The EMBO Journal vol.9 no.13 pp.4527-4533, 1990

Significance of the third tRNA binding site, the E site, on *E.coli* ribosomes for the accuracy of translation: an occupied E site prevents the binding of non-cognate aminoacyl-tRNA to the A site

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Communicated by K.H.Nierhaus

The E site (exit site for deacyl-tRNA) has been shown to be allosterically linked to the A site (aminoacyl-tRNA binding site), in that occupation of the E site reduces the affinity of the A site, and vice versa, whereas the intervening peptidyl-tRNA binding site (P site) keeps its high affinity. Here the question is analysed of whether or not the low affinity state of the A site caused by an occupied E site is of importance for the ribosomal accuracy of the aminoacyl-tRNA selection. In a poly(U) dependent system with high accuracy in poly(Phe) synthesis, the acceptance of the cognate ternary complex Phe-tRNA-EF-Tu-GTP (which has the correct anticodon with respect to the codon at the A site) was compared with the competing acceptance of ternary complexes with near-cognate Leu-tRNA^{Leu} (which has a similar anticodon) or non-cognate Asp-tRNA^{Asp} (which has a dissimilar anticodon), by monitoring the formation of AcPhePhe, AcPheLeu or AcPheAsp, respectively. Cognate (but not near-cognate) occupation of the E site reduced synthesis of the 'wrong' dipeptide AcPheLeu only marginally relative to that of the cognate AcPhe$_2$, whereas the formation of AcPheAsp was decreased as much as 14-fold, thereby reducing it to the background level. It follows that the allosteric interplay between E and A sites, i.e. the low affinity of the A site induced by the occupation of the E site, excludes the interference of non-cognate complexes in the decoding process and thus reduces the number of aminoacyl-tRNA species competing for A site binding by an order of magnitude. Furthermore, near- or non-cognate AcPhe-aminoacyl-tRNA molecules readily fall off the A site and can be efficiently chased from the A site by ternary complexes. This process might be related to the 'editing' mechanism proposed previously.

Key words: accuracy of translation/E site/non-cognate aminoacyl-tRNA/selection of aminoacyl-tRNA

Introduction

Three classes of aminoacyl-tRNAs, or the corresponding ternary complexes (aminoacyl-tRNA-EF-Tu-GTP), compete for the programmed ribosomal A site. The first class is represented by the cognate aminoacyl-tRNA, comprising a single species with an anticodon which is precisely complementary to the codon present at the A site. Secondly, there is a class of four to six near-cognate aminoacyl-tRNAs which bear anticodons similar to that of the cognate tRNA. The third class contains the majority of the tRNA species, comprising ~50 non-cognate aminoacyl-tRNAs with anticodons which are dissimilar to that of the cognate tRNA. In *Escherichia coli* 41 tRNA species with unique anticodons have been found (Komine *et al.*, 1990), about four of which can be assigned to the near-cognate class and 36 to the non-cognate class.

Cognate deacylated tRNA has an affinity for the A site which is about two orders of magnitude lower than that of cognate ternary complexes (0.2×10^7/M versus $10-20 \times 10^7$/M at 10 mM Mg^{2+}; S.Schilling-Bartetzko and K.H.Nierhaus, unpublished), indicating that strong interactions exist between the A site and the ternary complex, apart from the codon-anticodon interaction. Therefore, any ternary complex would be expected to interfere with the decoding process. The discrimination between cognate and near-cognate ternary complexes is thought to be governed by a proofreading mechanism. However, the fact that non-cognate ternary complexes do not interfere with the selection process is not yet understood. This problem is addressed in this paper.

The allosteric three-site model of the elongation cycle (Rheinberger and Nierhaus, 1986; Gnirke *et al.*, 1989) offers an explanation for the non-interference of non-cognate aminoacyl-tRNAs with the decoding process. According to this model (for main features see Table I) an occupied E site induces a low-affinity state of the A site. It follows that the A site occupation process must occur in two separate steps. The first step (presumably fast) represents the codon-anticodon interaction, and the second step (presumably slow) represents the allosteric transition from the post- to the pre-translocational state, involving a shift of the A site from low to high affinity. Only the first step (tRNA-mRNA interaction) is discriminatory, whereas the second step (tRNA-ribosome interaction) does not distinguish between 'wrong' and 'right' aminoacyl-tRNA. If the first step is the trigger for the second step, then the non-cognate aminoacyl-tRNAs are excluded from the process. They cannot interact with the codon, and are thus unable to bind firmly to the A site, i.e. the A site practically does not exist for non-cognate aminoacyl-tRNAs.

In this paper we demonstrate that the incorporation of non-cognate aminoacyl-tRNAs is indeed prevented by occupation of the E site (low-affinity A site) in contrast to ribosomal complexes with an unoccupied E site (high-affinity A site).

