Transfer RNA–Mediated Editing in Threonyl-tRNA Synthetase: The Class II Solution to the Double Discrimination Problem

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Summary

Threonyl-tRNA synthetase, a class II synthetase, uses a unique zinc ion to discriminate against the isosteric valine at the activation step. The crystal structure of the enzyme with an analog of seryl adenylate shows that the noncognate serine cannot be fully discriminated at that step. We show that hydrolysis of the incorrectly formed ser-tRNA^{Thr} is performed at a specific site in the N-terminal domain of the enzyme. The present study suggests that both classes of synthetases use effectively the ability of the CCA end of tRNA to switch between a hairpin and a helical conformation for aminoacylation and editing. As a consequence, the editing mechanism of both classes of synthetases can be described as mirror images, as already seen for tRNA binding and amino acid activation.

Introduction

From transcription of DNA to protein synthesis on the ribosome, each step of the translation of genetic information into proteins requires a high level of accuracy to express faithfully the genetic message. In that respect, the attachment of the correct amino acid to the cognate tRNA by a specific aminoacyl-tRNA synthetase (aaRS) is a critical point. Each synthetase has to sort out from a pool of very similar molecules: twenty amino acids and about sixty tRNAs. Mistakes are more frequent in the selection of amino acids (frequencies in the range of 10^{-4} to 10^{-5}), than in the selection of tRNA (in the order of 10^{-6}) (Jakubowski and Goldman, 1992). This is mainly due to the greater surface area of the tRNA,

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and the numerous specific contacts that are established upon the formation of the tRNA-aaRS complex (Giegé et al., 1998). aaRSs display two fundamentally different types of active sites, which led to a partition into two classes (Eriani et al., 1990). This partition reflects differences in the functional mechanism and the mode of substrate recognition. There is a class-characteristic ATP and amino acid binding site, and opposite directions for the tRNA approach (Arnez and Moras, 1997). The mode of amino acid binding shows class-specific features for the recognition of the carboxylic and amino groups, directly linked to the functional mechanism, but discrimination from the other amino acids can only be based on the characteristics of the side chain of the substrate. Crystal structures are now available for most of the twenty synthetases. However, the precise mechanism of discrimination among very closely related amino acids is just beginning to emerge on some systems.

The need of discrimination goes beyond the twenty amino acids normally used by proteins, since the amino acid pool in the living cell contains nonproteic amino acids, such as the by-products of amino acid synthesis. Some of these are closely related structurally or chemically to a given amino acid and this could be critical for selectivity. A major problem is posed by amino acids that are shorter than the cognate by one methylene group. According to Pauling, this would allow a discrimination ratio not better than 1/5, since the difference between the binding energies for such closely related molecules would be in the order of 1 kcal/mol (Pauling, 1957). Values for the discrimination between cognate and noncognate amino acids have been measured experimentally in several systems. The best documented cases are those of IleRS and ValRS, two synthetases specific for hydrophobic amino acids. In their case, the size of the substrate happens to be the only characteristic upon which the discrimination is based, and a double sieve model was proposed to account for the apparent discrepancy between the small differences in the binding energies and the overall accuracy of the aminoacylation reaction (Fersht and Dingwall, 1979; Fersht, 1985). The fundamental idea was that a synthetase discriminating against similar amino acids cannot fulfill efficiently its task with the use of a single binding site, and that a second site is required to correct errors, each site using a different structural property for selectivity. Since aaRSs function in two steps, amino acid activation and tRNA aminoacylation, editing could in principle also occur at both levels, upon the aminoacyl-adenylate or the aminoacylated tRNA (Jakubowski and Fersht, 1981). A structural evidence for the double sieve editing model came from the three-dimensional structure of IleRS, which showed a second binding site for valine (Nureki et al., 1998). It lies at a distance of about 34 Å from the catalytic site in a domain of the protein which was known to be responsible for editing (Schmidt and Schimmel, 1994, 1995; Lin et al., 1996). A functional model now generally accepted is that either the tRNA accepting end or the noncognate adenylate shuttles from the cata-
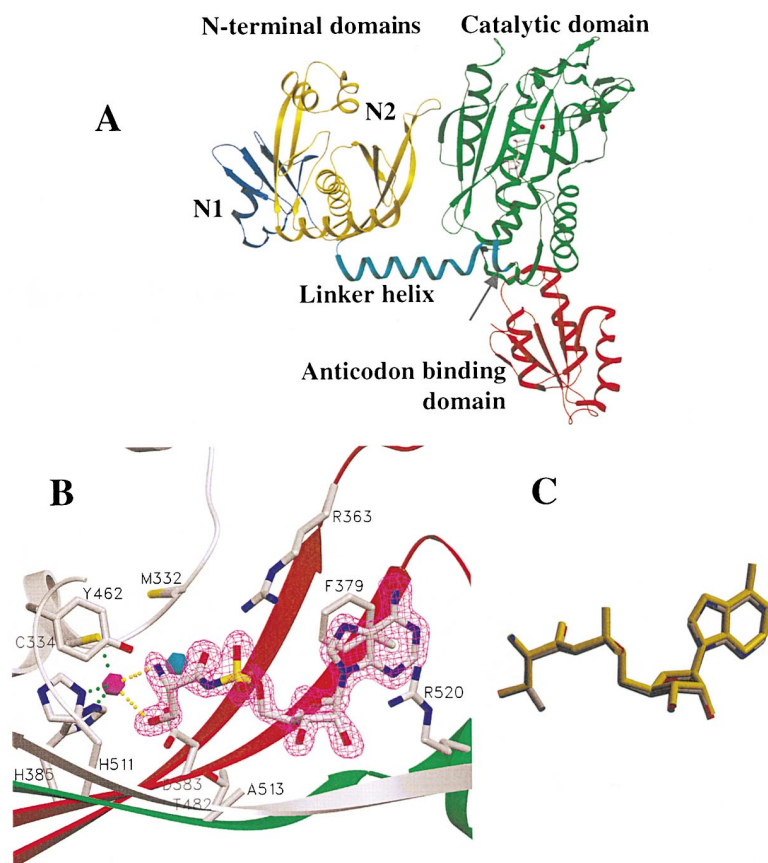
Figure 1. Serine Binding in the Active Site of ThrRS

