Hypothesis

A NEW MECHANISM FOR THE HYDROLYTIC EDITING FUNCTION OF AMINOACYL-tRNA SYNTHETASES

Kinetic specificity for the tRNA substrate

H. T. WRIGHT

Department of Biochemical Sciences, Princeton University, Princeton, NJ 08544, USA

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1. Introduction

The observed low frequency (≤0.03%) [1] with which an amino acid is incorrectly incorporated into proteins in vivo is due in part to the fidelity with which aminoacyl-tRNA synthetases catalyze aminoacyl-tRNA formation. The high specificity of these enzymes is not completely understood, but experiments show that discrimination between correct and incorrect substrates occurs in both the binding [2] and rate-determining steps [3].

One source of the exceptional specificity of these enzymes may be an editing function [4], in addition to discrimination arising from differential affinities of correct and incorrect substrates for the enzyme. Two types of editing function have been proposed:

(i) Kinetic proofreading [5] confers high specificity by coupling hydrolysis of activated amino acid to its dissociation from the enzyme and thus greatly increases the rate of this reaction relative to aminoacylation. The additional discrimination between correct and incorrect substrate amino acids at this step decreases the error fraction.

(ii) Chemical or hydrolytic proofreading [6] postulates a specific hydrolytic active site which discharges incorrectly aminoacylated tRNA, as has been observed for some aminoacyl-tRNA synthetases [7–10].

2. Aminoacyl-tRNA synthetase specificity

Aminoacyl-tRNA synthetases discriminate against non-cognate amino acid and tRNA to varying extents, which are dependent upon both the identity of the non-cognate substrate and the experimental conditions. For *Escherichia coli* Val-tRNA synthetase, the ratios of k_{cat}/K_m with respect to some non-cognate amino acids fall within the limits 1:200 to $1:10^7$ [11]. The lower values are observed for amino acids smaller than Val which possess sidechains, and the higher values for Ile and Gly. Ile is larger than Val and therefore likely to be excluded from the binding site, while the absence of sidechain specificity determinants in Gly greatly diminishes its affinity for the amino acid binding site of the synthetase.

On the basis of these data, Fersht has proposed that the specificity of aminoacyl-tRNA synthetases for their amino acid substrates plays a central role in the overall fidelity of the reaction [12]. This specificity is a simple lock-and-key type in which substrates larger than the cognate cannot fit into the binding site, while smaller ones do not make enough interactions to bind significantly at the active site. Those non-cognate amino acids which are not excluded by this simple discrimination are subsequently removed from the mischarged tRNA by a hydrolytic editing mechanism before dissociation from the synthetase.

Discrimination between cognate and non-cognate tRNA by aminoacyl-tRNA synthetases is generally not so great as that observed for the amino acid substrate. Equilibrium binding studies and steady state K_m-values show affinity ratios $\leq 10^4$ for pairs of cognate and non-cognate tRNAs [13,14], with values for most of the pairs in the range 10–100. Similar ratios of V_{max} for cognate and non-cognate tRNA substrate are also observed [15,16]. The relatively weak discrimination of aminoacyl-tRNA synthetases

toward their tRNA substrate is consistent with the hypothesis that all tRNAs have similar tertiary structures [17]. It is significant that the mischarging of $tRNA^{Trp}$ (*su7*) [18] and of mutants of $tRNA^{Tyr}$ (*su3*) [19] with Glu involve the correct recognition of Glu by Glu-tRNA-synthetase, but incorrect recognition of the tRNA. This is consistent with an important role for amino acid recognition in the specificity of this reaction, and implies that the specificity for tRNA substrate can be compromised under certain conditions. The fact that only a single tRNA is mischarged is also consistent with a common specificity determinant for the cognate and suppressor tRNAs.

Studies in which modified tRNAs have been presented to their cognate aminoacyl-tRNA synthetases show that important interactions occur between the synthetase and tRNA substrate, which are disrupted by the modification [20–27]. Such studies have implicated particular parts of the tRNA molecule in the recognition by the synthetase, but it is not clear whether these are the critical determinants of the ultimate specificity of the reaction or whether their modification results in changes which indirectly affect the specificity.

