	yes[236]	no	33, 42, 114, 236−240
IC								
	Tyr	α_2	424	yes	yes	no	no	23, 38, 169−171, 241−253
	Trp	α_2	334	yes	yes	no	no	25, 97, 250, 254−271
ID								
	Arg	α	577	yes	yes	yes[272]	no	32, 272−274
IE								
	Lys	α	533[h]	no	no	no	no	275
IIA								
	Ser	α_2	430	yes	yes	no	yes[e]	74, 93, 276−282
	Pro	α_2	572	yes[d]	yes	no	yes	72, 118, 283−285
	Thr	α_2	642	yes	yes	yes[286]	yes	71, 75, 286−294
	Gly	α_2	506[i]	no	yes	no	no	69, 134, 295−297
	His	α_2	424	no	yes	no	no	68, 118, 298−301
IIB								
	Asp	α_2	590	yes	yes	yes[302]	no	52, 62, 96, 119, 302−313
	Asn	α_2	465	no	yes	no	no	64, 314
	Lys	α_2	505	no	yes	no	yes[e]	63, 315−318
IIC								
	Phe	$(\alpha\beta)_2,\alpha$	327, 795	yes	yes	yes[319]	yes	84, 85, 103, 104, 127, 319−330
	Gly	$(\alpha\beta)_2$	303, 689	no	no	no	no	3UFG, 3RF1, 3RGL, 1J5W[l]
	Ala	α_2,α^m	876 (α_2)	no	yes	no	yes	91, 128, 130, 131, 331−334
	Sep	α_4	549[j]	yes[d]	no	no	no	82, 83
	Pyl	α_2	409[k]	yes	yes	no	no	92, 335−338

[a]Refers to the *Escherichia coli* enzyme except where otherwise indicated. [b]Refers to whether structures are available that, taken together, sufficiently illustrate productive binding of the ATP, amino acid, aminoacyl adenylate, and/or tRNA 3′-end (or analogues) so that the basis for aminoacyl adenylate (step 1) or aminoacyl-tRNA formation (step 2) can be deduced. In many cases, structures are available bound to an amino acid, but the basis for catalysis of step 1 and/or step 2 is unclear because productive ATP or tRNA 3′-end binding is not observed (see Figure 4). [c]The subclassifications of the aaRS are based on refs 4 and 92 (see the text). [d]For MetRS, ProRS, and SepRS, tRNA cocrystal structures illustrate anticodon binding only. These may represent initial docking complexes of the enzyme with tRNA. In each case, most of the enzyme−RNA interface is not formed. [e]For MetRS, SerRS, and class II LysRS, the editing activity is limited to pretransfer hydrolysis of noncognate aminoacyl adenylates within the synthetic active site. [f]For IleRS and ValRS, the tRNA 3′-end is visualized only bound in the editing site. [g]For CysRS, the tRNA 3′-A76 is bound unproductively in the ATP binding site. [h]The subunit size is for the enzyme from *Methanococcus maripaludis*.[29] [i]The subunit size is for the enzyme from *Thermus thermophilus*. [j]The subunit size is for the enzyme from *Methanocaldococcus jannaschii*.[5] [k]The subunit size is for the enzyme from *Methanosarcina barkeri*.[7] [l]RCSB PDB codes from http://www.rcsb.org. [m]Early studies reported a tetrameric organization for *E. coli* AlaRS,[339] but more recent work indicates that the enzyme is a dimer.[60] Eukaryotic AlaRS are monomers.

acylation mechanisms are significant in fully appreciating the roles of the added domains, because the entire enzyme architecture of a large eukaryotic aaRS may have adapted over evolutionary time to conduct both canonical and novel functions. The alternative view would be that the new domains are modular units with more segregated function. However, it is known that the added domains can improve aminoacylation kinetics,[19,20] while the novel functions can depend in part on canonical domains.[15] Thus, the former view may be more consistent with available data.

The intention of this review is twofold. First, we provide a comprehensive summary of all aaRS crystal structures determined to date. Here we distinguish the unique tRNA binding modes in each class and clarify which tRNA cocrystal structures provide suitable models for the tRNA aminoacylation reaction. After a brief review of the catalytic mechanism, we also describe all the distinct amino acid binding sites in the synthetic domains of the enzymes and analyze the need for editing reactions and the basis for structural discrimination in the post-transfer editing domains for the relevant enzymes. General themes regarding the origins of tRNA discrimination and the tRNA identity problem are also covered. Second, we describe the successes and limitations associated with the approach to expanding genetic codes in vivo, which primarily relies on genetic selections to create engineered tRNA synthetases containing novel amino acid binding pockets for nonstandard residues.[21] Finally, we assess the challenges to rational protein engineering of the aaRS as a complement to the genetic selections. Insight into the identities of the protein structure motifs that mediate selective amino acid−tRNA pairing may be

crucial to using rational design to improve the catalytic functioning of enzymes emerging from selections. A general approach that may be useful for achieving this goal is presented.

STRUCTURES OF AMINOACYL-TRNA SYNTHETASES

Classification, tRNA Binding Modes, and Evolutionary Relationships. *Class I aaRS.* The catalytic domains of all class I aaRS adopt a dinucleotide or Rossmann fold (RF), as first described for TyrRS.[22−24] The domain is located near the N-terminus and features a five-stranded parallel β-sheet connected by α-helices. There is pseudo-2-fold symmetry with the ATP and amino acid binding on opposite sides of the symmetry axis (Figure 1). In all class I aaRS, the RF is split by an inserted domain [connective peptide I (CPI)], which adopts a mixed α/β fold and binds the 3′-single-stranded end of tRNA in the monomeric enzymes (Figures 1 and 2 and Table 1). In the dimeric TyrRS and TrpRS, this inserted domain instead forms the dimer interface.[23,25] The CP1 domain is greatly enlarged in IleRS, ValRS, and LeuRS, to incorporate a distinct catalytic site for post-transfer editing hydrolysis of misacylated tRNA.[2,26] Two conserved sequence motifs, HIGH and KMSKS, are present in the first half of the RF and in a loop immediately following the second half, respectively, and are involved in ATP binding (Figure 3). The carboxyl-terminal domains of most class I aaRS (LeuRS is a notable exception) bind the tRNA anticodon region and contribute significantly to tRNA discrimination, but their structures are in general divergent, even within subclasses. Additional segments of the polypeptide chain at the N- and C-termini, and other insertions within or immediately after the RF, are present idiosyncratically among the class I aaRS.

The most definitive subclassifications of the aaRS are based on multiple structural alignments to construct accurate phylogenies.[4] Results of this work are largely consistent with prior sequence-based phylogenies,[3,27] albeit with a few significant discrepancies, and are summarized in Table 1. ArgRS is the most deeply rooted class I enzyme and occupies a separate subclass (ID), while CysRS is grouped with the class IB GlnRS and GluRS, which possess an interrelated evolutionary history (see below).[3,28] MetRS is grouped with the larger editing enzymes that are specific for amino acid substrates possessing aliphatic side chains, while dimeric TrpRS and TyrRS are more closely related to each other than to other class I enzymes and form a separate subclass. Finally, the class I LysRS also occupies a separate subclass (IE). LysRS is the only aaRS known to violate the class rule, as distinct enzymes are present in both class I and class II (Table 1).[29] The deduced coevolutionary scenario of LysRS-I and LysRS-II suggests that LysRS-I emerged first but was later replaced by LysRS-II in many lineages.[3,4,27] In contemporary organisms, LysRS-I remains in many branches of contemporary Archaea and in some Bacteria but is not found in eukaryotes. A subclass of methanogens in the Archaea, the *Methanosarcina*, possess both LysRS-I and LysRS-II.[30]

In the nine monomeric class I aaRS, a single tRNA molecule binds across a large portion of the enzyme surface from the anticodon stem−loop region to the active site (Figure 1).[31] The tRNA is often distorted from its unbound conformation in many local regions of its structure, particularly at the 3′-end and in the anticodon loop, but all tRNAs maintain the canonical L-shape tertiary structure when bound. The RF binds the acceptor stem of the tRNA from the minor groove side, and the

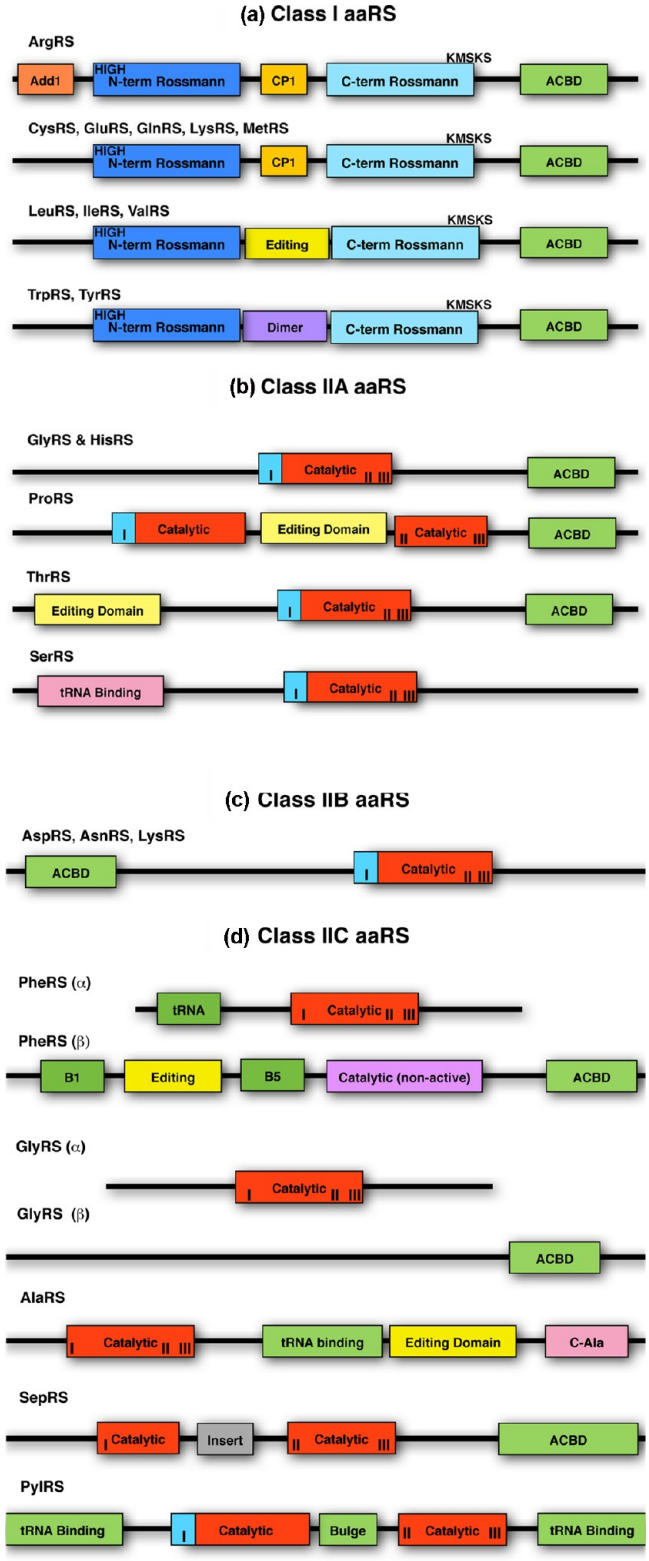

Figure 1. Primary structures and principal domains of tRNA synthetases. (a) Class I aaRS. The Add1 domain (orange) found in all ArgRS is responsible for D-loop recognition in the tRNA core.[32] The anticodon-binding domain (ACBD) forms a topologically identical α-helix cage in LysRS and some GluRS, and a pair of β-barrels in GlnRS and a separate subclass of GluRS.[31] ArgRS, CysRS, MetRS, ValRS, IleRS, and LeuRS possess a distinct structurally conserved α-helical anticodon-binding domain. The post-transfer editing domain is part of an enlarged CP1 in IleRS, ValRS, and LeuRS. TyrRS and TrpRS are dimers. Positions of the HIGH and