(A) The monomer of *E. coli* ThrRS, as seen in the structure of the complex with tRNA (Sankaranarayanan et al., 1999). The catalytic domain is in green. A molecule of AMP is shown in the active site. The anticodon binding domain is in red. The N-terminal domains are on the left, with N1 in blue and N2 in yellow. The arrow indicates where the truncation was made. The resulting δN-ThrRS comprises the catalytic core and the anticodon binding domain but not the N-terminal domains nor the linker helix. (B) The active site of δN-ThrRS with the adenylate analog ser-AMS. A ($2F_o$-F_c) map contoured at 2σ, calculated prior to the inclusion of ser-AMS in the model, is superimposed on the refined coordinates. The class II specific motifs 2 and 3 are colored in red and green, respectively. The zinc ion and water molecule are in pink and cyan. (C) A superposition of ser-AMS (yellow) on thr-AMS (Sankaranarayanan et al., 2000). This figure and Figures 3A and 3C were made using SETOR (Evans, 1993).

lytic site to the editing site (Nomanbhoy et al., 1999; Silvian et al., 1999).

Even though the editing function is well understood in some class I synthetases, very little is known about the mechanism of discrimination of similar substrates in class II enzymes. A very recent report on a class II enzyme, ProRS, indicates that both pretransfer and posttransfer editing pathways exist in that enzyme (Beuning and Musier-Forsyth, 2000; Musier-Forsyth and Beuning, 2000). However, this is not a classical case for editing, since the edited noncognate amino acid, alanine, has no structural or chemical similarity to proline. In fact, few amino acids corresponding to class II synthetases show similarities like isoleucine/valine or valine/threonine pairs. Problems may be encountered by AlaRS, which has to discriminate against glycine and serine, and by ThrRS which has to discriminate against serine and valine. In a study performed with AlaRS, an editing activity was indeed observed (Tsui and Fersht, 1981). However, no structural information is yet available on the AlaRS system.

In the case of ThrRS, the first crystal structure of the *E. coli* synthetase complexed with its cognate tRNA and AMP (Sankaranarayanan et al., 1999) showed that the protein is made of two limbs, one comprising the catalytic domain and the anticodon binding domain, and the other the two domains N1 and N2, corresponding to the characteristic N-terminal extension of ThrRSs. The two limbs are linked by a long helix (Figure 1A). In addition to the catalytic core, which makes contacts with the