3. A new editing mechanism

The results in [11] are consistent with the hypothesis that stereochemical specificity for the amino acid contributes to the overall specificity of the aminoacylation reaction. However, no comparable data exist to suggest similar stereochemical discrimination among potential tRNA substrates. Clearly, there must be strong specificity for the correct tRNA substrate, since the fidelity of the overall reaction cannot exceed that of the least specific step. While such specificity may occur as the result of subtle differences in structure among the tRNAs, the presence of the editing function suggests that the determinants of specificity for the tRNA substrate may differ in type from those responsible for specificity toward the amino acid substrate.

In the mechanism proposed here for some or all of those aminoacyl-tRNA synthetases possessing an editing function, the major contribution of the tRNA substrate to fidelity of product formation in the reaction comes through the binding and interaction of 2 tRNA molecules on the aminoacyl-tRNA synthetase. This recognition complex of 2 tRNA substrate molecules exerts its influence upon the specificity by kinetically controlling the rates of product formation and hydrolytic editing. A different type of model from that proposed here, but also involving interaction of 2 bound tRNA substrate molecules, has been proposed [28].

The proposed mechanism has 3 important features:

(i) The aminoacyl-tRNA synthetases bind 2 molecules of tRNA during the reaction;

(ii) The acceptor arm of the tRNA substrate is unwound as a result of its interaction with the synthetase;

(iii) A sequence of bases in the acceptor stem, possibly the positions 2–5 from the end of the acceptor arm in the tRNA substrate, is a specificity determinant in the reaction.

3.1. *Stoichiometry of tRNA binding to synthetases*

Most aminoacyl-tRNA synthetases occur as dimers or tetramers with at least one pair of identicial subunits [29]. Of those which are monomeric, several have been shown to have sequence redundancies and may be bifunctional monomers [30–33]. Although in some cases equilibrium binding studies find only 1 tRNA bound/synthetase molecule, the non-equivalency of substrate binding sites in both the monomeric Val-tRNA synthetase and the dimeric Tyr-tRNA synthetase from *Bacillus stearothermophilus* [34] suggests that at least some of the synthetases found to bind only a single tRNA may bind a second under reaction conditions. Furthermore, the aggregation state of synthetases during catalysis is not known in many cases, and may be of higher order than found under equilibrium conditions.

3.2. *Unwinding of the tRNA acceptor arm*

There is no direct evidence indicating that the acceptor stem of tRNA is unwound upon binding to aminoacyl-tRNA synthetase, but a precedent for such a loss of secondary structure exists in the reverse transcriptase-catalyzed synthesis of DNA in which the 3′-terminal 16 residues of $tRNA^{Trp}$ act as primer [35]. This activity undoubtedly requires opening of the acceptor and TψC stems of the tRNA primer, and the unwinding of this tRNA may be a function of the reverse transcriptase itself [36].

Changes in the acceptor stem of some aminoacyl-tRNA synthetases cause loss of activity. Photoinactivation of $tRNA^{Ala}$ occurs through transformation of residues in the 3′-strand, while UV inactivation of

yeast $tRNA^{Tyr}$ is a result of changes in the 5′-strand of this stem [24]. Such changes are unlikely to disrupt the tertiary structure substantially, and their inactivating effects suggest they may be exposed during aminoacylation. Mutants of *E. coli* $tRNA^{Tyr}$(*su3*), in which double base changes leading to replacement of a G–C pair by an A–U pair, result in mischarging with glutamine [19,37], a result that is more consistent with an effect upon the kinetics of the reaction than upon the recognition of a specific tRNA conformation.

The crystals of $tRNA^{Gly}$ in which the acceptor stem is observed to have lost its helical structure [38] are grown from solvent containing ~50% dioxane. The organic solvent concentration which is high relative to that used to crystallize yeast $tRNA^{Phe}$, is the likely cause of the observed helic destabilization. We have suggested that the aminoacyl-tRNA synthetases unwind the tRNA acceptor stem in the same way as they are unwound in a solution of 50% dioxane [38].

3.3. *Sequence specificity in the acceptor stem*

The hypothetical mechanism described here for the aminoacylation reaction brings into play a sequence of bases in the acceptor stem which is postulated to be exposed during the reaction. Of the 5 terminal basepairs of the acceptor stem, that segment of 4 or fewer basepairs which is most characteristic of a tRNA consists of basepairs 2–5 (table 1). In the published sequences of *E. coli* tRNAs [39], the only 2 tRNAs to have identical sequences at these positions in both strands are $tRNA^{Trp}$(*su*⁻ and *su7*) and $tRNA^{Gln}$. The in vivo suppression by $tRNA^{Trp}$(*su7*) occurs through insertion of Glu, which is transferred to the $tRNA^{Trp}$(*su7*) by Glu-tRNA synthetase [18]. The 3′-strand of the acceptor stem of $tRNA^{Ala}$ is identical to that of $tRNA^{Gln}$ and $tRNA^{Trp}$, but the 5′-strand has one base different, and the anticodon of $tRNA^{Ala}$ bears no relationship to the other two anticodons, so that in vivo mischarging, if it occurred, would not be detected through suppression.

