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The Allosteric Three-Site Model for the Ribosomal Elongation Cycle: Features and Future

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ABSTRACT: The ribosome contains three binding sites for tRNA, viz., the A site for aminoacyl-tRNA (decoding site), the P site for peptidyl-tRNA, and the E site for deacylated tRNA (E for exit). The surprising finding of an allosteric linkage between the E and A sites in the sense of a negative cooperativity has three consequences: (a) it improves the proper selection of aminoacyl-tRNAs while preventing interference from noncognate aminoacyl-tRNAs in the decoding process, (b) it provides an explanation for the ribosomal accuracy without having to resort to the proofreading hypothesis, and (c) it has deepened our understanding of the mode of action of some antibiotics.

The elongation cycle of the ribosome is a series of reactions in which the growing peptidyl chain is lengthened by one amino acid. In the early 1960s Watson (1963, 1964) and Lipmann (1963) suggested a model for this elongation cycle in which the ribosome contained two binding sites for tRNA, namely, the P site for the peptidyl-tRNA and the A site for the newly selected aminoacyl-tRNA.

Figure 1A demonstrates the three basic reactions of the elongation cycle in the frame of the two-site model. The left half of the ribosome represents the P site and the right half the A site. In reaction 1 the A site is occupied with an aminoacyl-tRNA cognate to the codon exposed at this site. Next, the peptidyltransferase, an activity associated with the large ribosomal subunit, cleaves off the peptidyl residue from the peptidyl-tRNA and transfers it to the aminoacyl-tRNA at the A site (reaction 2). The result is that the peptidyl-tRNA (extended by one amino acid) is now located at the A site and the deacylated tRNA is at the adjacent P site. Reaction 3 is the translocation step in which the deacylated tRNA leaves the ribosome, the peptidyl-tRNA moves from the A to the P site, and a new codon invades the A site. According to the two-site model, the translocation is necessarily coupled to the release of deacylated tRNA. As a corollary of the two-site model, the posttranslocational ribosome always contains one tRNA, whereas the pretranslocational ribosome contains two tRNAs.

The two-site model became generally accepted (and still survived in the textbooks) when the so-called puromycin reaction was unraveled (Traut & Monro, 1964). Puromycin is a structural analogue of the aminoacyl end of an aminoacyl-tRNA. The drug can bind to the A-site region of the ribosomal active center where the peptide bond is formed and accepts the peptidyl residue from peptidyl-tRNA via formation of a peptide bond. The peptidyl-puromycin then falls off the ribosome.

It is clear that the peptidyl residue can be transferred to puromycin only if the peptidyl-tRNA is present at the P site, whereas no transfer can occur if the peptidyl-tRNA is located at the A site. These facts are the basis for the operational definition of A and P sites; namely, a positive puromycin reaction indicates P-site location of a peptidyl-tRNA, and a negative reaction is evidence for an A-site location. A revised and extended definition of the ribosomal tRNA binding sites taking into account recent observations is given at the end of the next section (Table II).

The puromycin reaction is an exclusive test of the acyl residue of aminoacyl- or peptidyl-tRNA, but the tRNA moiety itself is not tested. Accordingly, we undertook a study of the number of tRNA binding sites on the ribosome. Since three different species of tRNA are found on the ribosome in the course of elongation, i.e., peptidyl-tRNA, aminoacyl-tRNA, and deacylated tRNA (Figure 1A), saturation experiments were performed with all three species. Surprisingly, different answers were found in each of the three cases.

tRNA Binding Sites on the Ribosome

70S ribosomes were saturated with peptidyl-tRNA^{Phe}, aminoacyl-tRNA^{Phe}, or deacylated tRNA^{Phe} in the presence

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Table I: tRNA Binding Capacities of 30S and 50S Subunits and of 70S Ribosomes from *E. coli*[a]

ribosomes	poly(U)	tRNA species	binding sites: no.	binding sites: sites	remarks
70S	–	AcPhe-tRNA	1	P	saturates at 0.5
	–	Phe-tRNA	0		
	–	$tRNA^{Phe}$	1	P	
	+	AcPhe-tRNA	1	P or A	exclusion principle
	+	Phe-tRNA	2	P and A	
	+	$tRNA^{Phe}$	3	P, E, and A	binding sequence: first P, then E, then A
30S	–	AcPhe-tRNA	0		
	–	Phe-tRNA	0		
	–	$tRNA^{Phe}$	0		
	+	AcPhe-tRNA	1	P*	P*: prospective P site (becomes part of P site upon 50S association)
	+	Phe-tRNA	1	P*	
	+	$tRNA^{Phe}$	1	P*	
50S	–	AcPhe-tRNA	0		
	–	Phe-tRNA	0		
	–	$tRNA^{Phe}$	1	E*	E*: prospective E site (saturates at 0.2; becomes part of E site upon 30S association)
	+	AcPhe-tRNA	0		
	+	Phe-tRNA	0		
	+	$tRNA^{Phe}$	1	E*	E*: prospective E site (saturates at 0.4; becomes part of E site upon 30S association)

[a] Data are taken from Rheinberger et al. (1981) and Gnirke and Nierhaus (1986).

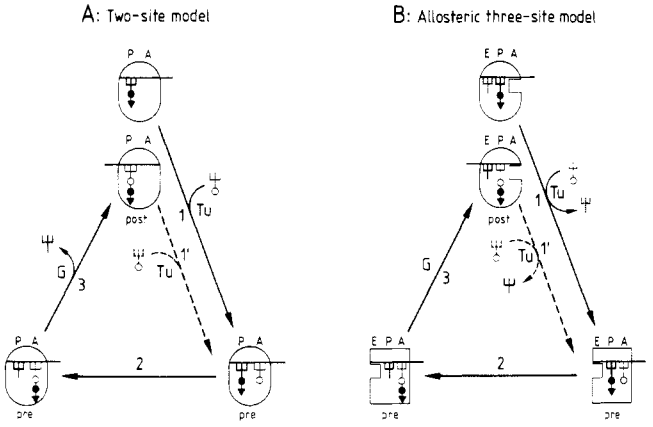

FIGURE 1: The three basic reactions of the elongation cycle: 1, A-site binding; 2, peptidyl transfer; 3, translocation. A, two-site model; B, allosteric three-site model. The pretranslocational conformer of the elongating ribosome is indicated by a rectangular ribosome and the posttranslocational conformer by an oval ribosome. Tu and G mean EF-Tu and EF-G.

or absence of poly(U), *N*-acetyl-Phe-$tRNA^{Phe}$ (AcPhe-$tRNA^{Phe}$) being taken as a simple analogue of peptidyl-$tRNA^{Phe}$. AcPhe-$tRNA^{Phe}$ saturated at one molecule per 70S, Phe-$tRNA^{Phe}$ at two molecules, and—most surprisingly—deacylated $tRNA^{Phe}$ at three molecules per 70S in the presence of poly(U) (Table I, 70S; Rheinberger et al., 1981).

The only not unexpected result was the experiment with Phe-tRNA. This species could obviously occupy the A and P sites of the same ribosome, and formation of dipeptides was observed (the actual saturation value was found to be 1.5 Phe-$tRNA^{Phe}$ molecules per 70S, due to contamination with deacylated $tRNA^{Phe}$, which we could not remove efficiently at that time).

The binding of AcPhe-$tRNA^{Phe}$ leveled off at one molecule per 70S, although AcPhe-$tRNA^{Phe}$ can be present at either the A or P site. This indicates that the binding of AcPhe-tRNA is governed by an "exclusion principle": Two sites (A or P) are available, but if one site is occupied by AcPhe-tRNA, then the second site cannot bind a further AcPhe-$tRNA^{Phe}$ molecule. The tunnel that has been observed in the large ribosomal subunit (Yonath et al., 1987), possibly harboring the growing peptidyl chain, could provide an explanation for this exclusion principle. The peptidyl residue is located in the tunnel regardless as to whether the corresponding tRNA moiety occupies the P or A site. Thus, once the tunnel is occupied, there is no possibility for a second peptidyl-tRNA to bind.

The validity of the exclusion principle has been questioned (Kirillov & Semenkov, 1982; Lill et al., 1984), but it could be verified with our preparations of ribosomes by means of quantitative puromycin reactions: Ribosomes were saturated with purified AcPhe-tRNA (saturation at about one molecule per 70S), and the bound AcPhe-tRNA was found to react quantitatively with puromycin. However, when AcPhe-tRNA was bound to the A site, no reaction took place. It follows that the P sites were exclusively filled and that the adjacent A sites did not bind an additional AcPhe-tRNA (Geigenmüller et al., 1986).