Results

Definition of the binding system

A system had to be devised which firstly guarantees a defined occupational state of the E site prior to A site binding, and which secondly allows the aminoacyl-tRNA bound to programmed A sites to be identified unambiguously and distinguished from low-level binding to unprogrammed P sites. Using poly(U) as mRNA, ribosomal complexes are

established which carry AcPhe-tRNA at their P site and expose either a free or an occupied E site. Occupation of the E site is achieved by direct binding of tRNA (Rheinberger and Nierhaus, 1986; Gnirke *et al.*, 1989). Next, a mixture of the cognate and a near- or non-cognate aminoacyl-tRNA is added in preformed ternary complexes with EF-Tu and GTP. Binding of aminoacyl-tRNA to the A site adjacent to an AcPhe-tRNA in the P site leads to peptidyl transfer and thus to incorporation of the correct or misincorporation of the wrong amino acid into an Ac-dipeptide.

However, the reaction does not stop at this stage. In the presence of EF-G, but also in its absence (spontaneous translocation; Bergemann and Nierhaus, 1983), translocation of the Ac-dipeptidyl-tRNA occurs, yielding ribosomal complexes with Ac-dipeptidyl-tRNA at their P sites and occupied E sites. Subsequent A site binding of aminoacyl-tRNA−EF-Tu−GTP complexes resulting in the formation of Ac-tripeptidyl-tRNA *only* occurs on ribosomes with a filled E site. Therefore, from this stage of synthesis on no information can be gained concerning the influence of E site occupation on the accuracy of A site binding.

Consequently, the relevant product, namely the Ac-dipeptidyl-tRNA, has to be separated both from longer products and from the aminoacyl-tRNA substrates. In addition, non-acetylated dipeptidyl-tRNA, which could result from aminoacyl-tRNA binding to 70S−poly(U)−Phe-tRNA complexes, has to be removed. Separation of products and substrates is achieved by reversed phase HPLC, and Figure 1 shows that good separation of all the relevant standard peptides can be obtained in this system.

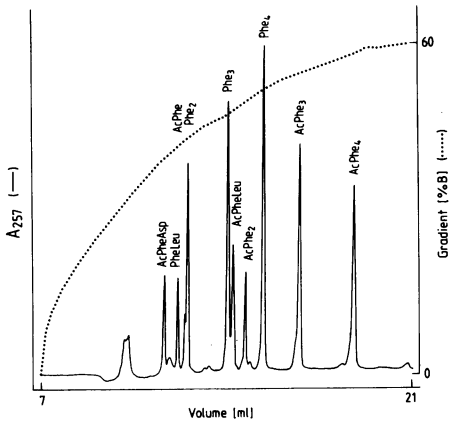


Fig. 1. Separation of peptides by reversed phase HPLC. 15 nmol e of Phe_2, Phe_3, Phe_4 and PheLeu and ≤15 nmol each of AcPhe, AcPhe2, $AcPhe_3$, $AcPhe_4$, AcPheLeu and AcPheAsp in H_2O/acetic acid were applied onto a Nucleosil 100-5 C18 column equilibrated with 0.1% TFA. Peptides were eluted with an acetonitrile gradient (%B) in 0.1% TFA, and were detected by absorption at 257 nm.

Table I. Features of the allosteric three-site model

1. A ribosome contains three tRNA binding sites A, P and E. In the cou of elongation a tRNA occupies the sites successively in the or A → P → E. The E site exclusively binds deacylated tRNA.

2. A and E sites are allosterically linked via negative cooperativity, i occupation of one site decreases the affinity of the other and vice ver

   Consequences: an elongating ribosome can adopt two states:
   - The pre-translocational state, where A and P sites have high affinit for tRNA.
   - The post-translocational state, where P and E sites have high affiniti

   Deacylated tRNA is released upon A site occupation and not duri translocation.

3. Both tRNAs present on the ribosome before and after translocati undergo codon−anticodon interaction simultaneously.

4. Both elongation factors EF-Tu and EF-G promote the transition fr one state to the other: EF-Tu that of the post- to the pre-translocatior and EF-G that of the pre- to the post-translocational transition.

Misincorporation into, and binding stability of Ac-dipeptides

Different mixtures of ternary complexes containing cognate [^{14}C]Phe-tRNA and near-cognate [^{3}H]Leu-$tRNA_2^{Leu}$ (codon CUU/C) were added to 70S−poly(U)−AcPhe-tRNA complexes. After binding, the 70S-bound and free tRNA were separated by ultracentrifugation, and both fractions individually analysed by HPLC. Table II compiles the errors in Ac-dipeptide found in the total material (70S-bound + free) and in the 70S-bound fraction, respectively. The high total misincorporation rates of 25%, 30% and 90% contrast markedly with the low translational error (~0.1%) in poly(U) dependent poly(Phe) synthesis under the same ionic conditions (Bartetzko and Nierhaus, 1989). This discrepancy is due to the rapid depletion of cognate substrate, which is added here only in limited amounts (0.2- and 1-fold with respect to 70S), and also to the high proportion of near-cognate substrate (1- and 20-fold with respect to the cognate substrate).