In the available yeast tRNA sequences, 3 pairs of different tRNA acceptors each have the same sequence at these positions. Of one of these pairs, $tRNA^{Ile}$–$tRNA^{Val}$, the $tRNA^{Ile}$ has been shown to be efficiently mischarged with Val catalyzed by yeast Val-tRNA synthetase [40]. Of the other 2 tRNA pairs, $tRNA^{Tyr}$–$tRNA^{Arg}$ and $tRNA^{Met}_{m}$–$tRNA^{Thr}$, the yeast tyrosyl-synthetase has been shown to have no editing function [10]. The same may be true of some or all of the other of these synthetases. Furthermore, each of these pairs is not strictly identical in sequence in the 4 residues of the acceptor stem proposed here to be a specificity determinant. Of the 4, 3 have a G–U basepair in this tetranucleotide sequence which results in a single base difference in one strand in each of the pairs. Superficially, this would not appear to be a sufficiently great difference in sequence to be the basis for discrimination. However, it is known that in the tyrosine suppressor tRNAs, *su3*(U80) and *su3*(U81), the mutations which results in a G–U basepair replacing a G–C basepair, leads to some mischarging with Glu [19].

Table 1
Acceptor stem sequences of *E. coli* tRNAs

			Anticodon 5′ 3′				Anticodon 5′ 3′
ala	5′	G G G G C A		lys	5′	G G G U C G U	
	3′	C C C C G C	U I C		3′	C C C A G C A	U* U U
arg	5′	G C A U C C G		met$_a$	5′	C G C G G G G	
	3′	C G I A G G C	I C G		3′	A C C C C C C	C A U
asn	5′	U C C U C U G		met$_m$	5′	G G C U A C G	
	3′	A G G A G A C	Q U U		3′	C C G A U G C	C* A U
asp	5′	G G A G C G G		phe	5′	G C C C G G A	
	3′	C C U U G C C	G U C		3′	C G G G C C U	G A A
gln	5′	U G G G G U A		ser	5′	G G A A G U G	
	3′	A C C C C A U	C U G		3′	C C U U C G C	U* G A
glu	5′	G U C C C C U			5′	G G U G A G G	
	3′	C A C G G G C	U* U C		3′	C C A C U C C	G C U
gly	5′	G C G G G C G		thr	5′	G C U G A U A	
	3′	C G C C C G C	C C C		3′	C G A C U A U	G G U
	5′	G C G G G C A		trp	5′	A G G G G C G	
	3′	C G C C C G U	U* C C		3′	U C C C C G C	C C A
	5′	G C G G G U A		tyr	5′	G G U G G G G	
	3′	C G C C C U U	G C C		3′	C C A C C C C	Q U A
his	5′	G U G G C U A		val	5′	G G C U G A U	
	3′	C A C C G A U	Q U G		3′	C C C A C U A	U* A C
ile	5′	A G G C U U G			5′	G G G U C C G	
	3′	U C C G G A C	G A U		3′	C G C A G G C	G A C
leu	5′	G C G A A G G			5′	G C G U U C A	
	3′	C G C U U C C	C A G		3′	C G C A A G U	C A C
	5′	G C C G A G G					
	3′	C G G C U C C	G A G				

4. Details of the proposed mechanism

The mechanism of aminoacylation proposed here confers high specificity on product formation by kinetic control of steps in the transfer and editing of amino acid and the dissociation of product. It utilizes the sequence information of basepairs 2–5 of the acceptor stem to control the rates of hydrolysis of aminoacyl groups from the bound tRNA substrate relative to the dissociation of aminoacyl-tRNA. This control is effected by the exchange of opposite strands of the acceptor stems of the 2 bound tRNA molecules.

It is also possible that primary enzyme–tRNA specificity occurs at the unwinding step through recognition by the enzyme of the cognate base sequence in the unwound acceptor stem. Affinity of this sequence for a binding site on the enzyme could help to drive the unwinding reaction. However, the variations in sequence among isoacceptors at these positions require that such specificity be weak.