Up to three molecules of deacylated $tRNA^{Phe}$ could be bound per 70S in the presence of poly(U). In the absence of poly(U) only one molecule per 70S was found, and it was known that this molecule was present at the P site. The two additional sites could be occupied only in the presence of poly(U)—but not of poly(A) (Rheinberger, 1984)—indicating that the additional sites can be occupied only if the cognate codons are available. Clearly, codon–anticodon interaction is a prerequisite for a stable occupation of the two additional sites. An "indicator reaction" was developed which demonstrated that in the presence of poly(U) first the P site, then a new site, and finally the A site were occupied (Rheinberger et al., 1981). The new site was termed E site [E for exit according to an early suggestion by Wettstein and Noll (1965)]. The E site exclusively binds deacylated tRNA, and this site was also found and confirmed by other groups (Grajevskaja et al., 1982; Kirillov et al., 1983; Lill et al., 1984, 1988).

The results obtained with *Escherichia coli* ribosomes were so unexpected and strange that we wondered whether or not they could be generalized. We therefore analyzed an unusual organism from another kingdom, the archaebacteria, namely, the extreme halophile *Halobacterium halobium*. This archaebacterium exists with an enormous intracellular concentration of monovalent cations (more than 4 M). For optimal poly(Phe) synthesis even higher amounts (6 M, i.e., saturated salt concentrations) are required. Under these conditions standard nitrocellulose filtration methods cannot be used, since the nonbound tRNA is also trapped quantitatively on the

Table II: Definitions of the Ribosomal tRNA Binding Sites A, P, and E

(1) P site	(1.1) the P site is the first site of programmed ribosomes to be occupied at 37 °C (1.2) an acylated tRNA at the P site reacts with puromycin (1.3) the P site can bind peptidyl-tRNA, aminoacyl-tRNA, and deacylated tRNA
(2) A site	(2.1) a tRNA binds to the A site when its anticodon interacts with the adjacent codon downstream from that at the P site (2.2) an acylated tRNA at the A site cannot react with puromycin (2.3) the A site can bind peptidyl-tRNA, aminoacyl-tRNA, and deacylated tRNA; the A site exclusively binds the complex of EF-Tu, GTP, and aminoacyl-tRNA
(3) E site	(3.1) a tRNA binds to the E site when its anticodon interacts with the adjacent codon upstream from that at the P site (3.2) the E site exclusively binds deacylated tRNA

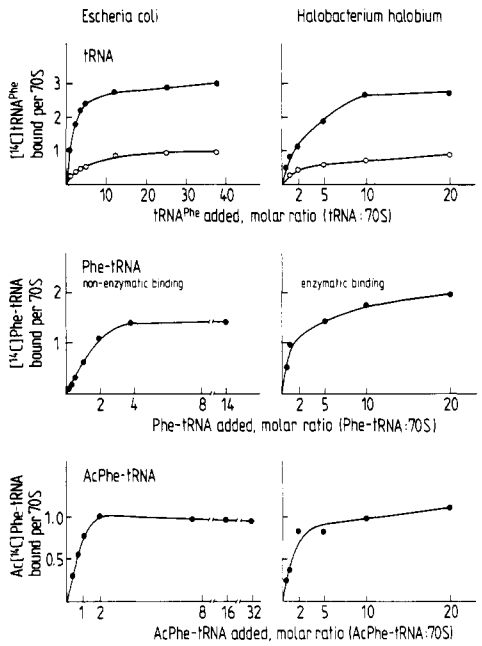

FIGURE 2: Comparison of tRNA-saturation experiments using ribosomes from *E. coli* (left column; Rheinberger et al., 1981) or *H. halobium* (right column; Saruyama & Nierhaus, 1986). Upper panels: ●, plus poly(U); ○, minus poly(U).

filters. Instead, centrifugation methods had to be applied.

In spite of the use of different organisms and different analytical techniques, practically the same results for tRNA binding were obtained (Figure 2, right half): deacylated $tRNA^{Phe}$ binding leveled off at three molecules per 70S, Phe-$tRNA^{Phe}$ at two, and AcPhe-$tRNA^{Phe}$ at one, thus confirming the exclusion principle (Saruyama & Nierhaus, 1986). A third binding site specific for deacylated tRNA has also been found in a eukaryotic system (rat liver; Rodnina et al., 1988). It is clear that the results concerning the tRNA binding sites on *E. coli* ribosomes can indeed be generalized. Table I also includes the binding features of the ribosomal subunits. For details and discussion, see Gnirke and Nierhaus (1986).

The features of A, P, and E sites described in this section complement and extend the definitions of the tRNA binding sites mentioned in the introduction. The revised definitions (Table II) rely—in addition to other features—on the position of the codon with respect to that at the P site, allowing a practicable and unequivocal assignment of the tRNA location. However, it would be mistaken to regard the sites as stable and rigid "moulds". We shall see in the next section that the ribosome is a dynamic reactor that oscillates between two major conformers, the pre- and the posttranslocational states (Table III). Therefore, it must be expected that some tRNA–ribosome contacts in one and the same site change during the course of the elongation cycle. Evidence for such changes has been presented already (Abdurashidova et al., 1986; Graifer et al., 1989; Moazed & Noller, 1989a).

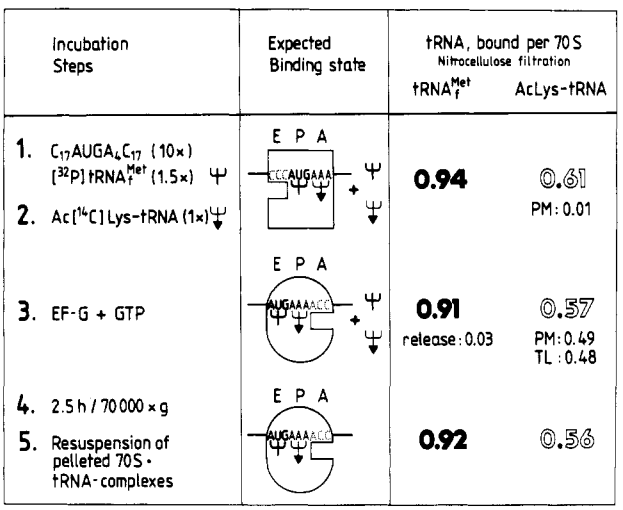

FIGURE 3: Translocation experiment using the heteropolymeric mRNA $C_{17}AUGA_4C_{17}$. Before translocation [^{32}P]$tRNA^{Met}$ was bound to the P site and Ac[^{14}C]Lys-tRNA to the A site (0.94 and 0.61, respectively, per ribosome). The A-site location of AcLys-tRNA is indicated by a negligibly low puromycin reaction (PM; 0.01). Upon EF-G-dependent translocation AcLys-tRNA reacted nearly quantitatively (0.49 out of 0.57), demonstrating a translocation (TL; 0.48 = 0.49 − 0.01) of almost all bound AcLys-tRNA from the A to the P site. During the translocation reaction practically no release of deacylated $tRNA^{Met}$ occurred (0.03 = 0.94 − 0.91); i.e., the $tRNA^{Met}$ was cotranslocated from the P to the E site. From Gnirke et al. (1989).

This view of the tRNA binding sites, which are dynamic but nonetheless still clearly defined, cannot be easily reconciled with the assumption of stable "hybrid sites" such as an A/P site (Moazed & Noller, 1989b). Furthermore, UV-cross-linking data (Abdurashidova et al., 1986) and affinity-labeling experiments (Graifer et al., 1989) do not support the concept of stable hybrid sites; both groups demonstrate that the A-site environment changes after EF-Tu-dependent GTP cleavage and peptide-bond formation, respectively, but the various A-site patterns are clearly distinguishable from those of the P site.

Features of the Third tRNA Binding Site, the E Site

An important step forward in establishing the properties of the E site and the principles of the elongation cycle was an analysis using a heteropolymeric mRHA, which exposes three different codons at the A, P, and E sites and thus allows an unequivocal assignment of the tRNA binding to any of the three ribosomal binding sites (Gnirke et al., 1989). The mRNA was 41 nucleotides long with the sequence $C_{17}AUGA_4C_{17}$, which contains in its central regions the three codons AUG(Met)-AAA(Lys)-ACC(Thr) or CAU(His)-GAA(Glu)-AAC(Asn). The third possible reading frame was not used here because it contains the stop codon UGA.

In the following we illustrate each of the features of the E site by preferentially describing an experiment using this heteropolymeric mRNA.