major groove, a tight binding of the tRNA acceptor arm is provided by the N2 domain, on the minor groove side. The structure of the ThrRS complex revealed the presence of an essential zinc ion in the catalytic site. To elucidate the role played by this ion in the catalytic domain, we solved two high-resolution crystal structures of an N-terminal truncated protein (δN-ThrRS) in the presence of either threonine or a threonyl adenylate analog (thr-AMS) (Sankaranarayanan et al., 2000). These structures showed that the zinc ion is involved directly in amino acid recognition by interacting with both the amino and the hydroxyl groups. This mode of interaction of threonine explained readily how the isosteric valine is rejected by ThrRS. However, it could not explain how serine could be rejected. The methyl group, which distinguishes threonine from serine, is interacting through two van der Waals contacts, of which only one involves a ThrRS invariant. Since serine and threonine are present in the cell at similar concentrations (Raunio and Rosenqvist, 1970), the discrimination factor may be estimated by comparing the ratio k_{cat}/K_m for serine to that for threonine, in the amino acid activation reaction. The experimental value, 10^{-3}, does not meet the accuracy level expected for the aminoacylation reaction, for which a maximum value of 10^{-4} has been measured (Loftfield and Vanderjagt, 1972). Therefore, the mechanism of discrimination of serine by ThrRS remained unclear. The presence of a water molecule as the fourth zinc ligand in the absence of the amino acid (Sankaranarayanan et al., 1999) suggests that the zinc ion could be involved

Table 1. Summary of Crystallographic Data and Refinement Statistics

Data Set	ser-AMS Complex
Space group	$P2_12_12_1$
Data collected at	BW7B, DESY, Hamburg
Wavelength (Å)	0.8439
Resolution (Å)	1.65
No. of observations	431,526
No. of unique reflections	127,385
Completeness (%)	95.6
R_{sym} (%)	5.3
Refinement Statistics	
Resolution range (Å)	20.0–1.65
No. of reflections (F $>$ 2σ)	116,879
No. of protein, ligand, solvent and metal atoms	6533, 58, 534, 2
R factor (%)	21.8
Free R factor (%)[a]	23.7
Rmsd in bond lengths (Å)	0.0076
Rmsd in bond angles (°)	1.34
Average B factors (Å^2) for protein, ligand, water and metal atoms	22.1, 15.7, 32.4, 14.0

[a] Calculated for a random set of 5% of reflections not used in the refinement.

in catalysis and hydrolyze an incorrectly formed seryl adenylate. This mechanism would require a different mode of binding of serine compared to threonine. We present here crystallographic and biochemical evidence, using the wild-type and different mutated versions of ThrRS, showing that serine binds in a way very similar to threonine and that the specificity is ensured by a two-step process, with the use of a separate editing site to remove a serine incorrectly attached to tRNA^{Thr}.

Results and Discussion

Structure of the Complex of δN-ThrRS with ser-AMS

Figure 1A shows the structure of the wild-type enzyme and the localization of the regions that are discussed in the present study. The truncated δN-ThrRS contains the catalytic and the anticodon binding domains and is fully functional in the activation of the amino acid (Sankaranarayanan et al., 2000). Our attempts to crystallize δN-ThrRS in the presence of free serine failed; however, crystals could be obtained in the presence of an analog of seryl-adenylate, seryl-sulfamoyl adenosine (ser-AMS), which diffracted to 1.65 Å resolution. The structure was solved using the coordinates from the structure of δN-ThrRS complexed with the threonyl adenylate analog as the starting model. The adenylate analog was not included in the initial model, but fitted in the electron density map (see Experimental Procedures section). Table 1 summarizes the data collection and refinement statistics. The binding of the serine moiety in ser-AMS is identical to that observed for the threonyl-adenylate analog or threonine. The zinc ion binds to both the amino and hydroxyl groups of the noncognate substrate (Figure 1B). The superposition of the two analogs is shown in Figure 1C. In the case of threonine, the methyl group was stabilized by two van der Waals interactions, with
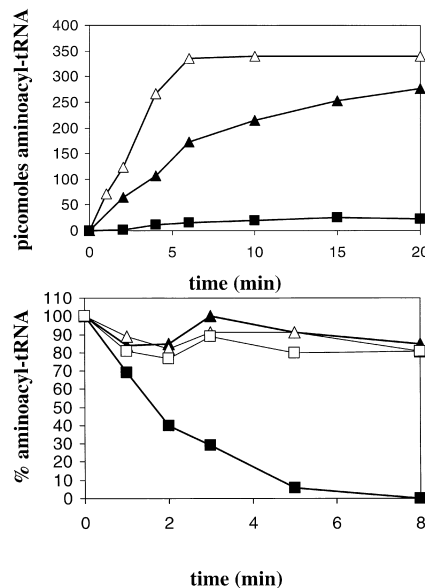