The error in the 70S-bound fraction of Ac-dipeptide is consistently smaller than that in the total Ac-dipeptide synthesized (e.g. 4.5% as compared with 25%). The reason for this is that the majority of the cognate $AcPhe_2$-$tRNA^{Phe}$ remains bound to the 70S (Table II, 51−94%), whereas significantly less of the near-cognate AcPheLeu-$tRNA_2^{Leu}$ remains stably attached to the ribosomes (4.3−50%). When the amount of ternary complexes added is raised from (0.2× + 0.2×) to (1× + 1×) with respect to 70S, and at the same time translocation is slowed down by omission of EF-G, then the drop-off of AcPheLeu-$tRNA_2^{Leu}$ increases sharply (50% residual binding falling to 13%). Thus, dissociation of the near-cognate dipeptide AcPheLeu-$tRNA_2^{Leu}$ is strongly stimulated by chasing with aminoac tRNA−EF-Tu−GTP from the A site, whereas the cogn dipeptide $AcPhe_2$ remains unaffected on the ribosome (and 94%) when cognate and near-cognate ternary comple are added in a stoichiometric ratio. Surprisingly, this chas is not dependent on the presence of cognate aminoac tRNA, since addition of an excess of the near-cognate tern complex Leu-$tRNA_2^{Leu}$−EF-Tu−GTP also results increased release even of cognate $AcPhe_2$-tRNA, althou the near-cognate AcPheLeu-$tRNA_2^{Leu}$ is preferentia released from the ribosome (residual bindings of 51 a 4.3%, respectively).

Influence of the occupational state of the E site on accuracy at the A site

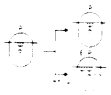
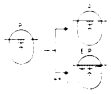
A mixture of the ternary complexes containing stoichiomet amounts of both [^{14}C]Phe-tRNA and [^{3}H]Leu-$tRNA_4$ (codon UUA/G) were added to 70S−poly(U)−AcPhe-tR complexes with free or occupied E sites, respectively, a

Table II. Comparison of total synthesis and 70S-bound fraction of Ac-dipeptides

Conditions				Results			
Initial complex	EF-G	Addition of ternary complexes		Error (%) [AcPheLeu/($AcPhe_2$ + AcPheLeu)] ×100		70S-bound dipeptides	
		[^{14}C]Phe-tRNA^{Phe}	[^{3}H]Leu-tRNA$_2^{Leu}$	Total	70S-bound	% of total $AcPhe_2$	% of total AcPheLeu
	−	1×	1×	25	4.5	92	13
	+	0.2×	0.2×	30	18	94	50
	−	0.2×	4×	90	45	51	4.3

50−60% of the ribosomes present in the mixture with 'initial complexes' carried an AcPhe-tRNA in the P site. The numbers in the columns 'addition of ternary complexes' give the molar ratio ternary complex:70S. The ternary complexes aa-tRNA−EF-Tu−GTP were added as a mixture of Phe-tRNA and Leu-tRNA. The specific activity of Ac[^{14}C]Phe in the 'initial complex' was 18-fold lower than that of [^{14}C]Phe in the ternary complexes (60 versus 1100 d.p.m./pmol). The HPLC analysis revealed that nearly exclusively oligopeptides primed with AcPhe were formed upon addition of ternary complexes. The amount of total AcPheLeu corresponding to 25, 30 and 90% were 3.7, 3.0 and 25.8 pmol, respectively. The HPLC patterns were highly reproducible, the relative precision of the given values is better than ± 5%. For further details see Materials and methods.

Table III. Error of tRNA reaction at the A site with and without occupation of the E site

Conditions					Results			
Experiment number	Initial complex	E site	Addition of ternary complex		Error (%) [AcPheAsp/($AcPhe_2$ + AcPheAsp)] ×100		Ratio error (E site free) to error (E site occupied)	
			[^{14}C]Phe-tRNA	[^{3}H]Asp-tRNA^{Asp}	Total	70S-bound	Total	70S-bound
1		free	0.2×	4×	2.4	0.72	9	14
		occ.			0.27	0.05		
2		free	0.2×	4×	2.3	1.2	1.0	0.75
		occ.			2.2	1.6		
	near cognate							

occ., occupied. The respective aminoacyl-tRNAs were added in ternary complexes with EF-Tu and GTP, and mixed before addition. In experiment 1 the E site has been occupied with cognate deacyl-tRNA^{Phe}, in experiment 2 with near-cognate tRNA^{Leu} (molar ratio tRNA:70S = 4:1). For further details see Materials and methods and footnote to Table II. Symbols are explained in the legend to Figure 3.

the formation of Ac-dipeptide at 0°C was monitored kinetically. As can be seen from Figure 2A, occupation of the E site slows down the synthesis of Ac-dipeptide considerably. At the same time, prefilling of the E site with deacyl-tRNA^{Phe} reduces the error in the production of Ac-dipeptide by a factor of up to two (Figure 2B). This E site effect on the A site error, however, is only observed initially and disappears during the course of the binding reaction. Probably, the effect is counteracted by the increasing depletion of the cognate substrate and by the delayed participation of functionally defective ribosomes in the binding process.