Cotranslocation of the Deacylated tRNA from the P to the E Site. The reading frame of the heteropolymeric mRNA is set with deacylated $tRNA^{Met}$, which binds to the P site (Figure 3, step 1). Next, an AcLys-tRNA is bound, for which the puromycin reaction (PM) indicates exclusive A-site binding. Now a translocation reaction is performed with the help of EF-G and GTP (step 3). In this step the amount of bound AcLys-tRNA hardly changes, and the puromycin reaction shows almost quantitative translocation (TL); i.e., AcLys-tRNA has moved from the A to the P site. The point of this experiment is that in the course of translocation the amount of deacylated $tRNA^{Met}$ also remains unaltered; i.e., the $tRNA^{Met}$ does not leave the ribosome from the P site but is rather cotranslocated from the P to the E site. The experiment clearly shows that there is no coupling of translocation and release of deacylated tRNA, in contrast to the prediction of the classical two-site model.

Similar results using the homopolymer poly(U) (Rheinberger & Nierhaus, 1983) were interpreted in a different way, namely, that the deacylated tRNA is released from the P site and rebinds quantitatively to the vacated A site (Baranov & Ryobova, 1988). This interpretation is refuted by the experiment shown in Figure 3, since here the A and P sites harbor different codons, thus preventing any possible rebinding to the A site.

The experiment in Figure 3 shows yet another detail. The posttranslocational complex was pelleted and resuspended, and the amounts of bound tRNAs were determined again. The binding values did not change, indicating that the deacylated tRNA does not easily dissociate from the E site but rather is stably bound at this site and has to be released by an active mechanism.

Wintermeyer and co-workers also observed an uncoupling of translocation and tRNA release, but they observed a labile E-site binding (Robertson et al., 1986; Robertson & Wintermeyer, 1987). The discrepancies are mainly due to technical differences, e.g., the use of different buffer systems and the use of NH_4Cl-washed ribosomes which have partially lost some ribosomal proteins and thus contain a "weak" E site [for details and discussion, see Gnirke et al. (1989)].

Codon–Anticodon Interaction at the Ribosomal E Site. Codon–anticodon interaction at the ribosomal A site provides the signal that is responsible for the selection of the correct amino acid. Therefore, this interaction is the central step in the decoding process and probably also the initial event in aminoacyl-tRNA binding to the ribosome (see next section).

Since a codon is about 10 Å long, and a tRNA has a diameter of about 20 Å, the formation of two adjacent codon–anticodon interactions presents a steric problem. It was thus a surprise when codon–anticodon interaction could also be demonstrated at the P site (Lührmann et al., 1979; Peters & Yarus, 1979; Wurmbach & Nierhaus, 1979; Ofengand & Liou, 1981). Clearly, the universal L-shape of the tRNA molecule has been evolved to solve the steric problem, i.e., to allow adjacent codon–anticodon interactions to occur and to bring peptidyl and aminoacyl residues into juxtaposition at the peptidyltransferase center. One might expect that simultaneous adjacent codon–anticodon interactions are of crucial importance for protein biosynthesis.

The early observation that the E site (just as with the A site) could be occupied with deacylated $tRNA^{Phe}$ only in the presence of poly(U) was taken as evidence that there is also codon–anticodon interaction at the E site (Rheinberger & Nierhaus, 1981). This point has been controversially discussed, and evidence both against (Kirillov et al., 1983; Lill et al., 1984) and in favor of codon–anticodon interaction at this site has been reported (Rheinberger et al., 1986; Lill & Wintermeyer, 1987).

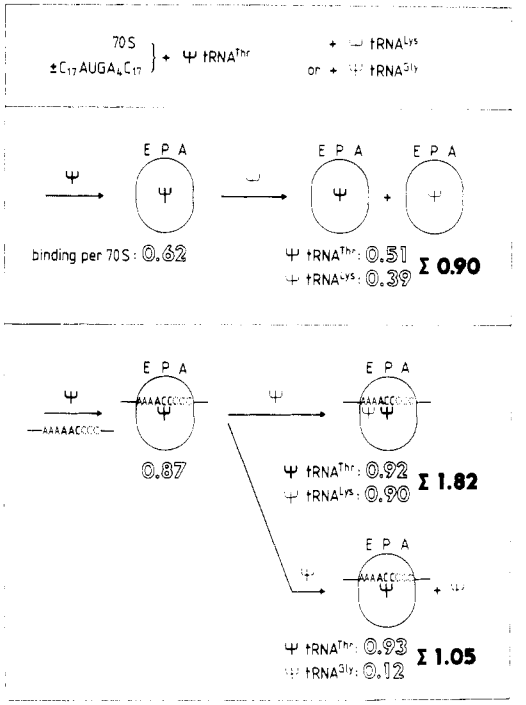

FIGURE 4: Codon-dependent binding to the ribosomal E site. [^{32}P]$tRNA^{Thr}$ was bound to the P site, and then either cognate [^{3}H]$tRNA^{Lys}$ (codon AAA) or [^{35}S]$tRNA^{Gly}$ (codon CCC) was added. In the upper box are shown results from a binding experiment in the absence of mRNA. Both tRNAs added obviously compete for one and the same binding site, since the total binding (0.90) does not exceed one tRNA per 70S (each tRNA was added in a molar ratio 70S:tRNA = 1:4). In the presence of mRNA both tRNAs bind almost stoichiometrically (total binding of 1.82 per 70S), but only if the second deacylated tRNA is cognate to the E-site codon [compare the relatively low binding of $tRNA^{Gly}$ (0.12, codon GGG) with the binding of $tRNA^{Lys}$ (0.90, codon AAA)]. From Gnirke et al. (1989).

The analysis with the heteropolymeric mRNA $C_{17}AUGA_4C_{17}$ gave a decisive answer to the question (Figure 4; Gnirke et al., 1989). In a control experiment without mRNA, two added tRNAs ($tRNA^{Thr}$ and $tRNA^{Lys}$) competed for one and the same binding site, and the total binding did not exceed one molecule per ribosome (0.90). However, when $tRNA^{Thr}$ was first bound to the P site in the presence of mRNA, thus exposing the codon AAA for $tRNA^{Lys}$ at the E site, then $tRNA^{Lys}$ [but not AcLys-$tRNA^{Lys}$; see Gnirke et al. (1989)] could fully bind to the E site, and the total binding approached two molecules per ribosome (1.82). In contrast, when $tRNA^{Gly}$ was added (which is noncognate to the AAA codon), negligibly low binding was found, whereas $tRNA^{Gly}$ bound normally to ribosomes in the presence of its cognate mRNA poly(I). It follows that quantitative filling of the E site depends on the presence of the cognate codon, strongly arguing for codon–anticodon interaction at this site.

Various tRNAs differ in their intrinsic affinities for nonprogrammed ribosomes (Gnirke et al., 1989). For example, $tRNA^{Lys}$ has a relatively low affinity and thus fills the P site but not the E site of nonprogrammed ribosomes. On the other hand, the strong binder $tRNA^{Phe}$ fills the P sites and 20–50% of the E sites of nonprogrammed ribosomes when added in high excess. In the presence of the cognate codons all tRNAs bind strongly to the P and E sites. It follows that the P sites and, in the case of strong binders, the E sites can be occupied in the absence of mRNA. However, in the presence of mRNA codon–anticodon interaction takes place, thereby significantly

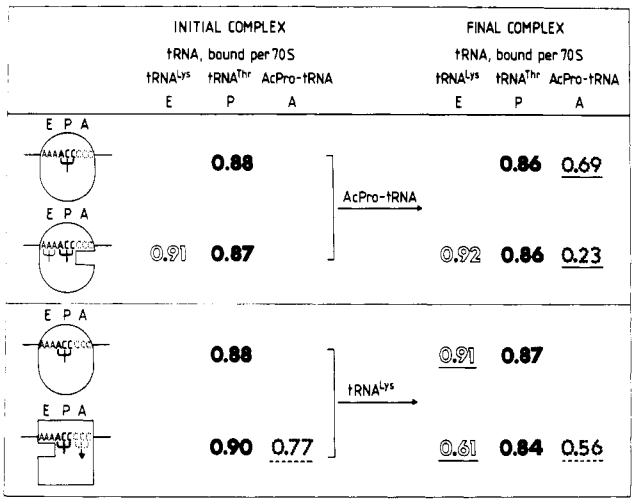

FIGURE 5: Allosteric interactions between A and E sites. When only the P site is occupied, the A site (first line) or the E site (third line) can readily be occupied as the second site. However, if the P and E sites are charged, the binding capacity of the A site is severely impaired (second line, from 0.69 to 0.23). Likewise, occupation of P and A sites reduces the E-site binding (fourth line, from 0.96 to 0.61). Note that in the course of the A-site/E-site interplay the binding at the P site remains unaffected (around 0.87). From Gnirke et al. (1989).