Figure 2. Aminoacylation and Deacylation by ThrRS

(Top panel) Aminoacylation of tRNA^{Thr} (4.5 μM) with ^{14}C-threonine or ^{14}C-serine. The experiment was performed at 37°C, in the presence of 10 mM ATP and 50 nM of wild-type ThrRS with 150 μM serine (filled squares), 9 nM δN-ThrRS with 50 μM threonine (empty triangles) or 90 nM δN-ThrRS with 150 μM serine (filled triangles). The background obtained from an incubation in the absence of tRNA was subtracted from the counts. A slight amount of radioactivity is systematically observed in the case of the aminoacylation test with serine by wild-type ThrRS. It can be due to trace amounts of ^{14}C-threonine present in the ^{14}C-serine stock, or to partial denaturation of the editing site. (Bottom panel) Incubation of 2 μM ser-tRNA^{Thr} or thr-tRNA^{Thr} with 2 nM ThrRS at 37°C in a buffer containing KCl 100 mM, HEPES-NaOH 50 mM, and MgCl$_2$ 5 mM at pH 7.0: thr-tRNA^{Thr} with wild-type ThrRS (empty squares) or δN-ThrRS (empty triangles); ser-tRNA^{Thr} with wild-type ThrRS (filled squares) or δN-ThrRS (filled triangles).

methyl groups from Ala513 and Thr482. The position of these two residues is unchanged in the ser-AMS structure, in spite of the absence of the methyl group. The displacement of the polypeptide chain 417–466, which was associated with the binding of threonine, is seen also in the case of serine, leading to the same interaction of Tyr462 with the amino group. Moreover, the water molecule that was observed in the threonine binding site is also present. Apart from the lack of the two weak interactions of the side chain methyl group of threonine, the structure shows that efficient discrimination of serine from threonine is not possible at this site.

Truncated δN-ThrRS Produces ser-tRNA^{Thr}

The truncated δN-ThrRS does not contain the N-terminal domains N1 and N2 but is still able to aminoacylate tRNA^{Thr}, albeit at a 20-fold reduced rate (Sankaranarayanan et al., 1999). The aminoacylation of *E. coli* tRNA^{Thr} with serine is shown in Figure 2, top panel. The experiment performed with wild-type ThrRS shows almost no incorporation of serine in the tRNA. Incorporation of threonine or serine was followed in parallel experiments with the truncated δN-ThrRS. The data show clearly that serine can be attached to tRNA^{Thr} at a significant rate.
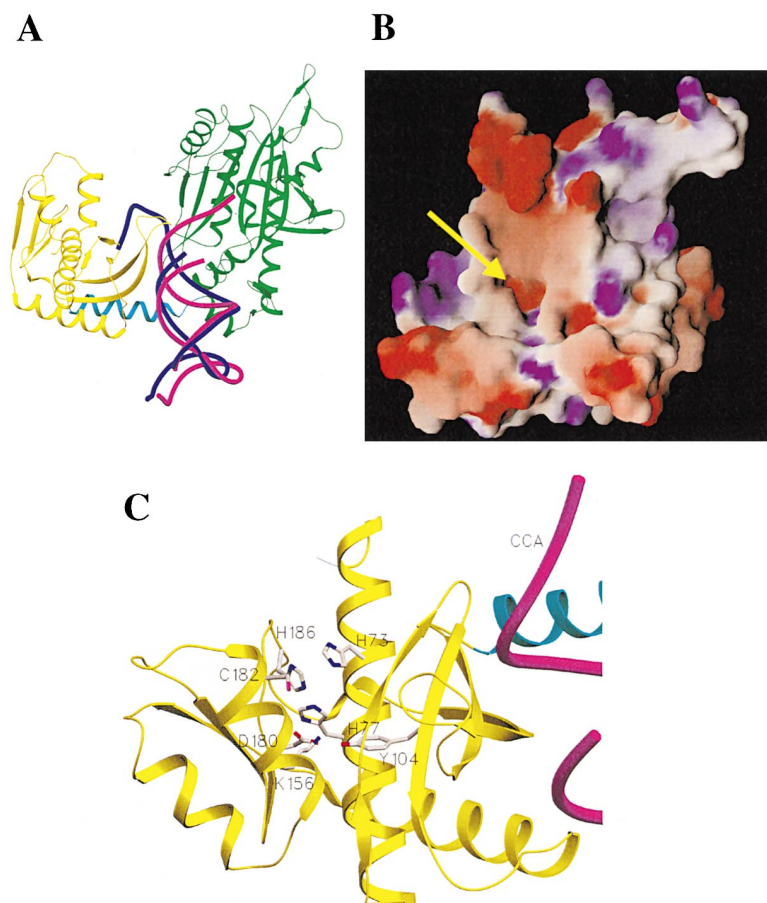
A