Next, the influence of E site occupation on the poly(U) dependent misincorporation of the non-cognate [^{3}H]Asp-tRNA−EF-Tu−GTP (codon GAU/C) was tested, using the same strategy as before. Here cognate and non-cognate ternary complexes are added in ratio of 0.2:4, in order to simulate the *in vivo* situation, where the cognate aminoacyl-tRNA has to compete with a large majority of non-cognate aminoacyl-tRNAs.

The results are shown in Table III, experiment 1. Initial E site binding reduces the relative misincorporation of total AcPheAsp 9-fold. In the 70S-bound fraction of Ac-dipeptide

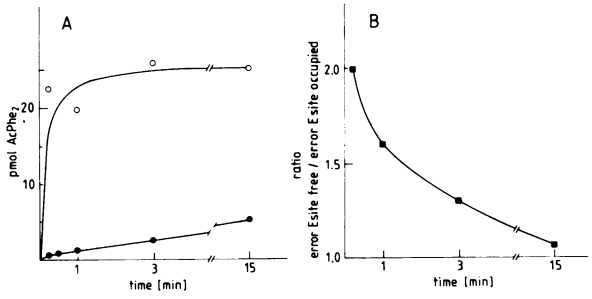


Fig. 2. A. Synthesis of $AcPhe_2$ after addition of a mixture of [^{14}C]Phe-tRNA−EF-Tu−GTP and [^{3}H]Leu-tRNA$_4^{Leu}$−EF-Tu−GTP (molar ratio of each to 70S = 1:1) to 70S−poly(U)−AcPhe-tRNA complexes with free (○) or occupied (●; 4× tRNA^{Phe}) E site, respectively, at 0°C. The binding reaction was stopped by addition of Na(acetate), pH 5.0 (final concentration 0.3 M). 70S-bound and free tRNA were not separated prior to HPLC analysis of the peptides formed. The specific activities were 1000 d.p.m./pmol ([^{14}C]Phe) and 16 500 d.p.m./pmol ([^{3}H]Leu). **B.** Ratios of error in Ac-dipeptide for free and occupied E site.

the decrease in error is even more pronounced, giving values of 14-fold. This rather dramatic effect of the E site occupation is illustrated in Figure 3. Whereas formation of

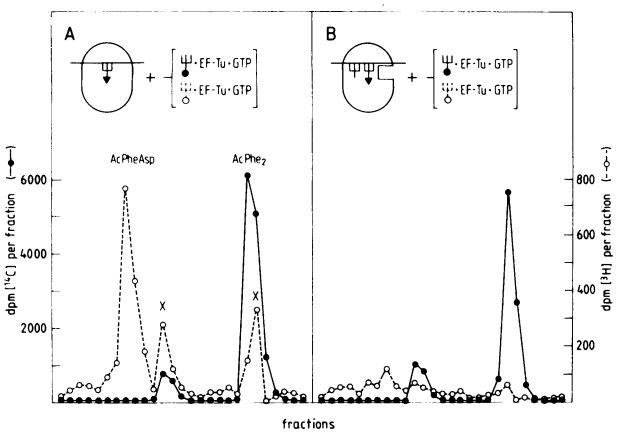


Fig. 3. Isolation of AcPhePhe and AcPheAsp by reversed phase HPLC. 70S-bound peptides after incubation of 70S−poly(U)−AcPhe-tRNA complexes with [^{14}C]Phe-tRNA−EF-Tu−GTP (molar ratio Phe-tRNA:70S = 0.2:1) and [^{3}H]Asp-tRNA−EF-Tu−GTP (ratio 4:1) with free (**A**) and occupied E site (**B**) in the absence of EF-G. The E site was occupied by addition of deacyl-tRNA^{Phe} (ratio 4:1) prior to binding of ternary complexes. For further details see Materials and methods. ●—●: [^{14}C]Phe; ○--○: [^{3}H]Asp. X: Asp-containing peptides, not further analysed. Symbols used in this paper are: ——, poly(U); Ψ , deacylated tRNA^{Phe}; Ψ , Ac[^{14}C]Phe-tRNA; Ψ , [^{14}C]Phe-tRNA, [^{3}H]Leu-tRNA$_2^{Leu}$, [^{3}H]Asp-tRNA^{Asp}; Tu, EF-Tu;

ribosome, not in a defined functional state of the elongation cycle

post-translocational ribosome (high affinity sites P and E; A with low affinity)

pre-translocational ribosomes (high-affinity sites A and P; E with low affinity)

AcPhe$_2$ is hardly affected by prior binding of tRNA^{Phe} to the E site, synthesis of AcPheAsp is practically abolished. Similarly, formation of other Asp-containing peptides is prevented when the E site has been occupied (X in Figure 3A).