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Figure 1. continued

KMSKS motifs are indicated. (b) Class IIA aaRS. The conserved class II catalytic domain possesses three conserved motifs (I, II, and III). The post-transfer editing domains of ProRS and ThrRS are located between motifs II and III of the catalytic domain and at the N-terminus, respectively. The ProRS editing domain is found only in the bacterial enzymes; archaeal and eukaryotic ProRS may rely more on pretransfer editing or utilize N- and/or C-terminal extension domains.[77,340] SerRS does not recognize the anticodon; in place of the ACBD, it possesses an N-terminal domain that binds the tRNA core region. The anticodon-binding domain (ACBD) forms a conserved mixed α/β structural topology. The positions of the dimer interfaces are colored light blue and include motif I. (c) Class IIB aaRS. The conserved N-terminal anticodon-binding domain in all three enzymes adopts an OB fold, and this is followed by the class II catalytic domain. The dimer interface is colored light blue and includes motif I. (d) Class IIC aaRS. Tetrameric $(\alpha\beta)_2$ PheRS possesses one active and one inactive subunit, as indicated; B1 and B5 are DNA binding-like domains. The quaternary structures of PheRS and SepRS (Figure 2F) are composed of peptides from many parts of each chain. Like PheRS, the $(\alpha\beta)_2$ GlyRS tetramer in this class also possesses two active and two inactive chains; parts of the protein responsible for quaternary structure and most tRNA binding motifs are not well characterized. In AlaRS, the dimer interface is associated with the C-Ala domain. The bulge and tRNA binding domains of PylRS are unique to that enzyme, which dimerizes similarly to the class IIA and class IIB aaRS.

3′-end of the tRNA adopts a hairpin structure to bind in the active site (Figure 1). Details of the 3′-hairpin conformation and its stabilization by protein differ among the enzymes: the closing 1−72 base pair of the acceptor stem is broken to facilitate binding in several cases,[31−33] although this is not required.[34] Binding of tRNA to IleRS, ValRS, and LeuRS involves two modes: binding to the RF domain by hairpinning of the 3′-end, for aminoacyl-tRNA synthesis, and binding to the CP1 editing domain, in which the path of the 3′-end follows the trajectory of the acceptor stem helix (Figure 1).[34−37] Translocation of tRNA between these two active sites is a critical part of the editing mechanism. Finally, the dimeric TyrRS and TrpRS exhibit cross-subunit binding of two tRNAs, forming a tetrameric complex (Figure 1). In contrast to the monomeric class I aaRS, the RF domains in the dimers bind the tRNA acceptor stems in the major groove; this feature is otherwise a characteristic of class II aaRS.[38] Cocrystal structures with tRNA are available for all the amino acid specificities in class I aaRS except for LysRS-I and a phylogenetically restricted variant of GluRS present in some bacteria (GluRS2), which is specific to Glu-tRNA^{Gln} synthesis (Figure 7).[39,40] The structure of a tRNA complex for the more common nondiscriminating GluRS (GluRSND),which is capable of both Glu-tRNA^{Glu} and Glu-tRNA^{Gln} synthesis,[41] is available bound in a ternary assembly with the tRNA-dependent amidotransferase GatCAB.[42,43] In a two-step pathway, GatCAB and its archaeal homologue GatDE convert Glu-tRNA^{Gln} to Gln-tRNA^{Gln} in organisms possessing GluRSND and lacking GlnRS. This occurs in all archaea, many bacteria, and eukaryotic organelles.[44]

Further examples of distinct tRNA binding modes in class I aaRS may occur in the dimeric forms of MetRS and CysRS. Some bacterial and archaeal MetRS possess an additional C-terminal domain appended to the core region,[45] which promotes dimerization and enhances tRNA binding affinity.[46] Dimeric MetRS have been observed to bind two tRNAs anticooperatively.[47] Eukaryotic CysRS also possess a C-terminal domain, which is structurally unrelated to that found in MetRS.[48,49] However, in these enzymes, the dimer interface is instead located in the common CP1 domain, as found for TrpRS and TyrRS.[19] It is not known whether eukaryotic CysRS bind one or two tRNAs. In both dimeric MetRS and CysRS families, the remainder of the enzyme is closely homologous to the core structure of the monomeric proteins, each of which exhibits the canonical class I tRNA binding mode.[50,51] Binding of two tRNAs to a class I aaRS dimer in the canonical class I mode has not yet been observed but remains possible in these cases.

Class II aaRS. The common catalytic domain found in all 13 class II aaRS families is organized as a seven-stranded β-sheet flanked by α-helices, as first described in the structures of SerRS and AspRS (Figures 1 and 2).[52,53] As in class I aaRS, this common domain binds and properly juxtaposes the amino acid, ATP, and the 3′-terminus of tRNA for the catalytic reactions. Most class II aaRS are homodimers, although examples of monomeric as well as α_4 and $(\alpha\beta)_2$ tetrameric quaternary organizations are known in subclass IIc, the most heterogeneous of the three subclasses (Table 1).[1,54−59] Three conserved motifs are present in the active site domains of class II aaRS.[8] Motif 1 forms a long α-helix followed by a short strand that terminates in a conserved proline and is involved in dimer interface formation in most of the enzymes (Figures 1 and 2),[60,61] while motifs 2 and 3 are found directly in the active site in a pair of antiparallel β-strands connected by a loop and in a strand−helix substructure, respectively (Figure 3).[8,53] Motif 3 binds ATP, while motif 2 plays a key role in coupling ATP, amino acid, and 3′-tRNA binding (Figure 3).[62] In its ATP binding function, motif 3 may be likened to the HIGH and KMSKS motifs of class I enzymes, but there is no analogous motif in class I aaRS that plays the central role attributed to motif 2.

The structure-based phylogenetic analysis of class II aaRS, also based on the structures of the conserved catalytic domain, reveals three subclasses (Table 1).[4] Less structural and sequence divergence exists in the class II catalytic domains than in class I, suggesting that the latter enzymes may be of more ancient origin. A notable feature of the class II phylogeny is the evolutionary path of the polyphyletic GlyRS, which, like LysRS-I and LysRS-II, is present as two forms in distinct classes that do not directly share a common ancestor of that specificity.[3,4] Unlike LysRS, however, both forms of GlyRS are class II enzymes. The α_2 homodimeric GlyRS in subclass IIA conforms well to the features of that subclass, while the $(\alpha\beta)_2$ tetramer in subclass IIC diverges greatly even within this subclass.[4]

Subclass IIA and subclass IIB aaRS possess subclass-specific anticodon-binding domains (Figure 1). Although located in distinct parts of the primary structures, they occupy a similar position in the tertiary architecture with respect to the catalytic domain. Subclass IIB enzymes are specific for charged and large polar amino acids and possess a common anticodon-binding domain at their N-termini, which forms a β-barrel of the OB fold variety (Figure 1).[52,63,64] Only relatively minor differences in overall structural organization exist within this subclass. As with class IB GluRS, the class IIB AspRS enzymes can also be divided into discriminating and nondiscriminating (AspRSND) subgroups.[65] The canonical discriminating AspRS catalyze synthesis of Asp-tRNA^{Asp}, while AspRSND synthesizes both Asp-tRNA^{Asp} and Asp-tRNA^{Asn}.[66] The phylogenetic distribution of AspRSND is more restricted than that of GluRSND: it is