B

C

Figure 3. The Editing Site

(A) Superposition of the acceptor arm of tRNA^{Gln} (in purple), from the class I complex of GlnRS (Rould et al., 1989) on the acceptor arm of tRNA^{Thr}(in pink) from the class II complex of ThrRS (Sankaranarayanan et al., 1999). The picture shows the CCA end of tRNA^{Gln} pointing toward the editing site. The catalytic domain of ThrRS is in green, the N2 domain in yellow. (B) A surface representation drawn using GRASP (Nicholls and Honig, 1991) showing the cleft responsible for the editing activity. (C) The cleft of the N2 domain displaying the cluster of His73, His77, His186, and Cys182 that are reminiscent of a metal binding site. The other labeled residues surround the cleft and are highly conserved in ThrRS.

With a 10-fold increase of the enzyme concentration and a 3-fold increase of the amino acid concentration, the plateau level with serine is comparable to that with threonine. This indicates that ThrRS deprived of the N-terminal domains has lost the capacity to discriminate serine against threonine, while being still active in the tRNA aminoacylation. In a previous study of the active site of ThrRS (Sankaranarayanan et al., 2000), we followed the activation reaction, which combines the amino acid and ATP to produce the aminoacyl-adenylate. The comparison of the rates of activation of threonine and serine at the first step of the aminoacylation showed a preference for threonine over serine of about 1000 to 1. Both wild-type and truncated ThrRSs were found to have similar kinetic parameters. Here, the two enzymes show a different behavior in the second step of the aminoacylation. This indicates that an important step in the discrimination function occurs in the presence of tRNA, and involves the N-terminal domains of ThrRS.

**Only the Wild-Type ThrRS Rapidly
Deacylates ser-tRNA^{Thr}**

Once ser-tRNA^{Thr} was available as the product of the aminoacylation with δN-ThrRS, the existence of a deacylation reaction could be checked. This reaction proceeds in the absence of ATP and amino acid, and is not a reversal of the aminoacylation reaction (Schreier and Schimmel, 1972). It is linked to the specific removal of an amino acid mistakenly bound to the tRNA (Eldred and Schimmel, 1972) and was used to follow the editing activity in IleRS and ValRS (Lin et al., 1996). The measure is direct and does not depend on the purity of amino acid stocks. Usually, the major difficulty with this test is to produce the substrate, the mischarged aminoacyl-tRNA, which can only be obtained with some synthetases handled in rather harsh conditions (Giegé et al., 1971). Here, δN-ThrRS provides an easy way for its synthesis. Figure 2, bottom panel, shows that when incubating either ser-tRNA^{Thr} or thr-tRNA^{Thr} with wild-type ThrRS, significant deacylation is observed only with ser-tRNA^{Thr}, and not with thr-tRNA^{Thr}. As expected, δN-ThrRS does not deacylate ser-tRNA^{Thr} . This experiment clearly indicates that the N-terminal region of ThrRS is involved in the deacylation process and that the deacylation is specific for seryl-tRNA^{Thr}. An indirect test for the presence of an editing mechanism would be the measure of the ATP consumption resulting from the hydrolysis of the noncognate adenylate or the mischarged tRNA. Such ATP consumption was detected in the case of IleRS (Schmidt and Schimmel, 1994). In the case of ThrRS, we could not detect any significant ATP consumption in the standard conditions, including 200 mM noncognate amino acid, 2.8 μM enzyme, and 14 μM tRNA. However, the direct observation of ser-tRNA^{Thr} deacylation gives a solid ground to the hypothesis of an editing function in ThrRS. It indicates that the editing
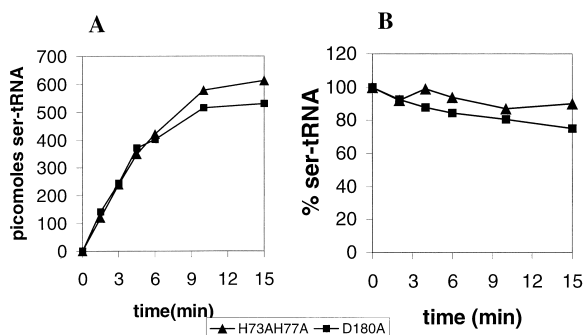
Figure 4. Editing Activity in ThrRS Mutants

(A) Aminoacylation of tRNA^{Thr} (4.5 μM) with ^{14}C-serine (150 μM) in the presence of mutated ThrRS: 50 nM H73A-H77A double mutant (black triangles) or 124 nM D180A (black squares). (B) Incubation of ser-tRNA^{Thr} (1.6 μM) with 5 nM H73A-H77A double mutant (black triangles) or 12.4 nM D180A (black squares), in the same conditions as in Figure 2. The concentrations of the mutant enzymes used in these tests were such as to produce equal rates of aminoacylation with threonine.

site is located in the N-terminal domain and recognizes serine while excluding threonine.