Next we tested whether or not the strong reduction of the formation of the 'wrong' product AcPheAsp was caused by the excess of added tRNA^{Phe} (molar ratio tRNA^{Phe}:70S = 4:1) which possibly could have chased the 'wrong' product more efficiently than the correct product AcPhePhe. An experiment was performed identical to that described in Table III, experiment 1, except that prior to the addition of ternary complexes the excess of non-bound tRNA^{Phe} was removed by a gel filtration step (AcA 34). Again the same results were observed, a strong reduction of the 'wrong' AcPheAsp in contrast to the correct AcPhePhe (data not shown), which clearly demonstrates that the prevention of the AcPheAsp formation was caused by the occupied E site rather than by the excess of non-bound tRNA^{Phe} .

tRNA is known to undergo codon−anticodon interaction at the ribosomal E site (Rheinberger *et al.*, 1986; Lill and Wintermeyer, 1987; Gnirke *et al.*, 1989). However, the E site can be occupied in a codon independent fashion by a tRNA showing a high intrinsic affinity for the 70S ribosome (Lill *et al.*, 1988; Gnirke *et al.*, 1989). Using such a tRNA, namely tRNA$_2^{Leu}$, the effect of misprogrammed occupation of the E site on the accuracy of aminoacyl-tRNA selection at the A site was studied. Addition of tRNA$_2^{Leu}$ (codon CUU/C) to 70S−poly(U)−AcPhe-tRNA complexes does not decrease the misbinding of Asp-tRNA−EF-Tu−GTP (Table III, experiment 2). Instead, the error in the 70S-bound fraction of Ac-dipeptide seems even slightly increased.

Discussion

Features of the system and preferential release of near-cognate oligopeptidyl-tRNAs

In this paper we analyse the significance of the occupational state of the E site for the accuracy of aminoacyl-tRNA selection at the A site. We start with poly(U) programmed ribosomes carrying AcPhe-tRNA at their P site, with or without deacyl-tRNA^{Phe} at the adjacent E site. Ternary complexes (aminoacyl-tRNA−EF-Tu−GTP) are added, and the accuracy of selection is assessed by the error in the Ac-dipeptides formed. A further distinction is made between 70S-bound Ac-dipeptides and those released from the ribosome.

It has been reported that near-cognate Ac-dipeptides are preferentially released from the ribosome, as compared with cognate Ac-dipeptides (Cabañas and Modolell, 1980; Gast *et al.*, 1987). We confirm this observation in that the error in the total fraction of Ac-dipeptide (70S-bound plus released) is always larger than in the ribosome associated fraction. With extremely limiting amounts of A site substrates the difference amounts to a factor of 1.7 (Table II, second line). However, the factor increases to 5.5-fold upon increase of the A site substrate concentration and concomitant delay of the translocation reaction (in the absence of EF-G; Table II, first line), which results in an extended time of residence at the A site. Two conclusions can be drawn. (i) At least part of the near-cognate Ac-dipeptidyl-tRNA easily falls off the ribosome. (ii) The near-cognate Ac-dipeptidyl-tRNA is efficiently chased from the A site by free ternary complex. Interestingly, cognate AcPhe$_2$-tRNA can also be chased even by a near-cognate ternary complex, although to a lesser degree, if the latter is present in high excess and the E site not occupied (high-affinity A site; Table II, third line). It follows that the presence of EF-Tu in the chasing complex under this condition (high-affinity A site) seems to be important *per se*, independent of the presence of a cognate anticodon in the aminoacyl-tRNA. This finding stresses the importance of contacts other than codon−anticodon interactions for binding ternary complexes to a high-affinity A site, and indicates the weak discriminatory power of the A site in the high-affinity state.

The preferential release of near-cognate peptidyl-tRNA should be restricted to short oligopeptidyl-tRNAs, since longer peptidyl-tRNAs are so firmly bound that they comigrate with the large subunits in sucrose gradients upon dissociation of ribosomes, which probably occurs with the concomitant loss of mRNA (Gilbert, 1963). In fact, release of peptidyl-tRNA has been observed *in vivo* (for review see Menninger, 1977), with a preference for shorter peptidyl-tRNAs (Menninger *et al.*, 1983). The energy drain resulting from the final check of the codon−anticodon fit after peptide bond formation would thus be limited by being confined to short oligopeptides (Gast *et al.*, 1987).

The preferential chasing of incorrect oligopeptidyl-tRNA is reminiscent of the 'editing' mechanism proposed by Menninger (1977). Editing, however, was considered to be an active correction process, which could not be specifically assigned to either A or P site. 'Drop-off' of oligopeptidyl-tRNA as observed here apparently represents a passive

process, due to the low binding stability of at least part of the near-cognate oligopeptidyl-tRNA, which results in spontaneous release or chasing by free ternary complexes in the A site. It might well be that the editing mechanism corresponds to this passive process which occurs at the A site.