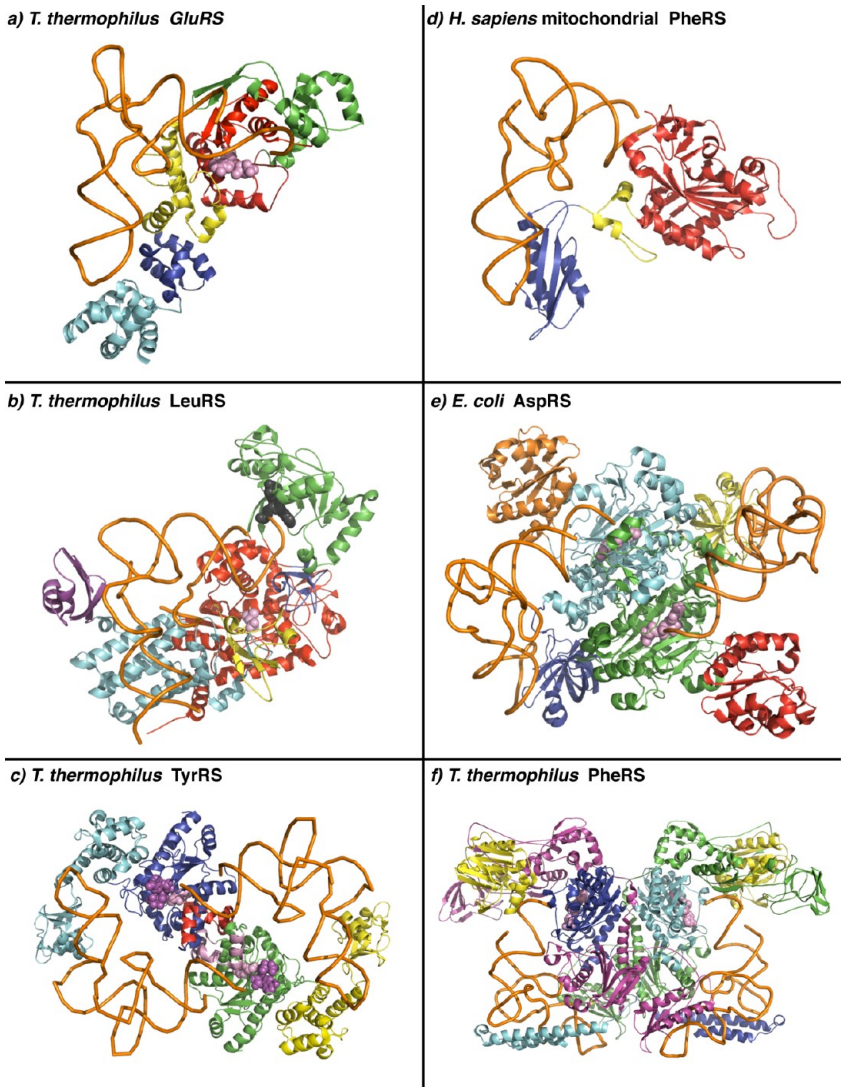

Figure 2. tRNA binding modes. (a) Structure of monomeric class I *T. thermophilus* GluRS bound to tRNA (orange) and ATP (pink, space filling) [Protein Data Bank (PDB) entry 1N77]. The tRNA 3′-end hairpins into the active site to bind adjacent to ATP. The catalytic Rossmann fold is colored red; the CP1 domain bridging the two halves of the Rossmann fold is colored green, and anticodon-binding domains are colored light and dark blue. A bridging subdomain (yellow) connects the catalytic and anticodon-binding domains. (b) Structure of monomeric class I *T. thermophilus* LeuRS bound to tRNA and the inhibitor AN2690 (PDB entry 2V0G). The inhibitor (black, space-filling) is bound to the green post-transfer editing domain, and the 3′-end of the tRNA is also oriented toward this domain, in a configuration suitable for editing. The synthetic active site is marked by the binding of leucine at the center (pink, space-filling). The Rossmann fold is colored red and the anticodon-binding domain light blue. An idiosyncratic C-terminal extension that binds the tRNA tertiary core is colored purple (top left). Several small subdomains specific to LeuRS and involved in tRNA translocation are colored yellow and dark blue. The tRNA anticodon loop and a portion of the D-loop are disordered. (c) Structure of dimeric class I TyrRS bound to two tRNAs, two ATPs, and two tyrisinols (space-filling) (PDB entry 1H3E). The Rossmann fold catalytic domains of the two monomers are colored dark blue and green, with the CP1 dimer interface domains colored red and pink. ATP and tyrosinol (space-filling in purple and pink, respectively) occupy the two active sites. The C-terminal anticodon-binding domains of each monomer are colored light blue and yellow, respectively. (d) Structure of monomeric class II human mitochondrial PheRS bound to tRNA (PDB entry 3TOP). The class II catalytic domain is colored red and the anticodon domain blue. (e) Crystal structure of dimeric *E. coli* AspRS bound to two tRNAs (PDB entry 1C0A).[302] Aspartyl-AMP, shown in pink space-filling representation, occupies each active site. One monomer chain is colored light blue (catalytic domain) and dark blue (anticodon-binding domain), while the other monomer is colored green (catalytic domain) and yellow (anticodon-binding domain). Each tRNA makes contact with only one monomer. An insertion domain specific to bacterial enzymes is colored red and pink in each subunit. (f) Structure of the class IIC PheRS $(\alpha\beta)_2$ tetramer bound to two tRNAs (PDB entry 2IY5).[319] The catalytic α-chains of each $\alpha\beta$ unit are colored dark blue and light blue, respectively. The larger, inactive β-subunits are colored purple and green. The editing domains on each β-chain (B3/B4 domain) are colored yellow. The dark blue α-chain associates primarily with the purple β-chain. The tRNAs bind across subunits; each tRNA makes contact with all four enzyme subunits. Active sites are bound by the phenylalanyl adenylate analogue PheOH-AMP (pink, space-filling).

present in many archaea and bacteria but is not found in eukaryotic organelles.[67] In organisms lacking both GlnRS and AsnRS, the GatCAB enzyme is generally of dual specificity, converting both Asp-tRNA^{Asn} and Glu-tRNA^{Gln} into their respective cognate products for use on the ribosome.[44,67]

Subclass IIA aaRS are specific for hydrophobic and small polar amino acids (Table 1). In these enzymes, the anticodon-binding domain is instead at the C-terminus and consists of a five-stranded mixed β-sheet surrounded by three α-helices.[68,69] The only enzyme that lacks this domain is SerRS, which does

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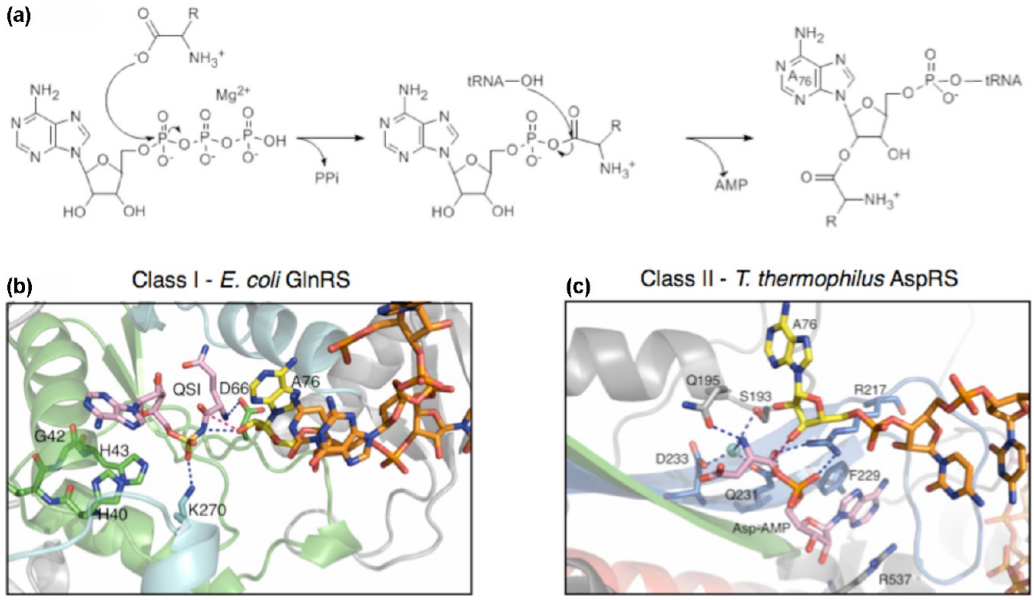

Figure 3. (a) Mechanism of aminoacyl-tRNA formation common to all aaRS. Mg^{2+} is required for the first step. In the second step, the nucleophilic oxygen that attacks the carbonyl carbon of the mixed anhydride of the aminoacyl adenylate is the 2′-OH of tRNA A76 in class I aaRS and in PheRS and the 3′-OH of tRNA A76 in the other class II enzymes. (b) Structure of *E. coli* GlnRS bound to $tRNA^{Gln}$ and the glutaminyl adenylate analogue 5′-*O*-[*N*-(L-glutaminyl)sulfamoyl]adenosine (QSI) (PDB entry 1QTQ).[135] In QSI, the phosphorus is replaced with sulfur and the bridging oxygen substituted with an amine. QSI is colored pink. The position and interaction of class I motif residues from HIGH (H40 and H43) and KMSKS (K270 represents the second Lys) are shown. Hydrogen bonds are shown as blue dotted lines. The red dotted line depicts the modeled position of the attack of 2′-OH on the carbonyl carbon of the mixed anhydride of Gln-AMP. The hairpinned tRNA 3′-end, a characteristic of class I monomers, is colored orange at the right with 3′-A76 colored yellow. (c) Crystal structure of *T. thermophilus* AspRS bound to $tRNA^{Asp}$ and aspartyl adenylate (PDB entry 1C0A).[302] The aspartyl adenylate is colored pink, the tRNA orange, and the 3′-A76 nucleotide yellow. Hydrogen bonds with the adenylate are shown as blue dotted lines. The position and interactions of class II aaRS invariant residues in motif 3 (Arg537) and motif II (Arg217 and Phe229) are shown. The red dotted line depicts the modeled position of the attack of 3′-OH on the carbonyl carbon of the mixed anhydride of Asp-AMP.

not use the anticodon to distinguish its cognate tRNAs.[70] Unlike class IIB synthetases, which all contain a uridine base at the center position of the anticodon, there are no common features shared among the anticodons recognized by class IIA aaRS, and it may therefore be expected that there is a greater diversity of structural detail in the class IIA anticodon interfaces. However, among the class IIA and class IIB aaRS (Table 1), tRNA cocrystal structures elucidating anticodon binding are available only for AspRS, ThrRS, and ProRS.[52,71,72] Therefore, more general insights are not yet possible.

A distinct SerRS is found in some methanogens,[73] which retains the class II catalytic domain architecture, but possesses both a unique tRNA recognition domain and amino acid binding determinants as compared with the canonical SerRS.[74] Like the canonical SerRS, the methanogen enzyme also lacks the class IIB-specific anticodon-binding domain. While both SerRS types feature an N-terminal domain that is involved in tRNA recognition (Figure 1), in the canonical enzymes this consists of a long antiparallel coiled coil,[53] while the methanogen SerRS possess a much larger mixed α/β domain.[74] ThrRS also possesses an N-terminal extension as compared with other class IIA aaRS; it forms a two-domain structure that contains the catalytic site for editing of misacylated Ser-$tRNA^{Thr}$ (Figure 1).[75] Another class IIA enzyme that conducts post-transfer editing of misacylated tRNA, ProRS, also possesses a distinct editing domain that is inserted into the catalytic domain between conserved motifs 2 and 3.[76,77] SerRS and LysRS also possess editing activities, but these are limited to tRNA-independent pretransfer hydrolysis of misactivated aminoacyl adenylates in the synthetic site (class I MetRS also edits by this mechanism).[78−80] GlyRS, HisRS, AsnRS, and AspRS are not known to possess editing activities.[2,81]

Unlike many of the other subclasses of class I and class II aaRS, the amino acids represented in subclass IIC do not share chemical or structural similarity. It has been suggested that, of the three ancestral proteins from which the three class II subclasses diverged, two were restricted in evolution because of constraints associated with amino acid substrate structure, while the third (subclass IIC) derived its selective value from a greater evolutionary plasticity.[4] The subclass IIC enzymes are the largest and most complex of the aaRS. While sharing the catalytic core domain and active site motifs of the other class II enzymes, they also exhibit the greatest diversity of additional idiosyncratic domains (Figure 1). AlaRS and PheRS exhibit diversity in quaternary structure, and each also possesses a distinct domain dedicated to post-transfer editing (Table 1 and Figure 1).

Two of the subclass IIC enzymes are present in only a limited number of organisms. Phosphoseryl-tRNA synthetase (SepRS) is most homologous to $(\alpha\beta)_2$ PheRS;[5] these two enzymes exhibit a very similar subunit structural organization despite the disparate tetrameric assemblies (Table 1) and are the most closely related pair among the five class IIC enzymes.[82−85] SepRS is responsible for the synthesis of phosphoseryl (Sep)-$tRNA^{Cys}$ in most methanogens and in *Archaeoglobus*. Sep-$tRNA^{Cys}$ is then converted to Cys-$tRNA^{Cys}$ by the pyridoxal phosphate-dependent enzyme SepCysS.[5] Pyrrolysyl-tRNA synthetase (PylRS) is present only in *Methanosarcina* and in some limited groups of bacteria, where it incorporates pyrrolysine (Pyl) in response to UAG stop

codons.[7,86−88] This process requires a unique $tRNA^{Pyl}$ suppressor tRNA of unusual architecture. Incorporation of Pyl is highly efficient in vivo in the native context but also functions in foreign genes containing UAG codons, suggesting that the mechanism is analogous to that underlying amber suppression generally.[89] Both SepRS and PylRS have been adopted as vehicles for genetic code expansion.[88,90]

The quaternary structure organization of class IIC aaRS is generally distinct from that of class IIA and IIB and is also idiosyncratic within this subclass. While little is known in the case of the $(\alpha\beta)_2$ GlyRS, in PheRS and SepRS the tetramers are highly interdigitated, and intersubunit contacts involve peptides from many portions of the structure (Figure 2F). In PylRS, the dimer interface includes motif I and is at the N-terminal end of the catalytic domain, so this enzyme is more similar to the class IIA and class IIB aaRS in this respect (Figure 1). AlaRS is unusual, because the C-terminal C-Ala domain, which bridges aminoacylation and editing functions and interacts with the outer corner of the tRNA L-shape, also forms a key part of the dimer interface.[60,91] All structures of AlaRS so far reported are monomeric fragments of the enzyme.