Localization of the Editing Site

A cleft in the N2 domain was identified previously as a potential candidate for editing (Sankaranarayanan et al., 1999). Indeed, a superposition of the acceptor stem of tRNA^{Gln}, a tRNA specific to a class I synthetase (Rould et al., 1989), upon that of tRNA^{Thr} shows the acceptor end to point toward this cleft in the N2 domain (Figure 3A). The cleft (shown in Figures 3B and 3C) is characterized by two groups of conserved residues. One group forms the cleft and includes residues Asp180, Lys156, and Tyr104. Another group of conserved residues, His73, His77, His186, and Cys182, lie adjacent to the cleft. The conservation of these residues is very high for both the bacterial and eukaryotic worlds, but has no equivalents in archaea, where the N-terminal extension of ThrRS is very different. A similarity of sequences between ThrRS and AlaRS was highlighted in the N-terminal region (Sankaranarayanan et al., 1999). The group of histidines and cysteine, in the disposition HxxxH, CxxxH, was a key to identify the domain of AlaRS homologous to the N2 domain. In AlaRS, the sequence conservation of this domain extends also to the archaeal world.

In order to precisely localize the editing site of ThrRS, point mutations were made in the two groups of residues surrounding the N2 cleft. A double mutant was engineered, where both His73 and His77 were replaced by alanines. As for the truncated δN-ThrRS, this mutant protein is no longer able to deacylate ser-tRNA^{Thr} and consequently charges tRNA^{Thr} with serine, as shown in Figure 4. This mutant enzyme is however as efficient as the wild-type enzyme in aminoacylating tRNA^{Thr} with threonine, with k_{cat}/K_m close to 10 s^{-1} μM^{-1} (20 s^{-1} μM^{-1} for the wild type). Thus, this mutation affects only the editing activity of ThrRS, showing that the region encompassing His73 and His77 is involved in the specific hydrolysis of the ser-tRNA^{Thr}. In a second experiment, Asp180, a highly conserved residue in nonarchaeal

ThrRS, was mutated to an alanine and the mutant protein was checked for the aminoacylation of tRNA^{Thr} with serine. Again, a clear incorporation of serine is observed, due to the fact that the enzyme is not able to rapidly deacylate the ser-tRNA (Figure 4). Those point mutations thus allow a clear localization of the editing site in the cleft of the N2 domain. Since AlaRS has also been shown to perform editing, the present observation suggests that the sequence similarity of AlaRS with the N2 domain of ThrRS could be linked to an analogous editing activity.

The group of three histidines and one cysteine adjacent to the editing cleft are in a spatial arrangement reminiscent of a zinc binding site (Figure 3C). Measurements of the zinc content of ThrRS by atomic absorption spectroscopy indicated the presence of 2.0 zinc ions per dimer, corresponding to the zinc ions in the catalytic domains (Sankaranarayanan et al., 1999). In the presence of 5 μM ZnCl$_2$, the measured zinc content of wild-type ThrRS increased to 3.8 zinc ions per dimer. The δN-ThrRS showed no increase of zinc content upon dialysis in the presence of the zinc salt, showing that the additional zinc ions bind to the N-terminal domains. In addition, in the recently determined crystal structure of ThrRS from *Staphylococcus aureus,* a metal ion is bound to the cluster of histidines and cysteine of the N2 domain in one of the two monomers in the asymmetric unit (A. Torres-Larios et al., unpublished data). The absence of a metal ion in the ThrRS-tRNA^{Thr} complex of *E. coli* could be linked to the presence of 0.1 mM of EDTA in the tRNA buffer and consequently in the crystallization drops, since this chelator has a very high affinity for zinc ($K_d = 10^{-16}$ M). Although the coordination suggests that zinc is the most probable candidate, at this stage we cannot exclude that another metal is the natural ligand. The function of this metal remains to be defined.