There are two general pathways which the proposed new editing mechanism could follow: In one, aminoacylation precedes acceptor stem unwinding, in the other, it follows it. Fig.1 shows schematically some of the steps likely to occur in each of these pathways.

In the case where aminoacylation precedes unwinding, hydrolytic editing would occur at the 3′-aminoacyl group of the cloverleaf-folded aminoacyl-tRNA (IV). Unwinding with complementary strand exchange (V) would remove the 3′-aminoacyl group from the hydrolytic editing site (IV) and lead to correct product formation (IX). Non-complementarity of the exchanged strands (VI) would allow the unwound 5′- and 3′-strands to rewind (III, IV), returning the aminoacyl group to the hydrolytic editing site (IV), where it is edited off. Alternatively, it is possible that non-complementarity of exchanged strands permits the 3′-aminoacyl terminus to bind to a hydrolytic editing site removed from the aminoacylation site while still unwound (VI) and there be hydrolyzed.

Aminoacylation of an already opened acceptor stem (II → III) presents the possibility of editing non-cognate amino acid which has been charged to cognate tRNA, in addition to discharging aminoacylated, non-cognate tRNA. The reduced affinity of the non-cognate amino acid for the aminoacyl binding site (II) might favor intramolecular strand recombination (IV) of the acceptor stem. If the hydrolytic editing site is positioned at the 3′-aminoacyl group of the rewound acceptor stem (IV), incorrect amino acid would be discharged. Such a mechanism would incorporate specificity for both amino acid and tRNA in its editing functions.

In the mechanism proposed here, there is no

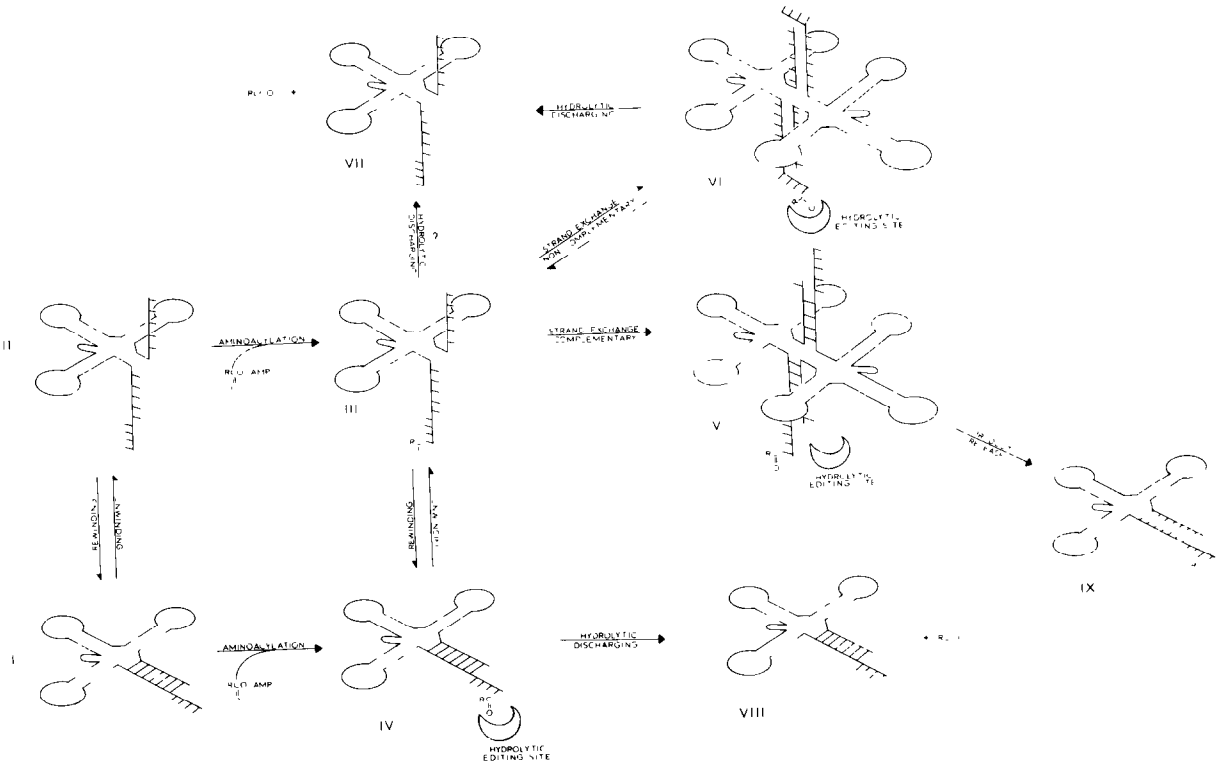

Fig.1. Hypothetical mechanism for hydrolytic editing in the aminoacylation reaction catalyzed by aminoacyl-tRNA ligases. I is a tRNA substrate molecule bound to aminoacyl-tRNA synthetase. Only basepairs in the acceptor stem of the cloverleaf are indicated.