All class II aaRS adopt a common mode of tRNA binding in which the major groove of the acceptor stem duplex is oriented toward the catalytic domain (Figure 2). This is the basis for a key class-specific distinction, as the monomeric class I enzymes approach the acceptor stem from the minor groove side (Figure 2).[52] The structural information for tRNA binding among class II aaRS is much less comprehensive than for class I: only seven of the 13 families are represented with cocrystal structures [further, for ProRS and SepRS, only anticodon binding is visualized (Table 1)]. The cocrystal structures show that the dimeric AspRS, ThrRS, and PylRS each bind two tRNAs (Figure 2). For the latter two enzymes, the binding of each tRNA involves amino acids from both subunits, while cross-subunit binding is not evident in AspRS.[52,71,92] Cocrystal structures of the SerRS−$tRNA^{Ser}$ complex reveal only one tRNA bound across the two subunits,[93] but solution studies showed that two tRNAs may bind cooperatively under some conditions.[94] The dimeric nature of most class II aaRS is important to function: even in AspRS, which does not exhibit cross-subunit tRNA interaction, mutations in motif 1 at the dimer interface were detrimental.[95] It was also shown that the reduced catalytic efficiency of bacterial AspRS toward eukaryotic $tRNA^{Asp}$ has its origin in asymmetric binding: this heterologous cocrystal structure, the only such example in the field, shows that only one of the two bound tRNAs is properly oriented in an active site.[96] It is worth noting, however, that asymmetric tRNA binding has also been observed in cognate tRNA complexes of ProRS and TrpRS,[72,97] and that half-of-sites reactivity is not uncommon in dimeric and tetrameric aaRS.[98−101] Hence, asymmetry in some aaRS−tRNA complexes may be a natural feature of their interactions.

The PylRS−$tRNA^{Pyl}$ and PheRS−$tRNA^{Phe}$ cocrystal structures each illustrate how idiosyncratic domains specific to these class IIC families contribute to formation of distinctive complexes. PylRS possesses a unique N-terminal domain and C-terminal tail, which together contribute approximately half of the intramolecular interactions with tRNA.[102] Further, $tRNA^{Pyl}$ possesses a unique architecture in its globular core domain,[7] and the enzyme structure has clearly adapted to make unique interactions with this region. Together, these features contribute to the ability of PylRS and $tRNA^{Pyl}$ to function as an orthogonal pair in *E. coli*: interactions with other aaRS and tRNAs in vivo are excluded, permitting the use of PylRS to incorporate noncanonical amino acids (see below).[88] The PheRS $(\alpha\beta)_2$ tetramer possesses a remarkable 10 structural domains per $\alpha\beta$ dimer, many of which are unique. The two structural domains within the α-chain are similar in structure to two of the domains in the β-subunit (Figure 2).[84] The α-chain carries the canonical class II catalytic fold and activity, but essential catalytic residues in the homologous β-chain subunits are missing. Hence, the tetrameric PheRS possesses only two active sites and is observed to bind two tRNAs.[85] Each tRNA makes contacts with all four enzyme subunits. A cocrystal structure of monomeric human mitochondrial PheRS bound to tRNA is also available.[103] The 415-amino acid mitochondrial enzyme is a chimera of the bacterial PheRS α-subunit and C-terminal anticodon-binding domain of the β-subunit and may represent a minimal protein structure capable of highly efficient tRNA aminoacylation. Allowing recognition of both the 3′-terminus of tRNA and the anticodon requires a very large reorientation of the anticodon-binding domain as compared with its position in the apoenzyme.[104]

Catalytic Mechanisms. All aaRS are thought to follow the two-step mechanism demonstrated explicitly by Fersht for IleRS and TyrRS:[105]

$$\text{ATP} + \text{amino acid} \rightarrow \text{amino acid}{-}\text{AMP} + \text{PP}_i$$

$$\text{amino acid}{-}\text{AMP} + \text{tRNA}^{aa} \rightarrow \text{amino acid} - \text{tRNA}^{aa} + \text{AMP}$$

In the first step, one α-carboxylate oxygen of the amino acid attacks the α-phosphorus of Mg-ATP, forming the mixed anhydride linkage in the aminoacyl adenylate with release of pyrophosphate (Figure 3). In the second step, either the 2′- or 3′-hydroxyl group of the *cis*-diol at the 3′-terminal A76 nucleotide of tRNA attacks the carbonyl carbon of the adenylate, forming aminoacyl-tRNA with release of AMP. All class I aaRS, including the dimeric enzymes, catalyze aminoacylation directly to the 2′-OH of tRNA-A76, while class II enzymes, with the exception of PheRS, catalyze aminoacylation directly to the 3′-OH. Thus, the initial site of aminoacylation is generally, but not strictly, correlated with an overall orientation of tRNA binding in which the enzyme associates primarily with the minor groove (class I) or the major groove (class II) of the tRNA acceptor stem (Figure 2). Another class-specific distinction in the mechanism is that class I aaRS are generally limited in rate by release of the aminoacyl-tRNA product ($GluRS^{ND}$ and IleRS are exceptions),[105−108] while class II aaRS are limited by an earlier step associated with formation of aminoacyl-tRNA on the enzyme.

GlnRS, GluRS, ArgRS, and LysRS-I are unable to catalyze aminoacyl adenylate formation in the absence of tRNA, as are all other aaRS.[109−112] While binding of ATP and/or amino acid may be possible without tRNA in these enzymes, productive juxtaposition of the reactive moieties does not occur.[33] Thus, these four aaRS are obligate ribonucleoprotein enzymes with the catalytic activity residing in the protein subunit. Crystal structures of unliganded and tRNA-bound ArgRS, GlnRS, and GluRS demonstrate how tRNA binding induces the functional active site conformation in each case.[32,113,114] All four enzymes are class I monomers, but they are dispersed among three of the five subclasses and so are not closely related by the structures of their catalytic domains (Table 1). The other five class I monomers catalyze aminoacyl adenylate formation with comparable kinetic parameters compared with those of dimeric

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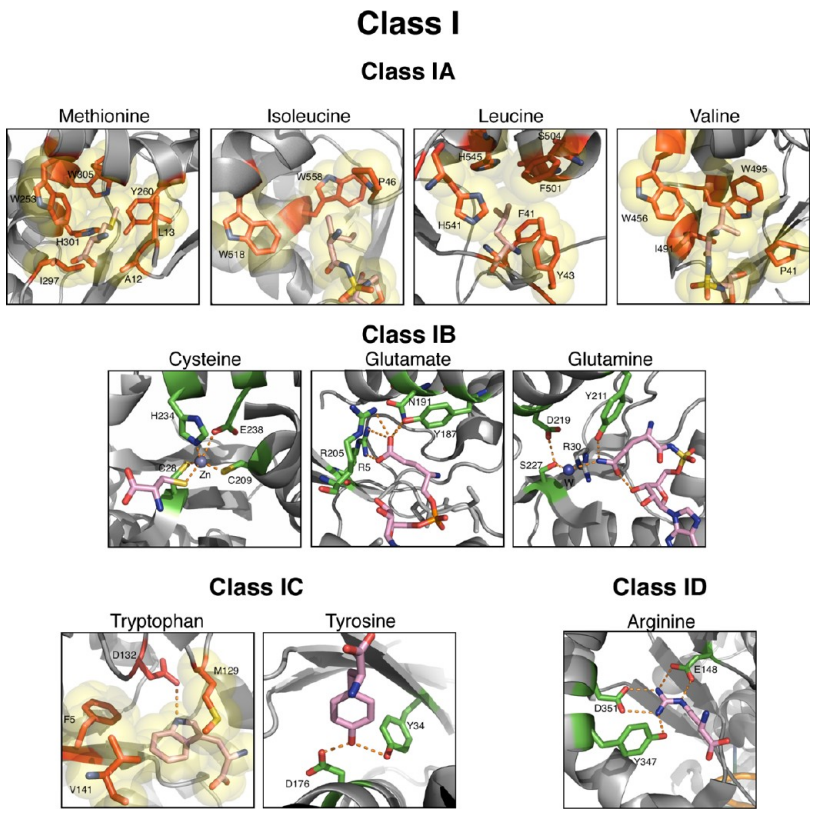

Figure 4. Structures and interactions of substrate amino acids in class I aaRS binding pockets. Interactions with hydrophobic residues are depicted as space-filling van der Waals surfaces. Residue numbers are specific to the crystal structure determined and are usually highly conserved among enzymes of a particular specificity. Substrate amino acids are colored pink, and hydrogen bonds are shown as dotted orange lines. Some structures depict free amino acid binding (Met, Leu, Cys, Trp, Tyr, and Arg), while others depict the aminoacyl adenylate (Ile, Val, Gln, and Glu). Figures were drawn from the following PDB coordinates: MetRS, 1PG2; LeuRS, 2V0G; CysRS, 1LI7; TrpRS, 1MB2; TyrRS, 4TS1; ArgRS, 1F7U; IleRS, 1JZQ; ValRS, 1GAX; GlnRS, 1QTQ; GluRS, 1N78. Coordinates for the published structure of class I LysRS bound to Lys were not deposited in the PDB. That structure may represent a nonfunctional complex because the enzyme requires tRNA for lysyl adenylate formation.[275]

class I or class II aaRS. It is probable that the origin of this distinction among class I monomers lies in the interactions of the catalytic domain with peripheral structural motifs that couple tRNA binding to active site formation. This is an important unsolved problem that also provides a clear functional goal on which to focus protein engineering experiments. In one study addressing this question, a MetRS hexapeptide in a structurally conserved surface loop that binds the inner corner of the tRNA was replaced with the equivalent peptide in GlnRS. This substitution did not cause methionyl adenylate formation to become tRNA-dependent.[115] Interconverting this functional property among the class I aaRS monomers will likely require much more extensive engineering, possibly in multiple parts of the protein−tRNA interface.