Cognate E site occupation prevents the interference of non-cognate aminoacyl-tRNA with the decoding process at the A site

Occupation of the E site improves the selection of cognate versus near-cognate aminoacyl-tRNAs (Figure 2B). The observed effects however, are small, and the absolute errors extremely high (up to 90%, see Table II) due to the conditions used, which were chosen to promote a high yield of Ac-dipeptides. These error frequencies are far from the accuracy values of about 1:1000 seen *in vivo* (Bouadloun *et al.*, 1983; Laughrea *et al.*, 1987; and references herein), as well as *in vitro* under steady-state conditions using either the polymix system (Jelenc and Kurland, 1979; Ehrenberg *et al.*, 1986) or the ionic milieu applied here (Bartetzko and Nierhaus, 1989). Thus, no conclusions can be drawn concerning the selection mechanism for cognate versus near-cognate aminoacyl-tRNAs.

However, the results reported here shed light on the discrimination against non-cognate aminoacyl-tRNAs at the A site. Figure 3A demonstrates that non-cognate Asp-$tRNA^{Asp}$ clearly interacts with high-affinity A sites (E site free) and allows the formation of AcPheAsp peptides. In contrast, we do not find any significant amounts of the non-cognate Ac-dipeptide when the A site affinity has been lowered by E site occupation (Figure 3B). The specific repression of the non-cognate AcPheAsp peptides is not caused by non-bound $tRNA^{Phe}$, since after removal of the non-bound material the specific repression was still found.

However, E site occupation *per se* is not sufficient. The tRNA at the E site clearly must undergo codon–anticodon interaction in order to reduce the error, since near-cognate $tRNA_2^{Leu}$, which can bind to non-programmed E sites under the conditions used (U.Geigenmüller and K.H.Nierhaus, unpublished observation), does not reduce the error in the Ac-dipeptides (Table III, experiment 2). This finding underscores the functional importance of codon–anticodon interaction at the E site.

It follows that *cognate* occupation of the E site reduces the A site affinity to such an extent that the A site practically does not exist for the 50 or so non-cognate ternary complexes. This reduces the problem of aminoacyl-tRNA selection by an order of magnitude. Furthermore, discrimination against the four to six near-cognate aminoacyl-tRNAs seems at least to be improved upon E site occupation as already mentioned above.

In agreement with these findings is the observation that increasing amounts of non-cognate (data not shown) or even near-cognate (Bilgin *et al.*, 1988a) ternary complexes do not reduce the speed of elongation. In contrast, even transient, EF-Tu promoted binding of non- or near-cognate aminoacyl-tRNA should lead to a delay in elongation if the A site were always in a high-affinity state, since A site binding has been identified as the rate limiting step in elongation (Bilgin *et al.*, 1988b; S.Schilling-Bartetzko, A.Bartetzko and K.H.Nierhaus, unpublished observation).

The improvement in the decoding accuracy induced by

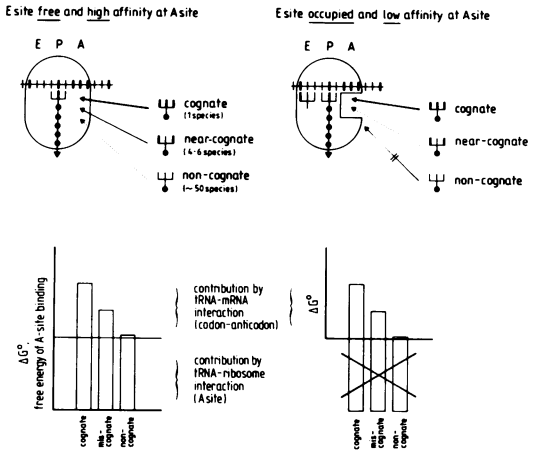


Fig. 4. Outline of the hypothesis that the E site induced lowering of the A site affinity prevents the binding of non-cognate aminoacyl-tRNAs (see text).

occupation of the E site is explained by a hypothesis depicted in Figure 4. Assuming that the E site is not occupied, thus resulting in a high-affinity A site (Figure 4, left half). The free energy of A site binding can be separated into two terms. The first term concerns the tRNA–mRNA interaction (codon–anticodon interaction), the second one of the tRNA–ribosome interactions. The second term does not discriminate between 'wrong' and 'right' aminoacyl-tRNAs (in contrast to the first term), but is responsible for a correct positioning of the aminoacyl residue at the peptidyl-transferase centre and thus is a prerequisite for peptide bond formation. Since the second term is operative (the A site being in its high-affinity state), even non-cognate aminoacyl-tRNAs will occasionally be accepted at the A site, giving rise to the incorporation of non-cognate aminoacyl residues into the growing peptidyl chain.