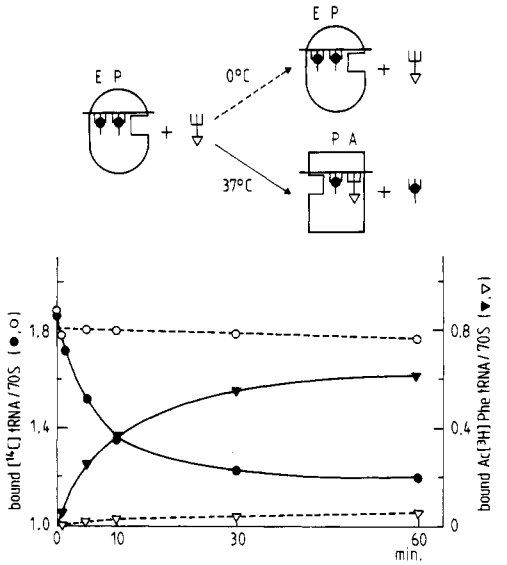

FIGURE 6: Allosteric interactions between A and E sites: kinetics of Ac[^{3}H]Phe-tRNA binding to the A site. When P and E sites are occupied with [^{14}C]tRNA^{Phe} in the presence of poly(U), Ac[^{3}H]-Phe-tRNA does not bind at 0 °C to the A site, indicating its low affinity. When activation energy is provided (incubation at 37 °C) AcPhe-tRNA does bind, and for each molecule of AcPhe-tRNA bound, one molecule of tRNA^{Phe} is released from the E site, demonstrating the negative cooperativity between A and E sites. From Rheinberger and Nierhaus (1986).

increasing the stability of the tRNA binding.

Allosteric Interactions between the A and E Sites in the Sense of a Negative Cooperativity. Evidence for an allosteric interplay between the A and E sites was first shown in the poly(U) system. When only the P site was filled with deacylated tRNA^{Phe}, the A site could readily be occupied with AcPhe-tRNA at both 0 and 37 °C. However, when the P and E sites were filled, AcPhe-tRNA no longer bound to the A site at 0 °C, but still bound at 37 °C. Clearly, filling of the E site induces a low-affinity A site, and activation energy is required to convert this low-affinity A site into a high-affinity state. Several further observations could be made: (a) when only the P site was filled with deacylated tRNA^{Phe}, charging of the A site with the ternary complex Phe-tRNA·EF-Tu·GTP did not affect the tRNA^{Phe} binding; (b) when both the P and E sites were filled, binding of one ternary complex induced a stoichiometric release of tRNA^{Phe}; and (c) when P, E, and A sites were filled with tRNA^{Phe}, the binding of one ternary complex to the A site induced the release of two molecules of tRHA^{Phe} from A and E sites caused by chasing and allosteric effects, respectively [Figure 1 and Table II in Rheinberger and Nierhaus (1986)]. The interpretation was that occupation of the E site induces a low-affinity A site (posttranslocational state) and vice versa, namely, that occupation of the A site induces a low-affinity E site, thus triggering the release of deacylated tRNA.

This interpretation was questioned, and the observed effects were reinterpreted as chasing effects by tRNA^{Phe} contaminants present in the preparation of the A-site ligand (Robertson & Wintermeyer, 1987). This criticism illustrates a difficulty that is intrinsic to the poly(U) system used both by us and by our colleagues; namely, an assessment of the site location of a deacylated tRNA is often difficult due to the presence of identical codons and tRNAs at each position. Again the use of the heteropolymeric mRNA, which exposes different codons at A, P, and E sites, solved this problem (Figure 5).

When the P site was filled with tRNA^{Thr}, AcPro-tRNA could readily bind to the A site. However, prefilling of the P and E sites with tRNA^{Thr} and tRHA^{Lys}, respectively, severely reduced the AcPro-tRNA binding (from 0.69 to 0.23). The opposite is also true; viz., a ribosome with a prefilled P site (tRNA^{Thr}) readily accepts tRNA^{Lys} at the E site, but prefilling of the P and A sites with tRNA^{Thr} and AcPro-tRNA, respectively, impaired the E-site binding (from 0.91 to 0.61). The effect is clear but less pronounced. The reason is that some of the prebound A-site ligand was released (from 0.77 to 0.56) by the occupation of the E site with tRNA^{Lys}, underlining the strength and stability of the tRNA binding at the E site. The essential outcome of the experiment shown in Figure 5 is that occupation of the E site triggers a response at the A site and, vice versa, that occupation of the A site triggers a response at the E site, but *the tRNA at the intervening P site remains unaffected* (at about 0.87). This clearly demonstrates the allosteric coupling between A and E sites in a sense of negative cooperativity.

We believe that the allosteric interplay between A and E sites is of central importance for protein biosynthesis. Therefore, this feature is demonstrated in another way by a simple experiment (Figure 6). The P and E sites of poly-(U)-programmed ribosomes are filled with [^{14}C]tRNA^{Phe}, and then the kinetics of Ac[^{3}H]Phe-tRNA binding are followed at 0 and 37 °C. At 0 °C no significant AcPhe-tRNA binding or tRNA^{Phe} release is observed over a period of 60 min, demonstrating the low-affinity A site upon E-site occupation. However, at 37 °C a slow AcPhe-tRNA binding is seen, and deacylated tRNA^{Phe} is released with the identical rate; i.e., for each AcPhe-tRNA molecule bound to the A site, a molecule of deacylated tRNA^{Phe} is released from the E site. It follows that A-site occupation induces a low-affinity E site, thus triggering the release of the deacylated tRNA from this site. Qualitatively the same picture is found when the ternary complex Phe-tRNA·EF-Tu·GTP is used as the A-site ligand instead of AcPhe-tRNA. However, in this case the reaction runs so quickly that a precise assessment of the binding data is difficult (Rheinberger & Nierhaus, 1986).

Recently, a diagnostic assay for the site location of tRNA was proposed, which was based on the protection patterns of specific bases in the rRNA against chemical modification in

Table III: Features of the Allosteric Three-Site Model

(1) the third site, the E site, is allosterically linked to the first site, the A site, in the sense of negative cooperativity (i.e., the oocupation of one site significantly decreases the affinity of the other); this feature has three corollaries
  (A) the elongating ribosome oscillates between two states, the pre- and the posttranslocational states: in the pretranslocational state the A and P sites have a high affinity for tRNA binding, whereas the E site has a low affinity; in the posttranslocational state the P and E sites are of high affinity, and the A site now has a low affinity
  (B) the elongating ribosome always carries two tRNAs, which are at the A and P sites in the pretranslocational state and at the P and E sites in the posttranslocational state
  (C) the deacylated tRNA is not released from the P site during translocation but is cotranslocated from the P to the E site; only occupation of the A site triggers the release of deacylated tRNA from the E site
(2) both tRNAs present on the ribosome before and after translocation contact the mRNA via codon–anticodon interaction (Rheinberger et al., 1986; Rheinberger & Nierhaus, 1986; Gnirke et al., 1989)

the presence of bound tRNA. The E-site pattern was derived from two experiments performed at very different Mg^{2+} concentrations (6–10 and 20–25 mM, respectively). Such a concentration change would affect the ribosomal conformation and also severely change the relative affinities of the three sites (Lill et al., 1986). The resulting complexes can thus hardly be compared. Moreover, they are not comparable for a second reason, namely, that one of the constructed ribosomal complexes represents a state not found during elongation (only one tRNA, an AcPhe-tRNA, present on the ribosome), in contrast to the other complex. An "E-site" pattern was even described in an experiment where it is certain that no E site was occupied [Figure 4; compare Figure 1B in Rheinberger and Nierhaus (1987)]. Furthermore, all the observed strong protections in 23S rRNA specific for P-site binding (as well as for the assumed E-site binding) were dependent on the presence of the 3′-terminal –CA residues of tRNA. A "diagnostic assay" for the location of the relatively rigid tRNA molecules should not rely on the position of the flexible –CCA end of tRNA.

In the same study the A-site assignments were derived from an atypical complex carrying a deacylated tRNA at the P site and a ternary complex at the A site; such a complex again does not occur during elongation. The site assignments are thus dubious and the hybrid-site model (Moazed & Noller, 1989b), which was based on these assignments, is therefore also questionable.

The following point should be noted concerning structural analysis of the tRNA binding sites: In any study of structural changes within elongating ribosomes by means of fluorescence studies, cross-linking or protection experiments, etc., it is a prerequisite to compare the different states of the elongation cycle. It is inappropriate and misleading to compare the structure of a ribosome carrying one tRNA (initation state) with that of a ribosome containing two tRNAs (elongation phase) and then to ascribe the structural differences observed to conformational changes in the elongating ribosome. During elongation the ribosome always contains two tRNAs, either at the A and P sites (pretranslocational state) or at the P and E sites (posttranslocational state).