For 20 of the 24 aaRS families, structural information is available for ATP, amino acid, and/or aminoacyl adenylate binding [or for binding by analogues of these substrates (Table 1)]. Together with biochemical data, this has provided good insight into the mechanism of aminoacyl adenylate formation in both classes (reviewed in detail in ref 21). The structural studies are consistent with earlier measurements of kinetic isotope effects showing that the reaction proceeds via in-line nucleophilic attack of an amino acid carboxylate oxygen to form a pentacovalent intermediate at the α-phosphorus of Mg-ATP, leading to inversion of stereochemistry at this phosphate.[116] In class I enzymes, this requires proper orientation of a mobile surface loop containing the KMSKS signature motif (Figure 3), which stabilizes the transition state.[117] Ordering and rearrangement of surface peptides, including the motif 2 sequence, are also features of aminoacyl adenylate formation in class II aaRS, although there are substantial distinctions in the different families.[118,119] Differences in the requirements for magnesium ions are also found between class I and class II aaRS as well as among the class II enzymes.[54]

Elucidating the structural mechanism of the tRNA aminoacylation step has proven to be considerably more challenging. This is a significant limitation for protein engineering efforts, because a clear structural basis for the reaction, in which flexible portions of the enzyme and tRNA adopt conformations likely to be similar to that of the transition state, is available for only seven of the 24 families (Table 1). In these seven systems, experimental structures, combined in some cases with careful and limited modeling efforts, and substantiated by biochemical data, give reasonable confidence that the manner in which the reactive moieties of the aminoacyl adenylate and the tRNA 2′- or 3′-hydroxyl group are properly juxtaposed is understood (Figure 3). tRNA binding information is available in 10 other cases, but the reactive 3′-end in these structures is disordered, unproductively bound, or oriented toward a post-transfer editing domain (Table 1 and Figure 2). In every system, extensive induced-fit transitions in enzyme and tRNA are required to reach a ground-state conformation from which the transition state is accessible. The rearrangements are highly idiosyncratic, even among enzymes in the same subfamily. One

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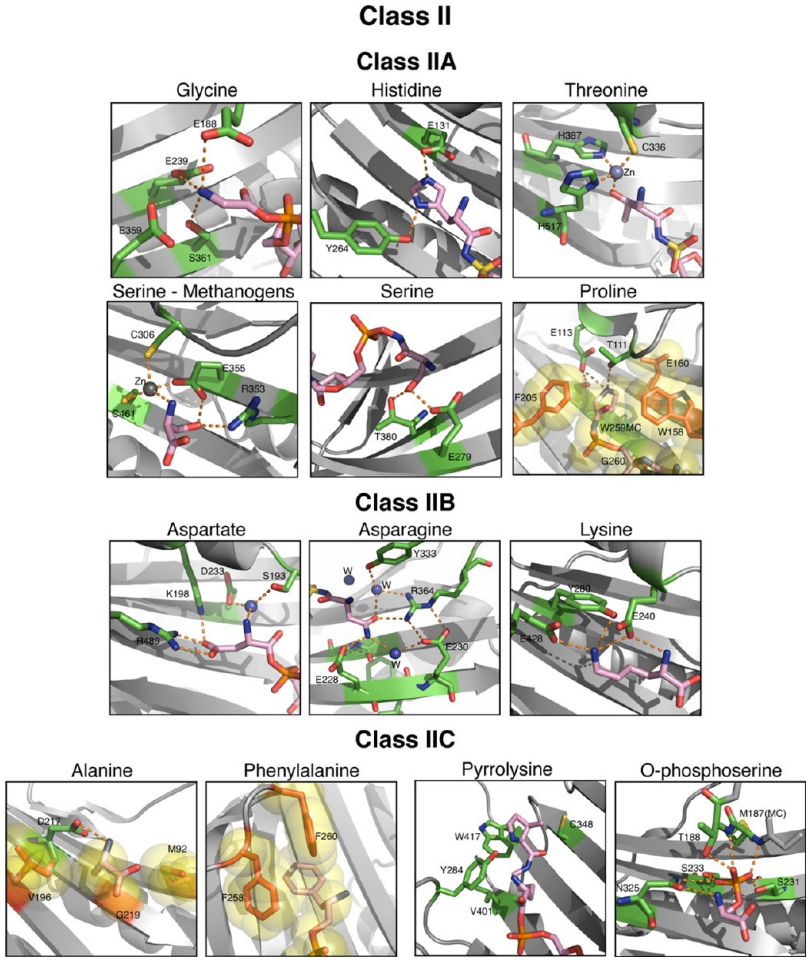

Figure 5. Structures and interactions of substrate amino acids in class II aaRS binding pockets. The depictions follow those for class I aaRS described in the legend of Figure 4. In GlyRS, binding of amino acids possessing a β-carbon is sterically blocked by a carboxylate oxygen of Glu359.[134] Some structures depict free amino acid binding (Ser-methanogen, Lys, Ala, and Sep), while others depict the aminoacyl adenylate (His, Thr, Ser, Gly, Pro, Asp, Asn, Phe, and Pyl). Figures were drawn from the following PDB coordinates: GlyRS, 1GGM; HisRS, 2EL9; ThrRS, 1NYQ; SerRS, 1SES; SerRS (methanogen), 2CJB; AspRS, 1C0A; AsnRS, 1X55; LysRS, 1BBU; AlaRS, 1YFS; PheRS, 2IY5; SepRS, 2DU3; PylRS, 2Q7H.

possible generalization is that the complexity of the structural changes is greater in class II enzymes, particularly among the very large enzymes in subclass IIC.

Among class I aaRS, it so far appears that the reaction mechanism first proposed for GlnRS, in which the phosphate of the aminoacyl adenylate abstracts a proton from the 2′-hydroxyl of tRNA-A76, may be general (Figure 4).[34,120] This mechanism was deduced on structural grounds from the geometry of the active site when tRNA and ATP are bound and is consistent with evolution from a ribozyme-based system and with the absence of conserved amino acids that could facilitate acid−base chemistry. The role of the phosphate group was elegantly confirmed by thio substitution experiments and density functional theory calculations in the class II HisRS system,[121,122] but there is no consensus that this mechanism necessarily also holds in other class II aaRS. Indeed, a novel mechanism has been proposed for ThrRS, in which the vicinal 2′-OH on tRNA-A76 promotes catalysis via proton relay (Figure 4).[123] The possibility that this mechanism may also operate in some other aaRS has not been studied. The existence of further distinct mechanisms also remains possible, because many of the enzymes have not been closely investigated with a view toward addressing this question.

Structural Determinants of Amino Acid and tRNA Specificities. *Amino Acid Specificity.* The current understanding of the origins of amino acid specificity is based mainly on crystal structures, which are available for all families except class IIC GlyRS and class I LysRS. Seven of the aaRS families, representing Ile, Val, Leu, Phe, Thr, Pro, and Ala, possess a second discriminating amino acid binding site in a spatially separate post-transfer editing domain dedicated to hydrolysis of misacylated tRNA (Figures 1 and 2).[2] The synthetic active sites in IleRS, ValRS, LeuRS, ProRS, ThrRS, and AlaRS have to discriminate against slightly smaller, mainly hydrophobic amino acids, where intrinsic binding free energies between cognate and structurally similar noncognate amino acids are too small to generate the roughly 10^4-fold specificity required to maintain adequate coding fidelity (Figures 4 and 5). In these enzymes, the post-transfer editing amino acid binding sites are generally built to exclude the larger cognate substrate, hence functioning by the classical "double-sieve" mechanism.[124]

In addition to Gly-tRNA^{Ala}, AlaRS also synthesizes Ser-tRNA^{Ala} at significant rates,[125] and PheRS rapidly catalyzes Tyr-tRNA^{Phe} formation,[126] even though it might be thought that these larger and more polar amino acids should be readily excluded. It is thus apparent that the synthetic active sites of these two editing aaRS are not optimized for amino acid

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discrimination. Crystal structures of PheRS and AlaRS bound to these noncognate amino acids have revealed how the synthetic active sites accommodate the additional substrate hydroxyl groups by hydrogen bonding, and that the overall orientations of cognate and noncognate substrates are similar.[127,128] An inherent capacity for some structural plasticity in the PheRS and AlaRS binding pockets, although small, is apparently sufficient to permit misacylation of larger noncognate substrates at rates sufficient to require editing. By contrast, rigid binding pockets would more likely preclude juxtaposition of the reactive moieties of the amino acid and aminoacyl adenylate with ATP and tRNA. Because the cognate amino acids of PheRS and AlaRS are smaller than the noncognate amino acids, the post-transfer editing active sites in these enzymes must rely on mechanisms other than steric exclusion. The PheRS editing domain makes hydrogen bonding interactions with the hydroxyl group of tyrosine to distinguish this substrate,[127] while the editing domain of AlaRS uses a zinc-dependent mechanism to bind serine.[129−131] Binding of zinc to the noncognate serine hydroxyl group in the editing domain of AlaRS and to the cognate threonine hydroxyl group in the synthetic domain of ThrRS is similar (Figure 5).

The three remaining editing aaRS, MetRS, class II LysRS, and SerRS, do not possess post-transfer editing domains but instead rely on synthetic site pretransfer editing hydrolysis of misactivated noncognate aminoacyl adenylates.[78−80] tRNA-enhanced pretransfer editing in the synthetic site also contributes significantly in IleRS; this occurs because the rate of transfer of the amino acid to tRNA is unusually slow for both cognate isoleucine and noncognate valine, permitting kinetic competition in the synthetic active site by a hydrolytic water molecule.[108] A similar reaction to hydrolyze cognate glutaminyl adenylate occurs in the otherwise nonediting GlnRS when the 2′-OH group of tRNA-A76 in the active site is substituted with hydrogen.[132] The capacity of aaRS active sites to conduct this hydrolytic reaction may be a significant consideration in protein engineering of novel activities. New enzymes emerging from genetic selections (see below) or rational engineering may well demonstrate suboptimal juxtaposition of reactive substrate groups in the active site, such that competition between water and the tRNA 3′-OH or 2′-OH nucleophile becomes a significant factor in limiting the efficiency of aminoacylation. Such an editing mechanism may indeed be present in a GluRS variant engineered toward Gln amino acid specificity.[133]

All 14 nonediting aaRS are able to discriminate against all possible noncognate amino acids in the synthetic reactions alone. GlyRS uses steric hindrance against any amino acid possessing a β-carbon to ensure selectivity,[134] and almost all of the other aaRS make hydrogen bonding electrostatic and salt bridge interactions with the cognate R group that, based on crystal structures, allow rationalization of the specificities (Figures 4 and 5). The sole exception is GlnRS, as X-ray structures show that the side chain amide makes only ambiguous hydrogen bonds with a tyrosine side chain and with water molecules in the pocket.[135,136] A crystal structure bound to noncognate glutamate shows that these interactions are preserved, with hydrogen bonding polarities reversed to accommodate the additional carboxylate oxygen.[136] Specificity against Glu instead may arise in part from a new interaction of the carboxylate group with a conserved arginine at the base of the pocket, which functions as a negative determinant to drive mispositioning. Further selectivity is possible from a hydrogen bonding interaction of the substrate Gln side chain amide with the ATP ribose; this is the only known example among the aaRS of a distal hydrogen bonding interaction between ATP and the side chain of the amino acid, and it is not formed in the noncognate complex with Glu.[136,137]

tRNA Specificity. Although there are only seven aaRS families in which tRNA−aaRS cocrystal structures permit structural details of the tRNA transfer step in catalysis to be deduced, in seven further cases (CysRS, ValRS, IleRS, TyrRS, TrpRS, SerRS, and PylRS) most of the tRNA interface is formed and likely reliably depicts a functional complex (Table 1). The interface is extensive in all 14 complexes (Figure 2), but complementarity is often not precise, as necessary to maintain only moderately tight K_d values ($\sim 10^{-7}$ M) to facilitate catalytic turnover. Also universally observed are a large number of contacts with the tRNA backbone, significantly more than the number of contacts with discriminating functional groups on the nucleotide bases. Alanine-scanning mutagenesis of 29 amino acids at the GlnRS−tRNA interface showed that removal of specific interactions made with nucleotide bases versus the sugar−phosphate backbone produced roughly equal diminutions in catalytic parameters.[138] Given the much larger number of contacts with the backbone, this indicates that indirect readout, where the tRNA base sequence determines the precise conformation and position of backbone moieties, dominates direct readout of base-specific functional groups.[139] Because all aaRS complexes reveal a preponderance of sugar−phosphate backbone interactions, this finding is probably general.