requirement that both tRNAs bound to synthetase be aminoacylated. It is conceivable that the first tRNA binds tightly and specifically and acts as the template for aminoacylation and editing of the second. Such a situation was shown to exist for the aminoacylation of yeast $tRNA^{Ile}$, which is uncompetitively inhibited by $tRNA^{Ile}$–C–C– 3′-deoxy-A [41]. This inhibition can be reversed by $tRNA^{Ile}$–C–C– which acts as an effector in this reaction without itself being aminoacylated [41].

It is also pertinent to note that the mechanism proposed here carries no implications of cooperativity of tRNA binding. The frequently observed anti-cooperativity of tRNA association is possibly related to the apparent effector function noted above and is consistent with different functions for the 2 bound tRNAs. The mechanism proposed here is based on kinetic rather than equilibrium properties of the enzyme–substrate complex.

The important feature of this scheme, in any case, is the kinetic stability of the complementary acceptor arm strands relative to non-complementary ones. The degree of basepair complementarity between exchanged strands determines the residence time of the aminoacylated 3′-strand of tRNA in the hydrolytic editing site of the aminoacyl-tRNA synthetase. A high degree of basepair complementarity decreases the probability of hydrolysis, lack of complementarity increases this probability. When complementary strands are matched, they slow the rate of hydrolytic editing relative to product dissociation. When strands are non-complementary, the hydrolytic reaction occurs at a higher rate relative to aminoacyl-tRNA dissociation.

5. Some observations explained

Certain observations may be understood in the light of this mechanism. The well-characterized mischarging of tRNA in the presence of organic solvents [3,40] may result from the decreased stability of double-stranded RNA in these solvent conditions. Any recognition complex between complementary acceptor arm strands would be destabilized and the editing function would be proportionately diminished, allowing increased proportions of incorrectly charged tRNA to dissociate.

Several stopped flow measurements of the aminoacylation reaction have found a rate-limiting step subsequent to amino acid activation and tRNA binding, but preceding product release [42,43]. Holler has shown that a slow rearrangement is correlated with displacement of the phenylalanyl group bound to tRNA from its substrate binding site [44]. Such rearrangements may be related to the conformational change for tRNA proposed here in forming the recognition complex.

The mischarging of *E. coli* $tRNA^{Trp}$(*su7*) [18] and the $tRNA^{Tyr}$(*su3*) mutants [18,19] provide experimental data relevant to the function of the base sequence in the acceptor stem. In the former, the complete identity of bases 2–5 in the acceptor stems of $tRNA^{Trp}$(*su7*) and $tRNA^{Gln}$ permits formation of stable $tRNA^{Trp}$–$tRNA^{Gln}$ recognition complex through acceptor arm strand exchange on the aminoacyl-tRNA synthetase (table 1).

The $tRNA^{Tyr}$(*su3*) mutants which can be mischarged with Glu show behavior consistent with changes in the kinetics of acceptor arm helix opening. Although similar to $tRNA^{Gln}$ in the sequence of acceptor stem recognition site proposed here and in anticodon, $tRNA^{Tyr}$(*su3*) is not mischarged with Gln by Glu-tRNA synthetase. However, mutations in the terminal 2 basepairs of the acceptor stem of $tRNA^{Tyr}$(*su3*) result in mischarging with Gln to varying extents, consistent with an influence upon the kinetics of the steps of strand opening and exchange. Table 2 shows that mutations in either of the last 2 positions of the acceptor stem of $tRNA^{Tyr}$ (*su3*) which would thermodynamically destabilize the double-strand, correlate with increased mischarging by Glu [37]. The effect is slightly more pronounced for the terminal basepair where the unwinding is likely to begin.