When the E site is occupied, the A site is present in a low affinity state (Rheinberger and Nierhaus, 1986; Gnirke *et al.*, 1989). We now assume that the second term is exclusively abolished (tRNA–ribosome interaction; Figure 4, right half), whereas the first term (codon–anticodon interaction) is either not affected or even improved. In this situation, non-cognate aminoacyl-tRNAs are no longer able to interfere with the decoding process; codon–anticodon interaction cannot take place, and a stable tRNA–ribosome interaction is prevented.

In thermodynamic terms the presumed mechanism can be described as follows. It is clear that the discrimination energy $\Delta\Delta G$ of the binding of cognate versus non-cognate substrate is not affected by the allosteric shift-down of the A site affinity. However, the important point is that a high-affinity A site (Figure 4, left half) results in significant sticking times with the tRNA substrate, even if it is non-cognate. Therefore, equilibrium between cognate and non-cognate substrates of the A site would only be attained after relatively long periods. Furthermore, the increment in ΔG of the high-affinity state over that of the low-affinity state is due to tRNA–ribosome interactions which are—as mentioned above—essential prerequisites for peptide bond formation. Both factors—the slow reaching of the equilibrium, and the readiness of the A site to bind the acceptor molecule for the peptidyl transfer—would make it possible for premature termination of the binding reaction by peptide bond formation to occur,

even with non-cognate substrates. In contrast, both factors are absent if the A site is in the low-affinity state; the codon–anticodon interaction quickly reaches equilibrium, and the peptidyl transferase centre is not yet ready to accept an aminoacyl-tRNA, thus resulting in an uncoupling of the selection process from the peptidyl-transfer.

The results presented here support the expectations derived from this hypothesis. It is clear that the low-affinity A site induced by cognate E site occupation prevents the interference of non-cognate aminoacyl-tRNAs with the decoding process, and thus plays an important role in both the accuracy and speed of translational elongation.

Materials and methods

$tRNA^{Phe}$, $tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA^{Asp}$ (*E.coli*) were purchased from Subriden RNA, Rollingbay, Washington. Bulk tRNA (*E.coli*), poly(U) and GTP was obtained from Boehringer Mannheim. All radioactive amino acids were from Amersham, and unlabelled amino acids and polyamines from Sigma. Standard peptides Phe_2, Phe_3, Phe_4 and PheLeu and AcA 34 were from Serva, Heidelberg, FRG, and PheAsp from Bachem, Bubendorf, Switzerland. Acetylation of standard peptides followed the procedure of Haenni and Chapeville (1966). Reversed phase column materials Nucleosil 300-5 C_4 and Nucleosil 100-5 C18 were obtained from Macherey-Nagel, Düren, FRG. All other laboratory chemicals were from Merck, Darmstadt, FRG.

70S tightly coupled ribosomes were isolated from *E.coli* K12, strain CAN/20-12E (Zaniewski *et al.*, 1984) according to the procedure of Rheinberger *et al.* (1989). One A_{260} unit of 70S was taken as 24 pmol. EF-G and EF-Tu from *E.coli* K12, strain D10 (Gesteland, 1966), were purified as described by Leberman *et al.* (1980).

Aminoacylation and purification of tRNA

$tRNA^{Phe}$, $tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA^{Asp}$ were aminoacylated, and Phe-tRNA was acetylated, as described (Wurmbach and Nierhaus, 1979). Initial charging levels amounted to 800 pmol/A_{260} (phe-tRNA), 750 pmol/A_{260} (Leu-$tRNA^{Leu}$) and 1500 pmol/A_{260} (Asp-tRNA). Phe- and AcPhe-tRNA were purified by reversed phase HPLC on a Nucleosil 300-5 C4 column, using a linear or step gradient of 0–30% methanol in 400 mM NaCl, 10 mM $Mg(acetate)_2$, and 20 mM NH_4(acetate), pH 5.0. After purification, charging levels of 1530 pmol/A_{260} were obtained.

Poly(U) dependent poly(phe) synthesis

Poly(Phe) synthesis was performed essentially as described, using an *in vitro* system displaying rates of polymerization and misincorporation near to those found *in vivo* (Bartetzko and Nierhaus, 1989), in the presence of 3 mM Mg^{2+}, 150 mM NH_4^+ and polyamines.

tRNA binding and oligopeptide synthesis

tRNA binding was performed under the ionic conditions of the poly(U) dependent poly(Phe) synthesis system described (3 mM Mg^{2+}, 150 mM NH_4^+; Bartetzko and Nierhaus, 1989), except that the concentrations of spermine and spermidine were 0.2 mM and 2 mM, respectively.

Formation of the initial complexes

20 pmol 70S, 100 μg poly(U) and 16 pmol Ac[^{14}C]Phe-tRNA (60 d.p.m./pmol) per aliquot (25 μl) were incubated for 20 min at 37°C. Direct occupation of the E site was achieved by subsequent addition of 80 pmol $tRNA^{Phe}$ (5 min/37°C), raising the aliquot volume to 50 μl.