Recently, it could be shown that the E site is also functional in vivo: Posttranslocational ribosomes in native polysomes contain an occupied E site (Remme et al., 1989).

The properties of the E site outlined in this section have led to the formulation of the allosteric three-site model (Figure 1B). The two key features of the model are summarized in Table III.

Significance of the Allosteric Interplay between A and E Sites for the Accuracy of Translation: An Occupied E Site Prevents the Binding of Noncognate Aminoacyl-tRNAs

Why does the elongation cycle follow the complicated allosteric three-site model, while the simpler two-site model has worked well at least in the textbooks for over 20 years? Two possibilities for the physiological significance of the allosteric three-site model can be envisaged, namely, proper translocations and correct A-site binding.

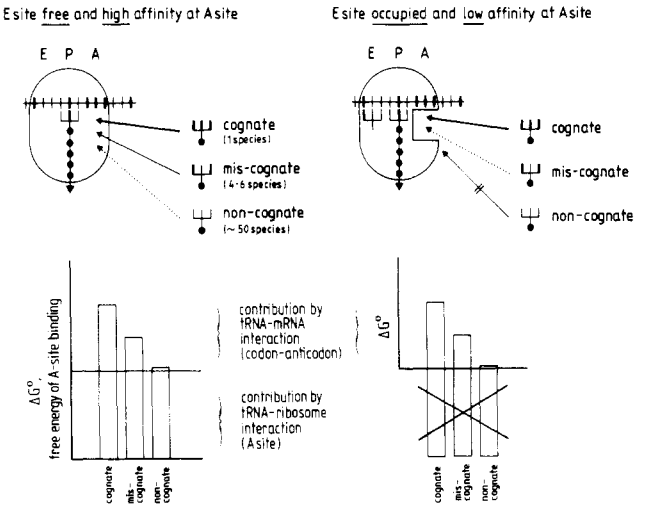

FIGURE 7: Outline of the hypothesis that the E-site-induced lowering of the A-site affinity prevents the binding of noncognate aminoacyl-tRNAs (see text).

Some observations indicate that the mRNA is pulled through the ribosome via the tRNA "handle", the evidence being that some tRNAs can translocate without mRNA (Belitsina et al., 1982). Furthermore, a tRNA mutant has been described which suppresses (+1) frameshifts via a tetraplet interaction between tRNA and mRNA instead of the usual canonical triplet interaction (Riddle & Carbon, 1973). Likewise, doublet interactions can induce (–1) frameshifts (O'Mahony et al., 1989). The "pulling" mechanism requires a tight coupling between tRNAs and ribosome, i.e., two adjacent codon–anticodon interactions are important for moving the mRNA and for maintaining the frame of the mRNA movement.

The second aspect concerns the accuracy of translation. Evidence will be presented that the most unexpected feature of the allosteric three-site model, namely, the low-affinity state of the A site induced by E-site occupation, plays an important role in discriminating against the binding of noncognate aminoacyl-tRNAs to the A site.

Three different classes of aminoacyl-tRNAs or ternary complexes compete for the programmed A site (Figure 7). The first class is represented by the *cognate* aminoacyl-tRNA, comprising only one species with an anticodon precisely complementary to the codon present at the A site. The second class contains four to six *miscognate* aminoacyl-tRNAs, which bear anticodons similar to that of the cognate tRNA. The third class contains the majority of the tRNA species, comprising about 50 *noncognate* aminoacyl-tRNAs, which contain dissimilar anticodons.

Let us assume that the A site is present in a high-affinity

state (Figure 7, left half). This is realized when only the P site is occupied and the E site is free. The respective free energy of A-site binding can be separated into two terms, one term reflecting tRNA–mRNA contacts (codon–anticodon interaction) and the second term tRNA–ribosome interaction. It is mainly the second term that is responsible for a correct positioning of the tRNA on the ribosome and hence for a successful peptide-bond formation. However, this term does not discriminate between wrong and right tRNA. The discrimination is achieved by the first term, i.e., codon–anticodon interaction, where the three classes of aminoacyl-tRNAs have to be distinguished. In the case of a high-affinity state of the A site where both terms play a part, one would expect significant A-site interaction of even the noncognate tRNAs, thus reducing the rate of protein synthesis and leading to an occasional incorporation of noncognate amino acids.

Now the E site comes in. We assume that E-site occupation induces the low-affinity A site by exclusively abolishing the second term (tRNA–ribosome interaction) but does not impair the effect of the first term (codon–anticodon interaction) and might even improve it (Figure 7, right half). The result is that the ribosome only has to discriminate between cognate and miscognate aminoacyl-tRNAs (which is thought to occur via proofreading mechanisms), whereas for noncognate tRNAs the A site practically does not exist. This mechanism reduces the problem of the selection of the correct aminoacyl-tRNA by an order of magnitude; the ribosome has to select one aminoacyl-tRNA out of five instead of one out of fifty.

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 vs that of noncognate substrate is not affected by the allosteric shift-down of the A-site affinity. However, the point is that a high-affinity A site (Figure 7, left half) results in significant "sticking times" (reciprocal of the dissociation rate constant) for even noncognate substrates. Therefore, equilibrium between cognate and noncognate substrates at the A site would only be attained after a relatively long time. Furthermore, the increment in $\Delta G°$ of the high-affinity state over that of the low-affinity state is due to tRNA–ribosome interactions, which are an essential prerequisite 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—render a premature termination of the binding reaction possible, by peptide-bond formation even with noncognate substrates. In contrast, both of these factors are nonoperative in the low-affinity A-site condition; the reaction of codon–anticodon interaction quickly reaches equilibrium, and the peptidyltransferase center is not yet ready to accept an aminoacyl-tRNA, thus resulting in a clear separation of the selection process and the peptidyl transfer.

Figure 8 shows a test of this hypothesis (U. Geigenmüller and K. H. Nierhaus, unpublished). AcPhe-tRNA was bound to the P site of poly(U)-programmed ribosomes, and the E site was free, leaving the A site in a high-affinity state. Next, a mixture of ternary complexes containing [^{14}C]Phe-tRNA or the noncognate [^{3}H]Asp-tRNA (codon GAC) was added, and the AcPhe dipeptides formed were analyzed by HPLC techniques. The analysis demonstrates significant AcPhe-Asp formation (about 1% of the total dipeptide formation; Figure 7, left half). In sharp contrast, when the P and E sites were prefilled with AcPhe-tRNA and deacylated tRNA^{Phe}, respectively, the AcPhe-Asp formation was reduced to background values, whereas the formation of the cognate AcPhePhe dipeptide was hardly affected. Clearly, an occupied E site

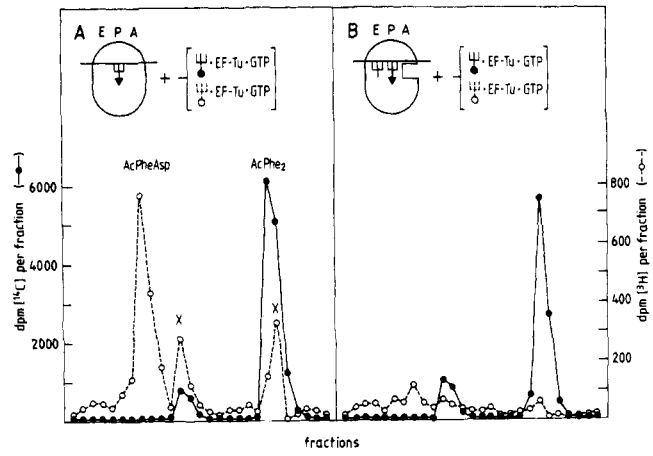

FIGURE 8: Test of the hypothesis outlined in Figure 7. (A) When AcPhe-tRNA is present at the P site in the presence of poly(U) and the E site is free, the A site has a high affinity. Addition of a mixture of cognate and noncognate ternary complexes containing [^{14}C]Phe-tRNA and [^{3}H]Asp-tRNA, respectively, leads to significant formation of "wrong" AcPhe-Asp dipeptides (detected by means of HPLC). (B) When P and E sites are respectively occupied with AcPhe-tRNA and deacyl-tRNA (A site with low affinity), the addition of the mixture of ternary complexes leads exclusively to cognate $AcPhe_2$ formation (U. Geigenmüller and K. H. Nierhaus, unpublished).

prevents a noncognate aminoacyl-tRNA from interacting with the A site, illustrating one important role of the E site during protein synthesis.