A Model for the Class II Editing Reaction

The finding of an essential zinc ion in the catalytic site of ThrRS and the coordination of that ion to a water molecule had suggested that the zinc could be a catalyst, and an appealing idea was that the metal ion played a role in correcting misactivations (Sankaranarayanan et al., 1999). This possibility is now ruled out since the crystal structure in the presence of ser-AMS shows that serine binds the zinc ion in the same manner as threonine. As a consequence, not only threonine but also serine is activated by ThrRS (Sankaranarayanan et al., 2000). This cannot be tolerated, since there are proteins in which a substitution of serine for threonine would be fatal to the cell, and a correction mechanism is therefore necessary. The existence of a correction mechanism at the level of the aminoacyl-tRNA has been shown by following directly the deacylation of mischarged tRNA. We have also shown that the serine-specific posttransfer deacylation activity depends on some key residues located around a cleft in the N2 domain of ThrRS, which is thus identified as the editing site. The structural data do not provide any evidence for hydrolysis at the pretransfer level in ThrRS. All protein residues involved in the transfer of the cognate or noncognate aminoacyl moiety on the tRNA are correctly positioned for the transfer to occur readily when the tRNA enters the cata-
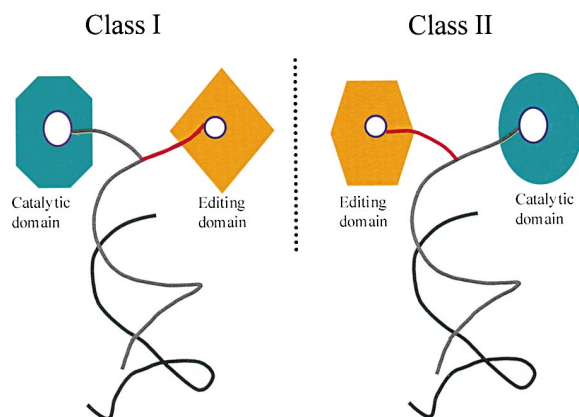
Class I   Class II

Figure 5. The Editing Model

A schematic view of the activation and editing mechanisms in class I (IleRS) and class II (ThrRS) emphasizing the mirror symmetrical character of the overall mechanism.

lytic site. Also, the ThrRS structure shows no path that the ser-AMP could follow to go from the catalytic site to the editing site without releasing in solution. Therefore, we conclude that the only way for the activated noncognate aminoacid to reach the editing site is by being attached to the tRNA CCA end. This implies that a shuttle mechanism is required for the CCA end of the misactivated ser-tRNA^{Thr} to come into the editing site.

Unimolecular substrate translocation is a process that has been discovered in DNA replication and translation to ensure the fidelity of protein synthesis. In the case of the editing of misincorporated nucleotides by DNA polymerase I, the nascent DNA strand shuttles between the active sites for synthesis and editing (Freemont et al., 1988; Joyce and Steitz, 1994). Extensive studies on the editing function of aaRSs, particularly on the IleRS system, have lead to the identification of the editing site (Lin et al., 1996; Nureki et al., 1998) and an editing model was proposed (Silvian et al., 1999). In this class I editing model, the acceptor end of the tRNA, once charged with the amino acid, changes from the hairpin conformation, which allowed the CCA end to go into the class I active site, to the helical conformation, typical of free tRNA or tRNA bound to a class II synthetase. In ThrRS, as shown in the superposition of Figure 3A, the CCA end of the class II tRNA needs to switch from the helical conformation to a bent conformation in order to reach the editing site, which is located in the N2 domain at about 35 Å from the catalytic site. In class I and class II systems, the catalytic modules approach the acceptor stem of their tRNAs in a mirror symmetrical way and hence their activation mechanisms may be considered as mirror images. Here we show that this concept can be extended to the tRNA-mediated editing mechanism. This is illustrated in Figure 5. Thus, class I and II synthetases utilize domains positioned symmetrically for activation and editing and use efficiently the ability of the CCA end to switch between helical and bent conformations.

The editing mechanism relies in both classes on the dynamics of the accepting end of the tRNA. That is, the movement of the aminoacylated end of the tRNA toward the editing site needs to be faster than the dissociation

of the tRNA core from the synthetase. In that respect, the interaction of tRNA^{Thr} with ThrRS at the level of the minor groove of the accepting arm may be important. The structure of the ThrRS-tRNA complex (Sankaranarayanan et al., 1999) pictured this specific interaction as a clamp that enhanced the tightness of the tRNA binding. In our editing model, we predict that this interaction would not be disrupted by the translocation of the CCA end, and, on the contrary, could act as a wedge allowing the CCA end to rotate so that the noncognate amino acid readily finds the editing site. A strong interaction of the class II synthetase at the level of the minor groove of the tRNA could even be a prerequisite for such a translocation mechanism. Interestingly, this model could as well apply to AlaRS, the other class II synthetase with a proven editing activity (Tsui and Fersht, 1981). In the class I editing model, an additional rotation of the editing domain is required to bring the editing site in contact with the charged amino acid (Nureki et al., 1998). This conformational change also opens a channel, which is required for pretransfer editing, allowing the adenylate to shuttle between catalytic and editing sites (Silvian et al., 1999). In ThrRS, analysis of the structure suggests that no such movement of the editing domain is necessary and the pretransfer mechanism has not been observed.