The notion of indirect readout as an organizing principle for understanding protein−nucleic acid recognition was first applied to simpler DNA duplexes but can be extended to tRNA systems, because all canonical tRNAs (some mitochondrial tRNAs are likely exceptions) possess 14 base stacking layers in the coaxially stacked D/anticodon arm of the molecule (Figure 6).[139] Even though the particular bases involved in the canonical and noncanonical base pairs and triples vary in different tRNAs, the conserved number of base stacking layers provides a basis for understanding how different base sequences alter the detailed path of the sugar−phosphate backbone, in a well-defined context that is analogous to (although more complex than) a DNA or RNA duplex. Combined in vitro mutational experiments with interacting enzyme and tRNA groups offer an approach to evaluating whether particular sugar−phosphate contacts provide specificity by indirect readout or not.[50] Another distinct recognition mechanism comes into play for tRNAs possessing long variable arms ($tRNA^{Ser}$, $tRNA^{Leu}$, and $tRNA^{Tyr}$), because the additional helical arm projects in different orientations from the tRNA body. In these systems, discrimination also occurs at the level of a more global tRNA shape.[38,93,139−141]

All tRNAs contain a set of nucleotides, specific to each amino acid isoaccepting group in a given organism, that defines the tRNA identity.[142,143] Hypotheses regarding which nucleotides are critical are formulated on the basis of genetics, cocrystal structures, and in vitro mutagenesis, but a definitive assessment requires transplantation of the proposed set of nucleotides into a noncognate tRNA framework. The hybrid tRNA is then functionally evaluated either in vivo or in vitro. Identity sets have been proposed and evaluated in all 20 *E. coli* aaRS systems, most yeast systems, and some systems in a few other organisms.[142] These experiments show that tRNA identity nucleotides are often found in the anticodon and in acceptor−stem base pairs nearer to the 5′- and 3′-termini of the molecule. There are also a number of enzymes for which the globular core

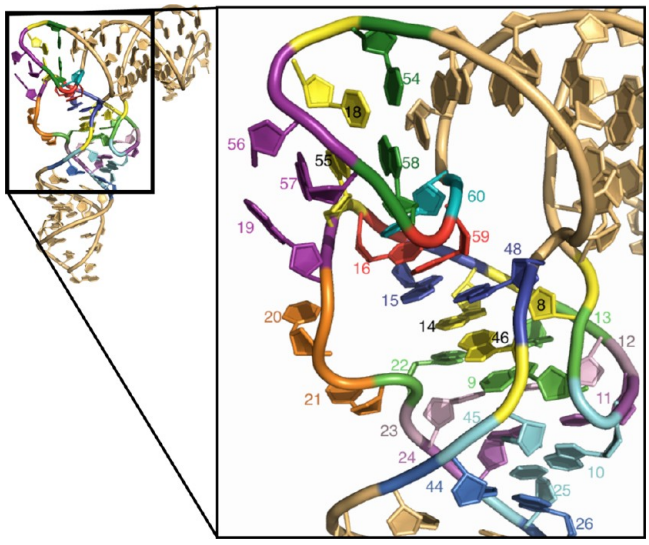

Figure 6. Crystal structure of *E. coli* $tRNA^{Cys}$ as visualized bound to CysRS (PDB entry 1U0B).[50] CysRS relies on shape selectivity among tRNA core regions as an important determinant of tRNA discrimination, with the unusual G15-G48 "Levitt" pair (dark blue at center) providing a key element of the distinct $tRNA^{Cys}$ core.[153] In general, details of base pair and base triple stacking in the tertiary core are controlled by the length and sequence of the D-loop (nucleotides 13−22) and variable loop (nucleotides 45−48). All canonical tRNAs possess 14 base stacking layers in the vertical D/anticodon arm. In $tRNA^{Cys}$, these stacks include U60 (light blue), C16-C59 (red), G15-G48 (dark blue), U8-A14-A46 (yellow), A9-A13-A22 (green), A12-U23 (pink), C11-G24 (purple), A10-U25-U45 (light blue), and A26-C44 (dark blue at bottom), followed by five Watson−Crick base pairs in the anticodon stem. The interdigitated D- and T-loops at the outer angle of the L-shape are shown at the top, with D-loop nucleotides colored yellow and T-loop nucleotides colored green. Detailed base stacking among all these bases ultimately controls the precise conformation of the sugar−phosphate backbone, and possibly also its capacity for deformability, at the interface with synthetase. In general, both positive and negative elements (determinants and antideterminants) for tRNA selection may reside in the tRNA tertiary core (see the text).

region is important. In several cases, post-transcriptional base modifications are crucial to identity, although this is not common.[144−146]

The number of nucleotides sufficient to specify tRNA identity varies in the different systems but may in some cases be 10 or more.[142] AlaRS recognition represents a lower limit, because a single G-U base pair in the acceptor stem (G3-U70), when introduced into noncognate suppressor tRNAs, fully converts tRNA identity in vivo.[147] (Unlike most aaRS, the anticodon is not a recognition element for AlaRS, so the necessary anticodon mutations for this experiment are not confounding.) However, while introduction of the G3-U70 pair improves alanylation of noncognate tRNAs in vitro, the efficiency of aminoacylation appears to be below that of a well-functioning WT aaRS system,[147,148] suggesting that other positive determinants may still be required to fully specify an alanine tRNA. The alternative is that antideterminants may exist in the particular noncognate tRNAs into which the G3-U70 pair was introduced. That is, some noncognate tRNAs may be rejected by AlaRS in part because they adopt a conformation that is incompatible with formation of a fully complementary interface. In such cases, alanylation would not be fully efficient even if all the essential positive signals were present. A common location for antideterminants is the tertiary core of the tRNA (Figure 6), as shown in transplantation experiments to define the identity of *E. coli* $tRNA^{Gln}$.[149,150] In this system, introduction of identity nucleotides into $tRNA^{Asp}$ generated a fully functional tRNA substrate for GlnRS in vitro, but the same experiment with $tRNA^{Glu}$ produced a hybrid tRNA that could not be efficiently glutaminylated. Antideterminants on noncognate tRNAs are important to fully understand tRNA discrimination by all of the aaRS. Moreover, there are no systems into which hypothetical identity sets have been introduced into more than a few noncognate tRNAs for in vitro studies. In this sense, the identity problem remains unsolved because comprehensive information regarding the scope of antideterminants on all noncognate tRNAs is not available for any aaRS system.

Coevolution of aaRS and tRNA sequences is another important theme emerging from tRNA identity experiments.[40,142,151] Enzyme and tRNA of the same amino acid specificity, but deriving from distant portions of the phylogenetic tree, often exhibit weak aminoacylation. However, efficient aminoacylation can be restored by mutating the tRNA, in experiments analogous to those used to determine tRNA identity.[152−154] Investigation of heterologous aminoacylation can be very insightful for structure−function correlations, because Nature has provided a myriad of functionally equivalent but structurally diverse solutions to the same problem.

PROTEIN ENGINEERING OF THE AMINOACYL-TRNA SYNTHETASES

Novel Enzymes Derived from Genetic Selections. Incorporation of unnatural amino acids (UAAs) into proteins in vivo has been conducted by several methods. Replacement of a standard amino acid with a structurally similar nonstandard analogue can sometimes be accomplished using wild-type tRNA synthetases, which have not been subjected to selective pressure against UAA's that are not present in the cell.[155] For example, MetRS is able to incorporate a variety of UAAs, including selenomethionine and homoallylglycine,[156,157] which contain useful functional groups not found in the standard set of amino acids. Site-directed mutagenesis of the synthetic or editing sites of several aaRS has also generated enzymes capable of UAA incorporation.[158,159] The major drawback of this residue-specific approach, however, is that the standard amino acid is replaced at every position where it occurs. Nonetheless, the capacity of an aaRS to tolerate UAAs, together with cocrystal structures of amino acids bound to the enzyme (Figures 4 and 5), can offer a guide to preferred sites at which to target protein engineering efforts, particularly when kinetic parameters for both cognate and UAA aminoacylation reactions are well-described.[160]

Site-specific incorporation of UAAs into proteins in vivo required the development of methods for integrating a novel aaRS−tRNA pair into the translation apparatus.[21] The strategy involves introduction of a new tRNA that typically reads a stop codon, thus making that stop codon into a sense codon for the new amino acid. In addition, a new aaRS is introduced into the host cell that is able to efficiently aminoacylate the new tRNA with an UAA. Two of the most difficult challenges for this experimental program are (i) mutational redesign of the aaRS binding pocket to specifically accommodate the desired new UAA and (ii) ensuring that the new aaRS−tRNA pair is orthogonal to all other such pairs in the cell. Orthogonality

demands that the new tRNA not be aminoacylated with a standard amino acid by one of the endogenous host aaRS, and also that the new aaRS not aminoacylate any host tRNA with the UAA.

These challenges were met with approaches based on positive and negative genetic selections, which generated new orthogonal tRNAs uniquely recognized by an orthogonal aaRS, and new binding sites in the aaRS complementary to the nonstandard amino acid.[21,161−163] More than 70 UAAs have now been incorporated into proteins expressed in bacterial, yeast, or mammalian cells, and the engineered proteins possessing novel functionalities usually produced in high yield. Despite these successes, however, many limitations remain. In particular, because of structural constraints, it is not clear whether the amino acid binding pockets of many of the aaRS can be successfully reengineered within the scope of the library size that can be used in genetic selections. A large number of the UAAs have been incorporated based on engineering a small number of aaRS, particularly the TyrRS from several organisms.[161,164,165] Thus, the library of incorporated nonstandard amino acids is heavily biased toward larger side chains that are structurally similar to tyrosine.[21] PylRS and LeuRS have been engineered by these approaches to incorporate some nonstandard residues,[166,167] but these are also biased toward large, structurally complex amino acids. A further success was obtained with *Pyrococcus horikoshii* class I LysRS, which was engineered to incorporate homoglutamine into proteins expressed in *E. coli*.[168] Despite the successful introduction of orthogonal pairs for a number of other aaRS, including GlnRS, GluRS, AspRS, and PheRS,[21] engineered variants derived from genetic selections that are able to incorporate UAAs in vivo have not been reported in these cases. The diversity of UAAs available for incorporation into cellular proteins would very likely be significantly enhanced if more aaRS could be adapted as platforms for amino acid specificity modification.