When miscognate tRNA^{Leu} was added instead of deacylated tRNA^{Phe} in order to fill the E site, no reduction of AcPhe-Asp formation at all was observed. This shows that only the presence of the cognate tRNA at the E site is a trigger for the reduction of the A-site affinity, underlining the functional importance of codon–anticodon interaction at the E site.

If a mutation were to change a tRNA in such a way that its affinity for the A site was increased without affecting the geometry of the anticodon loop, the allosteric three-site model would predict an increased misreading of this acyl-tRNA. Precisely this has been found. Hirsh (1971) described a mutation that causes a G24 → A change in the D-stem of tRNA^{Trp}, resulting in active misreading. A careful analysis revealed that most probably the geometry of the anticodon loop is not altered, whereas the affinity for the A site seems to be enhanced (Smith & Yarus, 1989a,b). The allosteric three-site model offers a simple explanation; the alteration of tRNA^{Trp} (or its UAG suppressor derivative) in the D-stem increases the affinity for the low-affinity A site, thus counteracting the codon-specificity effect and increasing the frequency of misreading.

The Inhibition Mechanisms of Some Antibiotics Are Better Understood in the Frame of the Three-Site Model

As mentioned in the preceding section, the elongating ribosome always contains two tightly bound tRNAs. During initiation, however, only one tRNA is present, viz., the initiator tRNA at the P site. Therefore, one has to distinguish between the first A-site occupation following initiation when the E site is free (which we refer to as A-site occupation of the i type; i for initiation), and the second and all subsequent A-site occupations where the E site is occupied (A-site occupation of the e type; e for elongation; see Figure 9). The two types of A-site occupation differ thermodynamically. The occupation of the i type occurs well at both 0 and 37 °C, whereas that of the e type requires temperatures above 0 °C; i.e., the allosteric transition (e type) needs higher activation energies.

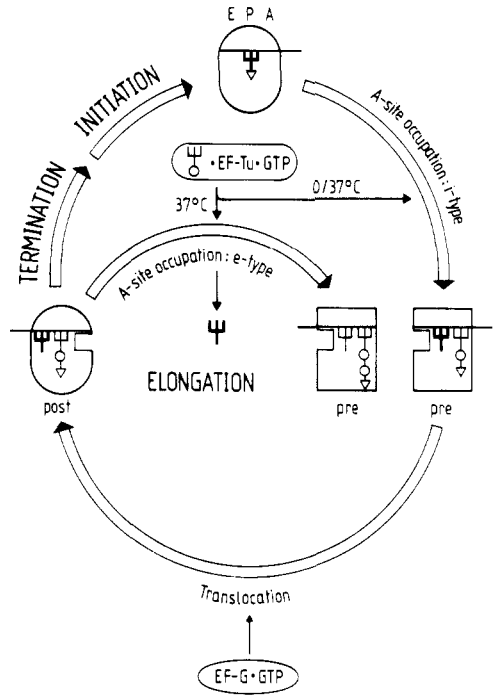

FIGURE 9: Two kinds of A-site occupation in the course of protein biosynthesis. i type: after initiation the E site is free, and therefore, the A site has a high affinity and can be charged even at 0 °C. e type: the second A-site occupation as well as all subsequent ones occur with an occupied P and E site (A site with low affinity). In this case A-site occupation requires a much higher activation energy, and the deacylated tRNA leaves the E site in the course of the allosteric transition from the posttranslocational to the pretranslocational state. From Hausner et al. (1988).

The respective activation energies are 23 kJ/mol (5.4 kcal/mol) and 83 kJ/mol (20 kcal/mol) at 10 mM Mg^{2+} (S. Schilling-Bartetzko and K. H. Nierhaus, unpublished).

The effects of a series of antibiotics were tested on the various reactions of the elongation cycle, in particular on both types of A-site binding and the subsequent EF-G-dependent translocation reaction (Figure 10; Hausner et al., 1988). Some control drugs were included to test the specificity of the system. Tetracycline (TET) inhibited the A-site occupation of both types as expected. Chloramphenicol (CAM) had marginal effects on A-site binding but blocked the puromycin reaction so that the translocation could not be assessed, and α-sarcin [which blocks the binding of elongation factors to the ribosome but has no effect on factor-independent reactions such as spontaneous translocation (Hausner et al., 1987)] did not impair nonenzymatic binding of AcPhe-tRNA to the A site but did block EF-G-dependent translocation.

It can be seen that both thiostrepton (THI) and viomycin (VIO) hardly affect A-site occupation of the i type but severely reduce A-site binding of the e type. In addition, the translocation is blocked by both drugs. It appears that these nonrelated antibiotics are inhibitors of the allosteric transitions in both directions, from the pre- to the posttranslocational state (i.e., translocation reaction) and from the post- to the pretranslocational state (A-site occupation of the e type). The inhibition of the factor-dependent GTPase reaction, which was thought to be the principal inhibition mechanism of thiostrepton (Cundliffe, 1980), is less pronounced (Hausner et al., 1988) and appears to be a consequence rather than the cause of inhibition, since the factor-dependent GTPase reaction follows the allosteric transitions. On the other hand, viomycin is not only an inhibitor of translocation as was hitherto accepted but it equally well blocks the allosteric transition in the reverse reaction [for references and discussion, see Hausner et al. (1988)].

The killing action of aminoglycosides is not caused by their well-documented misreading effect (Fast et al., 1987) but rather results from a not yet identified step shortly after initiation (Davis, 1987). Figure 10 demonstrates that these drugs have practically no effect on A-site occupation of the i type (as was known already) but totally block that of the e type; i.e., they block the second A-site occupation after initiation. Thus the simple picture that arises from the related structure of the aminoglycosides, together with a defined ribosomal region which has been determined at or near their binding sites (Moazed & Noller, 1987) and which harbors rRNA alterations conferring resistance (Cundliffe, 1987), is now complemented with a common point of interference, namely, the e-type occupation of the A site. Figure 11 summarizes the interference points in the elongation cycle of some antibiotics.

Hypothesis: Ribosomes Achieve Accuracy of tRNA Selection without Proofreading

It was shown in a preceding section that the allosteric three-site model explains why noncognate aminoacyl-tRNAs do not impair the rate and accuracy of protein biosynthesis (Figures 7 and 8). In this section the discrimination between cognate versus miscognate aminoacyl-tRNAs is considered. The allosteric three-site model provides a framework according to which the latter discrimination could be achieved via a one-step recognition. If this is so, then the assumption of a proofreading mechanism responsible for the accuracy of aminoacyl-tRNA selection would become unnecessary. A number of observations lend support to this view.

The term "proofreading" is used here in the strict sense that the anticodon of an aminoacyl-tRNA interacts not only once but twice or more with the codon before tight binding of the selected aminoacyl-tRNA at the A site occurs. It is widely accepted that such a proofreading mechanism is a prerequisite for explaining the accuracy values of about 1:1000 (incorporation of one wrong amino acid per 1000 amino acids) observed for protein synthesis in vivo and in vitro [see Bouadloun et al. (1983) and references cited therein]. The increased EF-Tu-dependent GTPase activity observed with a miscognate substrate at the A site is usually taken as evidence for a proofreading mechanism (Thompson & Stone, 1977; Ruusala et al., 1982), and it was suggested that EF-Tu is directly involved in the proofreading mechanism (Tapio & Kurland, 1986).