These data are consistent with a mechanism in which, when primary specificity determinants are equally satisfied by 2 different tRNAs, the propor-

Table 2
Acceptor stem sequences and specificity of aminoacylation of *E. coli* su7 suppressor and related tRNAs

	$tRNA^{Trp}$	$tRNA^{Gln}$	$tRNA^{Trp}$(su7)
acceptor stem 3′	U C C C C G C	A C C C C A U	U C C C C G C
5′	A G G G G C G	U G G G G U A	A G G G G C G
anticodon	A C C	C U C	A U C (U)
specificity of aminoacylation	trp	gln	gln

Table 3
E. coli tRNAs related to mischarging $tRNA^{Tyr}$ suppressors

tRNA	Acceptor Stem	Anticodon	Specificity of Aminoacylation
$tRNA^{Tyr}$	3' C C A C C C C 5' G G U G G G G	A U G*	Tyr
$tRNA^{Tyr}$(su3(A1))	C C A C C C C (A) G U G G G G	A U C	Gln
$tRNA^{Tyr}$(su3(A2))	C C A C C C C G (A) U G G G G	A U C	Gln > Tyr
$tRNA^{Tyr}$(su3(U80))	C (U) A C C C C G G U G G G G	A U C	Tyr > Gln
$tRNA^{Tyr}$(su3(U81))	(U) C A C C C C G G U G G G G	A U C	Tyr > Gln
$tRNA^{Tyr}$(su3(A1U81))	(U) C A C C C C (A) G U G G G G	A U C	Gln > Tyr
$tRNA^{Tyr}$(su3(A2U80))	C (U) A C C C C G (A) U G G G G	A U C	Tyr
$tRNA^{Gln}$	A C C C C A U U G G G G U A	G U C	Gln

tion of products formed will be determined by the kinetics of acceptor arm strand opening and exchange associated with the hydrolytic editing step. Extrapolation from rate constants of thermal melting and recombination of complementary RNA strands of $\geqslant$8 nucleotides give rate constants for such melting well above the ms^{-1} range for a pentanucleotide [45], so that such steps would not be inconsistent with the rate constants of the aminoacylation reaction.

It should be pointed out that the loss of double-strand helix postulated here is distinct from thermal melting phenomena. The former can arise from the effects of aminoacyl-tRNA synthetase structure upon the double-strand acceptor stem, whereas the latter is more nearly a reflection of thermodynamic stability of the polynucleotide double strand. Increasing temperature and increasing organic solvent concentration have different effects upon the specificity of the tRNA aminoacylation reactions [3].

6. Estimation of increase in specificity

Although it is difficult to quantitate the degree of specificity conferred by the editing mechanism proposed here, some estimates can be made. Consider the case of bound, activated, cognate amino acid to be transferred to enzyme-bound tRNA. A bound pair of tRNA molecules can be either 2 cognate (C2), 2 non-cognate (N2), or 1 cognate with 1 non-cognate (CN). If we assume that the affinity of the cognate tRNA for the enzyme is 10^2–10^3-times that of the non-cognate under the conditions of the reaction, and that there is always 19-times the concentration of non-cognate to cognate tRNA, then the ratio of (C2)/(N2) will be ~25. Then, the approximate relative proportions of the 3 types of pairs is (C2):(CN):(N2) = 500:20:1. If the CN and N2 pairs all went to mischarged product, the error rate would be 1 in 40. The mechanism of strand exchange control of the editing function proposed here would result in discharging of the CN pairs and so reduce the errors to <1 in 500. This value depends strongly upon the relative affinities of non-cognate and cognate tRNAs for the aminoacyl-tRNA synthetase, and taken with such other factors as the relative concentrations of tRNA, and the contribution, if any, of hydrolytic discharging of mischarged, non-cognate tRNA through recombination with its cognate synthetase, should be regarded only as illustrative. It is interesting that the frequency of mischarging by an aminoacyl-tRNA synthetase increases with increasing purity of non-cognate tRNA substrate available to it [3]. This may arise through an inflation of the number of enzyme-bound N2 pairs which, because of their acceptor stem complementarity, will drive the mis-aminoacylation in spite of the editing mechanism.

The mechanism proposed here exploits sequence information in the tRNA substrate to increase the fidelity of product formation. The means of accomplishing this is recognition through basepair complementarity. It does not require strong discrimination through enzyme–nucleic acid interactions, although some such recognition of either positive or negative character must occur. The mechanism also implies that not all ATP hydrolysis will lead to product, since correctly charged tRNA which occurs in CN pairs will be discharged. This sacrifice of a few % of correct product permits an increase of >10-fold in the fidelity of product formation.

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