Characterizing Evolved aaRS with Novel Amino Acid Specificities. Elucidation of structure−function parameters for evolved aaRS incorporating UAAs can provide fundamental information that should be quite useful in iterative rounds of rational and selection-based mutagenesis. For example, it would be of substantial interest to achieve a clearer understanding of why particular aaRS scaffolds, such as the *M. jannaschii* TyrRS, seem particularly well-suited for engineering, while other aaRS apparently are not. Crystal structures of a number of evolved aaRS have indeed been reported,[169−172] which generally show how the mutated binding sites are more complementary for the nonstandard versus the original cognate amino acid. However, functional characterization has been limited to analysis of the amino acid activation step, which is easily accomplished using the classic pyrophosphate exchange assay in which labeled pyrophosphate is incorporated into ATP.[161,173] A reporter assay based on incorporation of UAAs into green fluorescent protein (GFP) is also available, and this approach is useful for screening a variety of evolved aaRS against panels of UAAs.[169,174] An assay of expressed UAA-containing fluorescing GFP variants in a high-throughput format allows qualitative comparison of UAA incorporation efficiencies in vivo. However, this approach provides no insight into the kinetic properties of the evolved enzymes. Remarkably, no analysis of the aminoacylation efficiency of any evolved aaRS incorporating a UAA has yet been reported. The need for such work is underlined by a recent performance analysis of orthogonal pairs designed for expanding the genetic code in eukaryotes. The experiments suggested that metabolically inert UAAs may accumulate in the cell and thus be incorporated into proteins even if they are weak aaRS substrates.[173] Problems in experimental reproducibility in at least one case also suggest that characterization of the purified enzyme at the level of aminoacylation kinetics should be a standard part of the reported analysis for any engineered aaRS.[165]

The classic assay in which aminoacylation of a ^{3}H-, ^{14}C-, or ^{35}S-labeled amino acid is monitored by incorporation into acid-precipitable tRNA is not useful for measuring the incorporation of UAA by evolved aaRS, because UAAs are generally not available in radiolabeled form. However, the approach presented a decade ago by Wolfson and Uhlenbeck, in which the 3′-internucleotide linkage of tRNA is labeled with ^{32}P by the action of tRNA nucleotidyltransferase, is well-suited to the problem.[175] After aminoacylation with an unlabeled amino acid, the tRNA is hydrolyzed to its component 5′-phosphorylated nucleosides using S1 or P1 nuclease. Unreacted substrate tRNA (p*-A76-3′) and aminoacylated tRNA (p*-A76-aa-3′) are then separated by thin-layer chromatography (TLC) and quantitated.[175,176] This approach is general, requiring only modification of TLC conditions, and has been applied to a variety of aaRS.[106,175,177,178] The assay is capable of yielding highly reproducible steady-state and pre-steady-state parameters for aminoacylation, including substrate affinities derived from kinetics and first-order reaction rates on the enzyme.[179] Its application to evolved aaRS incorporating UAAs would allow comparative analysis of activities, and identification of defects present at specific reaction steps or for binding of ATP, amino acid, or tRNA substrates. Together with X-ray structures, this information would surely assist in the rational design of additional mutations or in choosing particular positions for further rounds of randomization and selection.

Challenges in the Rational Protein Engineering of the aaRS. As is apparent from inspection of Table 1, there are many aaRS systems for which the structural basis for tRNA aminoacylation is not yet understood, because available crystal structures do not depict a productive conformation of the complex in which reactive moieties from the tRNA and other substrates are properly juxtaposed. Thus, much fundamental X-ray work clearly remains to be done. However, there are also outstanding general issues that have yet to be well-studied in any system. First, mechanisms underlying long-range allostery, including identification of specific pathways of signal transduction from distal regions of the enzyme−tRNA interface to the active site, have only begun to be appreciated. The other major unresolved question relates to the interdependence of amino acid and tRNA specificities: the notion that there should exist conserved embedded elements of protein structure that have differentiated over evolutionary time, in each aaRS family, to provide the essential coupling between the RNA and amino acid that defines the genetic code. The challenges in addressing both of these fundamental issues are highly relevant to rational design.

Mechanisms of Allostery. Every tRNA−synthetase complex is thought to progress through a series of first-order conformational changes that follow an initial docking of the two macromolecules. It has long been known that discrimination against noncognate tRNAs in the steady-state aminoacylation reaction is usually manifested more in the maximal velocity than in $K_{m[tRNA]}$.[180] This observation leads to the notion, consistent with more contemporary notions of induced

fit in RNA−protein interactions,[181] that the discrimination against noncognate tRNA arises in part from the adoption of a nonproductive conformation of a noncognate tRNA−enzyme complex that is off-pathway for catalysis. Thus, the binding energy that is liberated to drive the catalytic steps in a cognate complex is not available for noncognates, and the induced-fit rearrangements may be viewed as an element of substrate specificity.[182] The heterologous cocrystal structure of bacterial AspRS bound to eukaryotic $tRNA^{Asp}$ shows clearly that the origins of weak transdomain aminoacylation efficiency in this system reside in a global deformation of the RNA−protein complex.[96] The situation is actually more complex because the induced-fit rearrangements may also be influenced by the proper binding of the ATP and/or amino acid in the catalytic site(s). For example, the findings that the binding affinity of GlnRS for $tRNA^{Gln}$ is some 30-fold weaker when noncognate glutamate occupies the amino acid binding site[179] and that k_{cat} for aminoacylation is also reduced by approximately the same degree suggest that the structural rearrangements of the enzyme−RNA complex may be influenced by which amino acid is bound.

Long-range communication has also been postulated in many aaRS systems based on the identification of tRNA identity elements in the anticodon and tertiary core of the tRNA.[31,183] However, it is important to recognize the nature of the kinetic data required to infer the existence of long-distance signal propagation. Thus, while mutation of anticodon identity elements often leads to significant decreases in k_{cat},[142] this cannot be interpreted as evidence of intramolecular communication because k_{cat} may represent a product release step for the WT enzyme−RNA complex, the mutant complex, or both.[107,177] Thus, a local mutation at the anticodon interface, for example, may have no influence in the catalytic site and yet still affect the magnitude of k_{cat}. In contrast, single-turnover kinetics yields an observed rate that signifies the chemical events in the active site or a preceding linked first-order conformational change associated with active site assembly. This has provided good functional evidence of allostery in several systems.[19,177,184−186] Titration of the amino acid concentration in the single-turnover regime further yields a kinetic K_d for amino acid binding, a derived parameter that reflects a molecular event that is clearly localized in the active site.[138,177]

Molecular dynamics calculations have provided another avenue for studying allostery in the aaRS.[187,188] These approaches can define physical networks of interacting amino acids that partition into local structural communities that are highly intraconnected but communicate with each other by a more limited number of interactions.[187] Analysis of the networks for the degree of evolutionary conservation in apparently critical amino acids can help to validate the computations. In TrpRS, molecular dynamics simulations conducted together with mutagenesis and analysis of divalent metal-dependent kinetics demonstrated that a conserved motif ("D1 switch") located in the distal portion of the Rossmann fold some 20 Å from the active site mediates long-range conformational effects, resulting in stabilization of the transition state for tryptophanyl adenylate synthesis.[189−191] Thus, allostery is a key component of catalytic efficiency in the activation step as well. This was also shown by the classic experiments on tyrosyl adenylate formation by TyrRS, which showed that active site amino acids on mobile surface loops can have selective effects on the transition state for tyrosyl adenylate formation, thus offering evidence of the importance of local structural changes in catalysis.[117] This analysis has been extended to include synthesis of Tyr-$tRNA^{Tyr}$ as well.[192]

Identifying the pathways by which allosteric signals are transmitted, particularly over long distances between the tRNA core region/anticodon arm and the active site, is a related and difficult endeavor. One approach to this is to measure thermodynamic free energy cycles, in which catalytic parameters for enzymes containing single, double, or even triple mutations at distant positions in the structure are compared.[193,194] Although so far mainly used to study amino acid activation, an application of the general approach to studying allostery in CysRS has recently been reported.[195] Of interest also are molecular genetics approaches, by which structural elements far from the anticodon loop enhance suppressor efficiency in vivo, suggesting the importance of particular motifs that should be testable by more detailed in vitro and in silico analyses.[196,197] Finally, more direct observation of dynamics through sophisticated spectroscopic approaches such as NMR has yet to be applied to the aaRS. These approaches may ultimately have the greatest potential to define the pathways of signal transmission more precisely.

Interdependence of Amino Acid and tRNA Specificities: An Approach to Rational Design. Nearly 25 years ago, before the first glimpse of an aaRS−tRNA cocrystal structure, de Duve made the provocative suggestion that the tRNA portion of the aaRS RNP particle might directly interact with the aminoacyl adenylate.[198] This was inspired by the finding that the G3-U70 acceptor−stem base pair of $tRNA^{Ala}$, not far from the alanine attachment site at the 3′-terminus of tRNA, is crucial to alanine identity.[147] Although there are today no examples of direct RNA−amino acid substrate contacts within aaRS complexes, de Duve's proposal also included the possibility that more complex recognition patterns could be involved. His affirmation was that a unique, probably nondegenerate "second genetic code" is written into the structures of the contemporary aaRS, involving conserved structural features perhaps persisting since the origins of the coding apparatus in pre-LUCA cells. Coding is a confusing metaphor for a complex problem of macromolecular stereochemistry,[31,199] but the key insight nevertheless remains. Today, more than 25 years after the functioning of aaRS began to be addressed in molecular detail, we still know almost nothing about the unique structural features, whether protein or RNA, that are responsible for specific RNA−amino acid pairings.

The difficulty in identifying these structural features can be contrasted with the much more straightforward nature of the tRNA identity problem, which is made tractable by the relative simplicity and conformity of tRNA structure. This allows transplantation experiments to be performed and identity sets to be determined, while circumventing the much more detailed and difficult question of precisely how particular identity sets actually function as they do. In contrast, both amino acid specificity and amino acid−tRNA pairing can be understood only in terms of protein structure. Thus, analogous engineering experiments involving specificity swaps are much more challenging. However, they are certainly possible in principle, at least among aaRS of the same class, and should be more practicable among more closely related subgroups within classes.

An approach that we have taken for approaching this central problem is to focus first on asking whether the amino acid specificities of related aaRS can be interconverted by sequence-

and structure-based mutagenesis in and around the active site. Very few prior studies along these lines have been conducted,[200] perhaps mainly because of technical limitations: K_m values for amino acids are fairly high, typically in the range of 10 μM to 1 mM, while concentrations and specific activities of commercially available radiolabeled amino acids are low. Thus, the standard assay involving attachment of a radiolabeled amino acid to tRNA cannot be usefully employed to analyze mutants in which amino acid binding affinities are significantly weakened. Even systematic mutagenesis studies of amino acid binding pockets to assess the roles of individual interactions in conferring efficient cognate aminoacylation have generally not been conducted: our recent study of GlnRS along these lines, studying a variety of point mutants in amino acids that bind glutamine (Figure 4), had little precedent.[137] By contrast, the approach proposed above for the study of new aaRS derived from selections, in which the tRNA is ^{32}P-labeled at the 3′-internucleotide linkage,[175] resolves this issue and allows very high amino acid concentrations to be used. This assay thus makes rational protein engineering experiments to redesign amino acid specificity feasible as well.