At least two facts cannot easily be reconciled with the proofreading hypothesis. (1) RNA polymerases without proofreading synthesize RNA with an accuracy of up to 1:1000 (Libby et al., 1989, and references cited therein). If these enzymes are so accurate in checking the pairing precision of only single pairs of nucleotides without proofreading, why should the ribosome not be able to achieve about the same accuracy without proofreading, while checking the pairing precision of the three pairs of nucleotides that comprise the codon–anticodon interaction? (2) Not one mechanism has been proposed up to now that satisfactorily explains a repeated melting and rejoining of codon–anticodon interactions. Even if such a mechanism does exist, the EF-Tu-dependent GTPase activity is not necessarily involved. It has been shown with the hardly cleavable GTP analogue GTPγS that, in principle, the ribosome can select ternary complexes with a precision of better than 1:1000 without needing this GTPase activity (Thompson & Karim, 1982). This precision was due to the dissociation (k_{-1}) of the ternary complexes, and since the rate of the next reaction—the GTP hydrolysis—was comparable to k_{-1}, the authors concluded that "the potential specificity

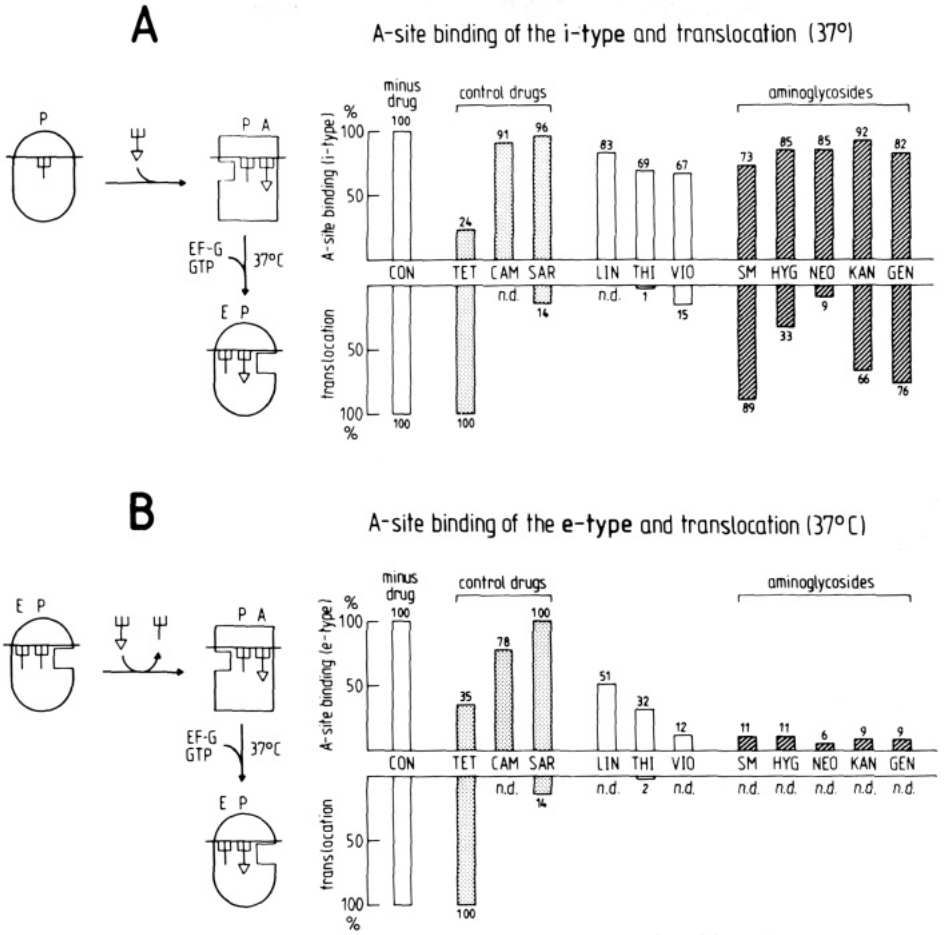

FIGURE 10: A-site occupation of the i type (A) and e type (B) in the presence of various antibiotics: Con, control; TET, tetracycline; CAM, chloramphenicol; SAR, α-sarcin; LIN, lincomycin; THI, thiostrepton; VIO, viomycin; SM, streptomycin; HYG, hygromycin; NEO, neomycin; KAN, kanamycin; GEN, gentamicin. From Hausner et al. (1988).

of the initial selection process (k_1/k_{-1}) is never fully realized". Finally, a lack of correlation has been reported between the efficiencies of a suppressor tRNA in vivo and the predictions of the ribosomal proofreading hypothesis (Faxén et al., 1988). The experiments were performed in a mutant with hyperaccurate ribosomes, which had earlier been described as excessive ribosomal proofreaders (Ruusala et al., 1984).

We assume in the framework of the allosteric three-site model that a successful codon–anticodon interaction at the A site is the trigger for the allosteric transition from the post- to the pretranslocational state (Gnirke et al., 1989). Thus, the A-site occupation separates into two consecutive steps. In the first step codon–anticodon interaction takes place (complex 2 in Figure 12). Apart from this, no significant interaction between the A site and the ternary complex occurs, since the A site is still in its low-affinity state. A successful codon–anticodon interaction then triggers the second step—the allosteric transition—which leads to a tight binding of the aminoacyl-tRNA (complex 3).

The A-site occupation is a relatively slow process, which in fact is the rate-limiting reaction of the whole elongation cycle (Bilgin et al., 1988). The "lion's share" of the whole process of A-site occupation is most probably represented by the second step, since this step calls for gross structural changes involving the shift from high to low affinity at the E site (tRNA release) and from low to high affinity at the A site. Because this step is slow, and the preceding step of codon–anticodon interaction is quick, the codon–anticodon interaction runs practically under equilibrium conditions during the course of protein biosynthesis (Figure 12). Therefore, the precision of the initial step (k_1/k_{-1}) observed by Thompson and Karim (1982) can become effective during translation. The poor accuracy of ternary complex selection they observed in the presence of GTP is probably caused by the presence of an empty E site under their conditions, and thus the lack of an allosteric transition during A-site occupation.

The EF-Tu-dependent GTPase activity functions in this scenario after the first step of A-site occupation and before tight binding of the aminoacyl-tRNA at the end of the second step (Figure 12). The precise trigger for the factor-dependent GTPase activity is not yet clear; it could be a successful codon–anticodon interaction that is sensed by EF-Tu via a change in the tRNA conformation, or on the other hand, it could be the incipient conformational change at the A site or the contact between EF-Tu and the P-site-bound tRNA (Bosch et al., 1985) during the allosteric transition. In any case, the EF-Tu-dependent GTPase reaction (k_a) is not coupled with the allosteric transition, nor is it a prerequisite for this transition (k_2), since the latter occurs equally well with a ternary complex containing the noncleavable GTP analogue GMPPNP or even with AcPhe-tRNA (Rheinberger & Nierhaus, 1986). Regardless as to what triggers the GTPase reaction, one can easily imagine that in the case of a miscognate ternary complex the signal from the suboptimal codon–anticodon interaction is sufficient to induce the GTPase reaction (and thus the dissociation of EF-Tu·GDP) but is too weak for the allosteric transition (complex 3′ in Figure 12). The result is that the miscognate aminoacyl-tRNA is loosely bound and thus quickly dissociates from the A site. This mechanism explains the increased GTP turnover in the case of miscognate ternary