Binding of Ac[^{14}C]Phe-tRNA was determined by nitrocellulose filtration of two individual aliquots. P site location of Ac[^{14}C]Phe-tRNA was confirmed by a puromycin reaction. For this purpose, two aliquots minus EF-G and two plus EF-G, respectively, received puromycin at a final concentration of 0.7 mM in a final volume of 70 μl. Two aliquots serving as background control received buffer instead. After 2 h at 0°C, the reaction was stopped by addition of 65 μl 0.3 N NaAC, pH 5.0, saturated with $MgSO_4$, and the Ac[^{14}C]Phe-puromycin was extracted with ethyl acetate.

Removal of non-bound $tRNA^{Phe}$

After filling the E site of the initial complex the non-bound $tRNA^{Phe}$ was removed in some cases prior to the addition of the ternary complexes. 300 μl of the initial complex with an occupied E site were prepared as described above except that the sample contained 300 pmol of 70S. The sample was gel filtrated through AcA 34 as follows: a 5 ml syringe was used as column and was filled with ~4 ml preswollen AcA 34 after covering the bottom with glass beads (Sigma, no. G-9268). The material was equilibrated with the sample buffer (see tRNA binding) by centrifugation for 2 min at 800 *g* in a swing-out rotor (HB-4, Sorvall) and by refilling with buffer, these steps were repeated twice. The last centrifugation was for 5 min at 800 *g*, then the sample was applied to the column which then was centrifuged again for 5 min at 800 *g*. The eluted fraction (250–300 μl) contained tightly coupled ribosomes freed of non-bound tRNA, the yield of 70S in this fraction was 50–80% of the input. A sample containing 130 pmol of 70S was mixed with 100 μl of a ternary complex mixture containing 25 pmol of [^{14}C]Phe-tRNA, 500 pmol of [^{3}H]Asp-tRNA and EF-Tu (for further details see following section). After an incubation for 5 min at 37°C two aliquots (each with 10 pmol 70S) were withdrawn for nitrocellulose filtration, an aliquot with 100 pmol of 70S were adjusted to 0.3 M NaAc, pH 5.0, and treated as described under Identification of peptides.

Binding of aminoacyl-tRNA·EF-Tu·GTP

20–400 pmol [^{14}C]Phe-tRNA (1100 d.p.m./pmol) and [^{3}H]Leu-$tRNA_4^{Leu}$, [^{3}H]Leu-$tRNA_2^{Leu}$ (16 000 d.p.m./pmol) or [^{3}H]Asp-tRNA (21 000 d.p.m./pmol), respectively, were incubated with 160–3200 pmol EF-Tu (an 8-fold excess with respect to tRNA) and 40–50 nmol GTP in 75 μl (5 min/37°C), to form the ternary complex aminoacyl-tRNA–EF-Tu–GTP. This was then (if not otherwise indicated) added at 37°C to the preformed 70S–poly(U)–AcPhe-tRNA(–tRNA) complexes (100 pmol 70S in 325 μl, corresponding to five standard aliquots) and incubated for 5 min. Subsequently, 70S-bound and free tRNA were separated by ultracentrifugation of the binding aliquot (now 400 μl) for 2.5 h at 70 000 *g* (1°C).

Identification of peptides

After centrifugation of binding aliquots (see above), the pellets were resuspended in 400 μl 0.3 M NaAc, pH 5.0 and both the resuspended material and the supernatant were phenolized. tRNA was collected from the aqueous phases by ethanol precipitation followed by low-speed centrifugation, and the dried pellets were resuspended in 60 μl of 0.2 M NaOH. After hydrolysis of the RNA (30 min/50°C), 16 μl 1 M HCl and a mixture of non-radioactive acetylated standard peptides were added. The sample was applied onto a Nucleosil 100-5 C18 column (250 × 4 mm) equilibrated in 0.1% TFA. After the amino acids had been washed out, peptides were eluted by acetonitrile in 0.1% TFA, using a convex gradient from 0 to 60% (flow rate 1 ml/min). The eluate was monitored at 257 nm and 250 μl fractions were collected, and quantified by liquid scintillation counting of the [^{14}C]Phe, [^{3}H]Leu or [^{3}H]Asp residues, respectively. Recovery from the column was ~85%. Hydrolysis of peptidyl-tRNA in the absence of NaOH was not observed. Up to ~90% of the 70S–poly(U)–AcPhe-tRNA complexes could be shown to participate in the formation of Ac-peptides.

Acknowledgements

We thank Dr R.Brimacombe for support and advice. We are indebted to Doris Finkelmeier for valuable technical help and to Drs A.Gnirke and H.-J.Rheinberger for many helpful discussions. We are grateful to Dr M.Deutscher for sending us the *E.coli* K12 strain CAN/20-12E which lacks a number of RNases.

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Received on August 3, 1990; revised on September 17, 1990