By straightforward mutagenesis based on analysis of sequence and structure phylogenies, we have successfully introduced amino acid specificity for glutamate into *E. coli* GlnRS, providing the first example in which the amino acid specificity of an aaRS has been rationally engineered to generate an efficiently functioning enzyme[200,201] (Figure 7). GlnRS first arose in eukaryotes from the duplication of a GluRS gene and subsequent diversification, to replace the more ancient system in which Gln coding was accomplished by the two-step pathway involving the misacylated Glu−tRNA^{Gln} intermediate.[28,202] The specificity conversion was accomplished by swapping in 24 amino acids from human GluRS, including the primary binding site and two bridging loops that do not contact the substrate. Together with subsequent fine-tuning mutations, these experiments generated a set of catalytically robust hybrid enzymes that synthesize Glu-tRNA^{Gln} with high k_{cat}, and with K_m values for glutamate in the low millimolar range. These values are comparable to the equivalent parameters measured for a naturally occurring archaeal GluRSND homologue.[201,203] In contrast, more local mutagenesis of residues that directly bind the amino acid generated only weakly functioning enzymes. Thus, amino acid specificity in GluRS and GlnRS is not solely a property of directly interacting residues but is derived from a larger context of protein structure in which distal residues influence the precise conformation and/or dynamics of the binding site.

By design, none of the mutations introduced in this study were located in the tRNA^{Gln} binding site. Thus, the experiment effectively asks whether amino acid specificity is solely a property of the protein structure. The alternative is that the protein requires assistance from tRNA to generate a fully selective amino acid binding site, the scenario envisioned by de Duve when he suggested a role for tRNA nucleotides in specifying the amino acid.[198] In the case we have studied, K_m for Glu in the hybrids appears to be high (2−5 mM) but does match that measured for the archaeal GluRSND, which also synthesizes Glu-tRNA^{Gln}. In contrast, the glutamate K_m value for the canonical discriminatory *E. coli* GluRS is reported to be 50 μM,[110] while $K_{m[Glu]}$ in human GluRS, from which the Glu binding site was imported, is not known. Regardless of the proper benchmark that should be used, it is at least clear that in this case the protein structure is doing much of the work of amino acid discrimination.

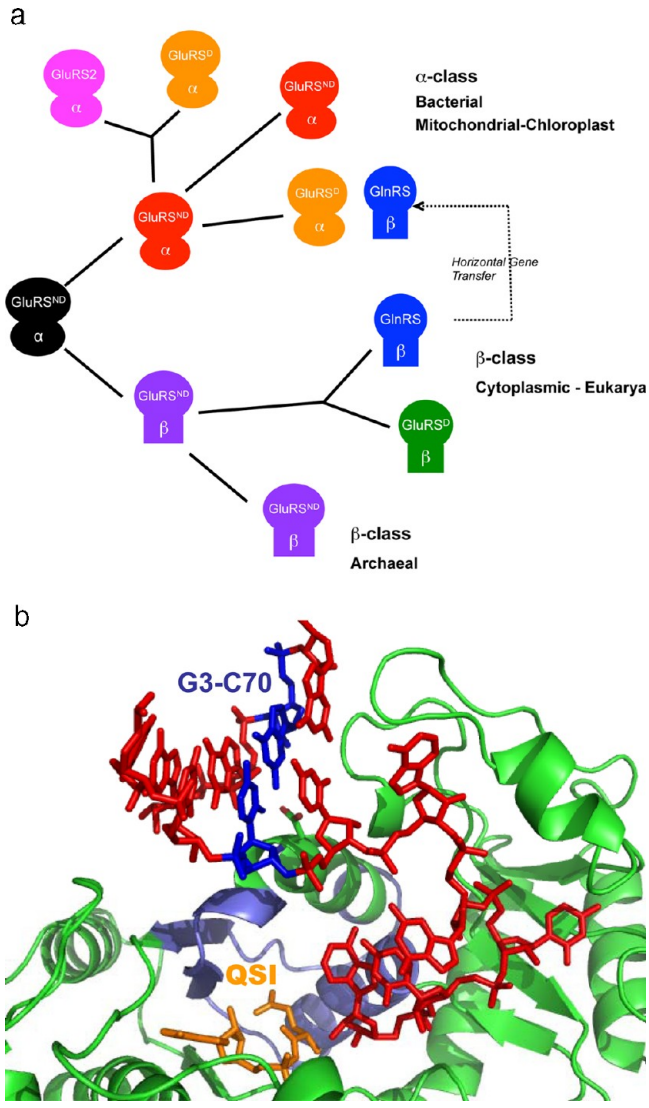

Figure 7. (a) Phylogenetic tree depicting the evolution of GluRS subtypes and the emergence of GlnRS.[3,238] For each depicted enzyme, the larger top oval represents the conserved Rossmann fold catalytic domain while α and β denote α-helical and β-barrel anticodon-binding domains, respectively. The ancestral GluRSND (black) may have possessed an α-helical cage anticodon-binding domain,[238] which was replaced in the archaeal/eukaryotic lineage by a pair of β-barrels. GlnRS is found in contemporary bacteria because of horizontal gene transfer. GluRS2 indicates a variant of GluRS found as a second enzyme in a subclass of bacteria that also contains a GluRS^{D}. GluRS2 functions as a specific misacylating enzyme to synthesize Glu-tRNA^{Gln}. This GluRS subtype appears to be distinct from GluRSND, which aminoacylates both tRNA^{Gln} and tRNA^{Glu}, although a quantitative analysis is lacking.[39,40] (b) Crystal structure of *E. coli* GlnRS depicting binding of the QSI adenylate analogue (orange, at bottom) to the Rossmann fold (green). The proximity of tRNA identity determinants at G3-C70 to the amino acid binding site is evident. The region of protein colored purple was swapped with equivalent sequences from human GluRS to generate hybrid enzymes capable of efficient Glu-tRNA^{Gln} synthesis.

The next step is to inquire about the capacity of the engineered enzyme to bind tRNA. Given the known interdependence of amino acid and tRNA binding, and the

proximity of binding sites in the protein structure, perturbation of tRNA interactions might well be expected in a hybrid enzyme with converted amino acid specificity. Indeed, in this case, we found that K_m values for tRNA are elevated by 100-fold as compared to GluRSND, even though no amino acids at the tRNA binding interface were mutated.[203] Along with much other data, this certainly indicates that the amino acid and tRNA binding sites of GlnRS are in communication with each other through the protein.[113,179,204,205] Detailed consideration of the protein structure, including experimental testing by the introduction of fine-tuning mutations at the interfaces between the swapped-in motif and the surrounding protein structure, was used to provide reasonable assurance that the deficit in tRNA complementarity does not arise from internal lesions in the proteins but rather is a property of still-deficient engineered enzymes in which amino acid and tRNA binding functions are not fully complementary.

These experiments then set the stage for the final and perhaps most challenging step of the rational engineering, which here must involve mutation of tRNA^{Gln} toward tRNA^{Glu} specificity, together with the further interconversion of protein structure motifs that bridge these tRNA^{Glu} identity elements to the active site, thus recapitulating long-range signaling as well. It is these envisioned experiments that have the potential to identify protein−RNA motifs that are essential to the selective matching of the amino acid and tRNA. If the K_m value for glutamate decreases further in these new RNP complexes, then the mutated RNA nucleotides would be considered part of the apparatus determining amino acid substrate selectivity. As with AlaRS, in GlnRS key identity determinants also reside in the acceptor stem, where they are separated from the amino acid binding pocket by just a small peptide motif from the Rossmann fold (Figure 7B). Thus, one possibility is that reconstituting a fully efficient engineered system in this case will require introduction of acceptor−stem tRNA nucleotides that coevolved with the protein-based Glu binding site that has already been swapped in.[40] It is possible that the large number of tRNA and enzyme sequences available from genome sequencing projects could significantly assist in identifying key RNA nucleotides, by employing computational approaches to detect otherwise difficult-to-detect covariations in the RNA and protein. This strategy has been useful in detecting communication between distant residues in the ribosomal protein and rRNA.[206]

tRNA nucleotides found to play important roles in amino acid selection would have to be considered as identity elements in the particular system. It may be likely that these nucleotides will comprise a subset of the full identity set and thus play dual functional roles in both tRNA and amino acid selectivity. Other identity set nucleotides may have no role in amino acid selection, while a third category of tRNA nucleotides may be neutral for tRNA selection but important for amino acid selection. An important earlier study in the GlnRS system established that mutation of known tRNA identity nucleotides influences K_m for glutamine;[204] however, neither affinities nor K_m values for noncognate glutamate were determined, and no tRNA identity swap experiments in any system have been conducted in the context of enzymes with altered amino acid specificity. Thus, the concept of tRNA identity has thus far been limited to effects of nucleotides on tRNA selectivity alone. A role for tRNA nucleotides in amino acid selection is particularly cogent in class I monomeric enzymes such as GlnRS and GluRS, where tRNA is required for aminoacyl adenylate formation. However, multiple roles for tRNA identity determinants may well be a general property of many or all aaRS systems. It is worth noting that the sets of tRNA nucleotides important for aminoacylation of tRNA^{Ile} and editing of Val-tRNA^{Ile} by IleRS are not the same and that, by virtue of influencing the editing reaction, the tRNA in this system is playing an important de facto role in amino acid selection.[207] Thus, in editing enzymes, tRNA nucleotides have multiple opportunities to influence the amino acid selectivity.

The likely need to introduce further protein elements together with RNA, in creating improved hybrid enzymes that fully embody the genetic code for the new amino acid, may seem daunting. However, the aaRS do seem to possess some elements of modularity that may mitigate the challenges. Minimal enzymes (urzymes) consisting of just portions of the catalytic domains are active for amino acid activation in both class I and class II systems[208,209] and may model ancestral core proteins before they became embellished with peripheral domains. In the anticodon region, a single amino acid change of a conserved Arg that binds anticodon nucleotide C36 in tRNA^{Glu} relaxes discrimination to permit recognition of both C36 and the tRNA^{Gln}-specific G36.[114] This amino acid substitution shifts the enzyme's tRNA specificity from GluRS^{D} toward GluRSND. Similarly, exchange of an anticodon-binding loop from AspRS into the homologous LysRS generates a hybrid LysRS capable of lysylating tRNA^{Asp}.[210] Thus, this feature of the protein structure is readily transplanted between enzymes of distinct amino acid specificity.

The approach that we have taken to examine the divergent evolution of selectivity in GluRS and GlnRS is certainly applicable to pairs of related aaRS from other subfamilies. Of particular interest may be an analysis of the TyrRS−TrpRS pair, because the tyrosine enzyme has been very useful as a scaffold for generating new enzymes specific for UAAs. Studies of the SepRS−PheRS pair may also be of interest, because the class IIC active site is broadly capable of recognizing structurally and chemically diverse amino acids, and an orthogonal pair involving PheRS has been constructed. In general, however, it appears likely that construction of orthogonal pairs, although not yet accomplished for many aaRS scaffolds, will not limit the incorporation of further UAAs. Given how little is known about the fundamental structural basis for selective amino acid−RNA pairing in any natural system, the study of many related aaRS pairs appears likely to be well worth the effort. A deeper appreciation of the evolution and detailed structural differentiation of the aaRS families can certainly be anticipated. The insights gained should also allow much more insightful use of rational mutagenesis to improve the activities of enzymes emerging from genetic selections. Alternatively, the rational mutagenesis experiments can be used to design engineered variants that can then be used as the starting point for further selections. For those interested, there is indeed a great deal to do.

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■ ABBREVIATIONS

aaRS, aminoacyl-tRNA synthetase; LUCA, last universal common ancestor; MARS, multi-tRNA synthetase complex; UAA, unnatural amino acid.

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