The current study presents clear evidences for the localization of the editing site of ThrRS in the cleft of the N2 domain. An interesting observation relates to the cluster of residues (His73, His77, His186, and Cys182) that could coordinate a metal ion. The fourth ligand cysteine exhibits a weaker electron density, which suggests an inherent mobility. The question remains whether the metal ion plays any role in the editing reaction. A comparison with other zinc binding proteins indicates some structural similarities with matrix metalloproteases (Bode et al., 1999), which encompass the helix bearing the two histidines His73 and His77, and the adjacent β sheet. Further experiments will however be required to define the residues which are involved in serine recognition, positioning of the terminal adenosine and catalysis.

In summary, this study allows a comprehensive understanding of how a high specificity is achieved by ThrRS. For the specific recognition of threonine, the enzyme has to solve two different problems of discrimination: one based upon size and the other based upon chemistry. The other known cases, IleRS and ValRS, illustrate solutions for either the size problem (IleRS), or the chemistry problem (ValRS). Both use editing to correct misactivated or mischarged species. ThrRS solves the chemistry problem at the activation step by involving a zinc ion in the catalytic site. This applies a negative selection to valine, which cannot bind. The size problem is then tackled by positive selection of serine in the editing site, where ser-tRNA^{Thr} binds and is hydrolyzed. The spatial organization of the different molecular components involved in the overall process is a mirror image of that observed in the class I IleRS. It is remarkable that even though both the classes of synthetases have evolved independently, they have arrived at similar mechanistic solutions to solve the problem of amino acid discrimination. The implication of that observation is that the tRNA molecule with its structural and physicochemical prop-

erties is the key player in the evolution of this family of enzymes.

Experimental Procedures

X-Ray Data Collection and Structure Determination

The crystals of δN-ThrRS complexed with ser-AMS belong to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions a = 87.0 Å, b = 109.5 Å, c = 115.4 Å. The asymmetric unit consists of a dimeric molecule of the truncated enzyme. The crystals were cryocooled for data collection. Diffraction data were processed using the HKL suite of programs (Otwinowski and Minor, 1997). The structure was solved by the molecular replacement method using the program AMORE (Navaza, 1994). The coordinates from the structure of the complex of δN-ThrRS with thr-AMS (Sankaranarayanan et al., 2000) were used as a starting model for solving the structure. The resulting model was adjusted using the program O (Jones et al., 1991) and refined with CNS (Brünger et al., 1998). All the routine crystallographic calculations were performed using the CCP4 suite of programs (CCP4, 1994). The data collection and refinement statistics are given in Table 1.

Enzymatic Measurements

The mutant enzymes were prepared from a *thrS*-deleted strain, as in Sankaranarayanan et al. (1999). The purifications were made according to Brunel et al. (1993). Aminoacylation assays were carried out under standard conditions (HEPES-NaOH 50 mM, pH 7.7, $MgCl_2$ 20 mM, β-mercaptoethanol 5 mM, KCl 30 mM, ATP 10 mM) in the presence of 4–5 μM $tRNA^{Thr}$. For threonylation assays, C^{14}-threonine concentration was 50 μM; the concentration of enzymes was 5 nM for wild-type ThrRS, δN-ThrRS or the H73A-H77A double mutant, and 12.4 nM for the less pure D180A mutant. These concentrations gave superposable kinetics for the four enzymes. For serylation assays, the serine concentration was 150 μM and the concentration of enzymes systematically 10 times larger than the optimal concentration for threonylation assays. Amino acid incorporation was followed by liquid scintillation counting of TCA-precipitated aliquots. ser-$tRNA^{Thr}$ was prepared by phenol extraction, followed by several ethanol precipitations, of a serylation mix containing 10 μM $tRNA^{Thr}$. Deacylation was measured in HEPES-NaOH 50 mM, pH 7.2, $MgCl_2$ 5 mM by incubation of 1–2 μM aminoacyl-$tRNA^{Thr}$ with the enzymes at the concentration used for threonylation. Aliquots were TCA-precipitated, and the remaining aminoacyl-tRNA measured by liquid scintillation counting.

Acknowledgments

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Protein Data Bank ID Code

The ID code for the coordinates of the structure reported in this paper is 1FYF.