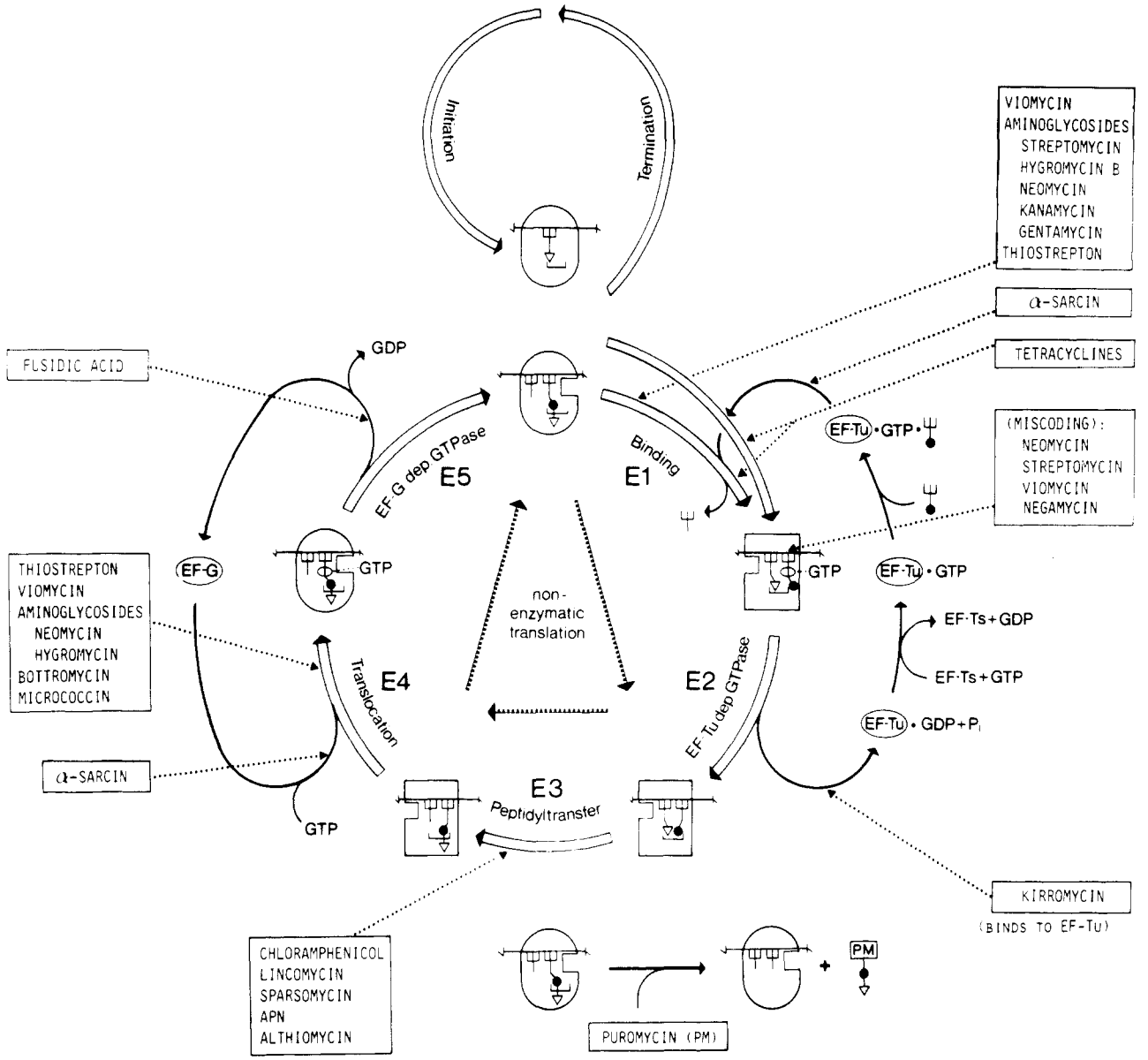

FIGURE 11: Points of interference of various antibiotics inhibiting the ribosomal elongation cycle. From Hausner et al. (1988).

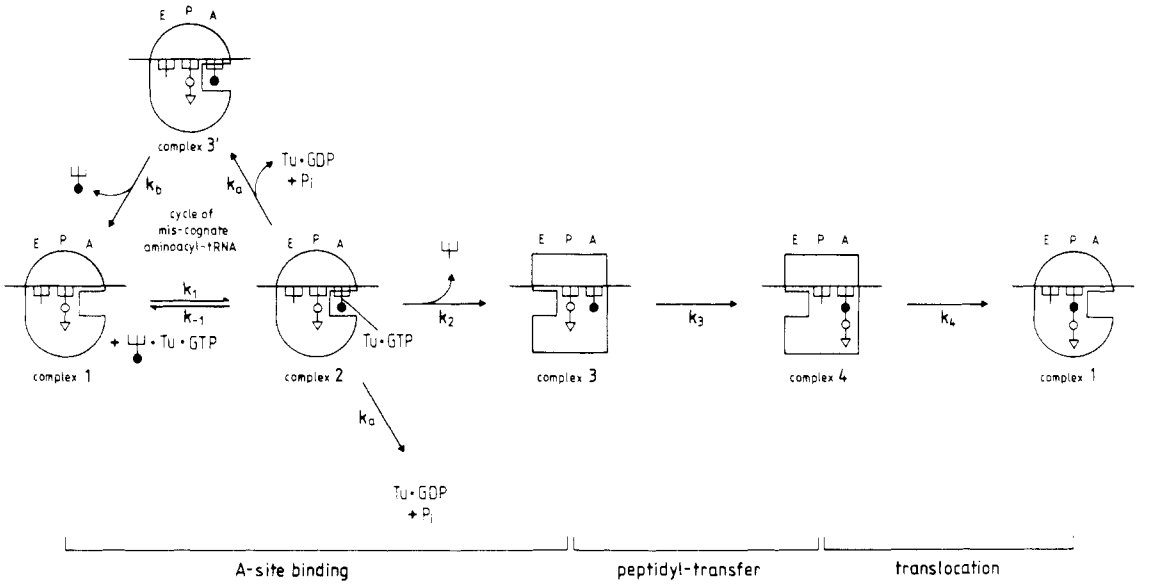

FIGURE 12: Hypothesis for the precision of A-site occupation in the framework of the allosteric three-site model, without proofreading. Complex 3′ indicates an intermediate in the course of binding of a miscognate aminoacyl-tRNA. The aminoacyl-tRNA present in complex 2 and complex 3′ at the A site is loosely bound to the low-affinity A site.

complexes, although codon–anticodon interaction occurs only once.

The mechanism depicted in Figure 12 lacks a proofreading step according to the above-mentioned definition, but it nevertheless represents a branched reaction. Thus, a branched reaction is not indicative of a proofreading mechanism per se, although all proofreading mechanisms necessarily represent branched reactions (Hopfield & Yamane, 1980).

It is not yet clear whether or not proofreading occurs in the ribosomal selection of an aminoacyl-tRNA. Nevertheless, there are good reasons to believe that it does not, and without proofreading ribosomal life would be much easier.

Acknowledgments

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Articles

Fluorescence Study of the Binding of m^7GpppG and Rabbit Globin mRNA to Protein Synthesis Initiation Factors 4A, 4E, and 4F†

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ABSTRACT: The interactions of protein synthesis initiation factors eIF-4E from human erythrocytes and eIF-4A and eIF-4F from rabbit reticulocytes with the cap analogue m^7GpppG and rabbit globin mRNA were investigated. The equilibrium binding constants for the binary complex formation of eIF-4E–eIF-4A, m^7GpppG–eIF-4E, m^7GpppG–eIF-4F, globin mRNA–eIF-4E, globin mRNA–eIF-4F, and globin mRNA–eIF-4A were measured by direct fluorescence titration experiments. The binding of eIF-4E to globin mRNA was found to be 5.5-fold tighter than its binding to m^7GpppG; the binding of eIF-4F for globin mRNA and m^7GpppG was similar to that of eIF-4E. Association equilibrium constants were determined for the ternary system mRNA–eIF-4E–eIF-4A; four thermodynamically independent equilibria characterize the system. These equilibrium binding constants were used to calculate coupling free energies, which provided an estimate of the cooperativity of the interaction of eIF-4E, eIF-4A, and mRNA. These coupling energies were all found to be small and positive, indicative of anticooperative binding.

The recognition of mRNA by the components of the translational machinery is a crucial step in the initiation of protein synthesis. Several mRNA structural features are important in this recognition process; one such feature is the 5′-terminal $m^7G(5')ppp(5')N$ moiety,[1] termed the cap structure. Several initiation factors have been shown to interact at or near the cap, including eIF-4A, eIF-4B, and eIF-4E [for reviews, see Shatkin (1985), Rhoads (1988), and Sonenberg (1988)]. In addition, the complex of eIF-4E, eIF-4A, and a 220-kDa polypeptide, termed eIF-4F, binds to the cap (Edery et al., 1983; Grifo et al., 1983). eIF-4E and eIF-4F, unlike eIF-4A and eIF-4B, bind directly to the m^7G cap (Tahara et al., 1981; Sonenberg, 1981; Sonenberg et al., 1981; Hellmann et al., 1982; Grifo et al., 1983; Webb et al., 1984; Goss et al., 1987). The eIF-4E component of eIF-4F has been shown to be responsible for the cap binding activity of eIF-4F, while the 46-kDa component, which has been shown to be a mixture of eIF-4AI and eIF-4AII (Nielsen and Trachsel, 1988; Dever and Merrick, unpublished), is responsible for ATP-dependent mRNA unwinding activity (Grifo et al., 1984; Lawson et al., 1989).

The interaction of eIF-4E and eIF-4A with mRNA has been extensively studied. eIF-4E, either separately or as part of eIF-4F, binds to the cap in the absence of ATP (Sonenberg, 1981; Hellmann et al., 1982) and independent of the degree of mRNA secondary structure (Pelletier and Sonenberg, 1985; Lawson et al., 1986). eIF-4A binds to single-stranded regions of mRNA with concomitant hydrolysis of ATP and may unwind mRNA secondary structure (Grifo et al., 1982; Ray et al., 1985; Goss et al., 1987). However, cross-linking studies have shown that eIF-4F binds more tightly to mRNA caps than eIF-4E alone (Abramson, 1989), implying that other polypeptides are involved in the mRNA recognition process. In the presence of ATP, eIF-4A is also capable of cross-linking to the mRNA cap, but only as part of the eIF-4F complex or in the presence of eIF-4E and eIF-4B (Grifo et al., 1983; Edery et al., 1983; Abramson, 1989). The inability of eIF-4A to bind the mRNA cap in the absence of eIF-4F or eIF-4E may indicate that eIF-4A does not interact with the cap per se but rather with the cap-binding component of eIF-4F (Rhoads, 1988). eIF-4A thus may influence cap binding as part of the eIF-4F complex.

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[1] Abbreviations: m^7G, 7-methylguanosine; kDa, kilodalton; eIF, eukaryotic initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol.

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