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The "allosteric three-site model" of elongation cannot be confirmed in a well-defined ribosome system from *Escherichia coli*

(protein synthesis/exit site/translocation/elongation factor G)

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ABSTRACT **For the functional role of the ribosomal tRNA exit (E) site, two different models have been proposed. It has been suggested that transient E-site binding of the tRNA leaving the peptidyl (P) site promotes elongation factor G (EF-G)-dependent translocation by lowering the energetic barrier of tRNA release [Lill, R., Robertson, J. M. & Wintermeyer, W. (1989) *EMBO J.* 8, 3933–3938]. The alternative "allosteric three-site model" [Nierhaus, K. H. (1990) *Biochemistry* 29, 4997–5008] features stable, codon-dependent tRNA binding to the E site and postulates a coupling between E and aminoacyl (A) sites that regulates the tRNA binding affinity of the two sites in an anticooperative manner. Extending our testing of the two conflicting models, we have performed translocation experiments with fully active ribosomes programmed with heteropolymeric mRNA. The results confirm that the deacylated tRNA released from the P site is bound to the E site in a kinetically labile fashion, and that the affinity of binding, i.e., the occupancy of the E site, is increased by Mg^{2+} or polyamines. At conditions of high E-site occupancy in the posttranslocation complex, filling the A site with aminoacyl-tRNA had no influence on the E site, i.e., there was no detectable anticooperative coupling between the two sites, provided that second-round translocation was avoided by removing EF-G. On the basis of these results, which are entirely consistent with our previous results, we consider the allosteric three-site model of elongation untenable. Rather, as proposed earlier, the E site-bound state of the leaving tRNA is a transient intermediate and, as such, is a mechanistic feature of the classic two-state model of the elongating ribosome.**

Textbook models of protein elongation distinguish two main states of the elongating ribosome: (*i*) the posttranslocation state with peptidyl-tRNA in the peptidyl (P) site and an empty aminoacyl (A) site, and (*ii*) the pretranslocation state with deacylated tRNA in the P site and peptidyl-tRNA in the A site. The latter state is formed by peptidyl transfer from the P site-bound peptidyl-tRNA to the aminoacyl-tRNA brought to the A site by the action of elongation factor Tu (EF-Tu). Translocation, that is the displacement of peptidyl-tRNA from the A site to the P site and the release of deacylated tRNA from the P site into solution, is brought about by elongation factor G (EF-G) that, during the reaction, hydrolyzes GTP (1, 2).

For the molecular mechanism of tRNA translocation, a number of transient intermediate states have to be considered. Most significantly, a third tRNA binding site, the exit (E) site, has been demonstrated on *Escherichia coli* ribosomes that is implicated in the release of the deacylated tRNA from the P site during translocation (3–6). The E site has been described also for ribosomes from an archaeon (7), from rabbit (8, 9), and from yeast (10). The E site of *E. coli* ribosomes specifically binds deacylated tRNA with intermediate affinity (K_d = 0.1 to 50 μM, depending on conditions) in a kinetically labile fashion. The binding to a large extent depends on the interaction of the 3′-terminal adenosine with the ribosome (4, 11), presumably with 23S rRNA. In comparison, the contribution of cognate codon–anticodon interaction to the free energy of tRNA binding to the E site is small (12–14) and difficult to assess, since the intrinsic affinities of various tRNAs for binding to the E site vary by about the same order of magnitude, about 10-fold (13).

For the functional role of the E site, two fundamentally different models have been put forward. We have provided evidence suggesting that transient E-site binding promotes the exit of the deacylated tRNA from the P site during translocation (13, 15, 16). The E site-bound intermediate state of the tRNA develops from the "hybrid" P/E state (17) by the action of EF-G and initiates translocation (18). In this model, the E site-bound state of the leaving tRNA, due to its low kinetic stability, is considered a transient intermediate rather than a stable product of translocation, and, therefore, a feature of the molecular mechanism of translocation.

In contrast, Nierhaus and colleagues (review ref. 19) have proposed that, after translocation, the deacylated tRNA remains stably bound to the E site in a codon-dependent fashion, and that its release into solution requires occupancy of the A site, which lowers the affinity of the E site by negatively cooperative coupling. Hence, the model of the elongation cycle was extended to include the E site as a stable intermediate tRNA binding site ("allosteric three-site model"; ref. 19). While the model was developed on the basis of experiments with homopolymeric mRNAs, the same conclusions were reached on the basis of results obtained with heteropolymeric mRNAs (20). As to the function of E-site binding, Nierhaus' group has postulated a role in reading frame maintenance (21) and, more recently, in aminoacyl-tRNA selection at the A site (19, 22), questioning the existence of kinetic proofreading during aminoacyl-tRNA binding to the A site.

The basic features of the allosteric three-site model, namely stable, codon-dependent binding of tRNA to the E site and negative cooperativity between E and A sites, have already been refuted by several groups including ours. Nevertheless, since far-reaching claims have been deduced from the model, we have now repeated the pertinent experiments in a protein synthesis system programmed with heteropolymeric mRNAs consisting of fully active components (23). To allow direct comparisons, we have used various buffer conditions, including polyamine-containing buffers (20, 22) and polymix buffer

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Abbreviation: EF, elongation factor.
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(24). To avoid the problem of "second-round translocation," which we have identified as one of the artifacts on which the idea of allosteric coupling between E and A sites was based (25), we have developed a novel procedure for the removal of EF-G from the ribosomes after translocation. Thereby we were able to study the influence of A-site binding on the occupancy of the E site without interference of second-round translocation. Another possible artifact is the competition for E-site binding by deacylated tRNA contaminating aminoacyl-tRNA, which we avoided by using purified, fully charged aminoacyl-tRNAs or purified aminoacyl-tRNA·EF-Tu complexes for filling the A site.

Confirming previous results, we found that the extent of E-site occupancy after translocation, that is the affinity of the E site for deacylated tRNA, varied with the buffer conditions. From low affinity at low Mg^{2+} concentration (7 mM), it increased with the concentration of Mg^{2+} or upon adding polyamines; in polymix buffer, which contains different polyamines and low Mg^{2+}, the affinity is relatively low. At any condition, most of the E site-bound tRNA was rapidly chased upon addition of unlabeled competitor tRNA. Working in spermine/spermidine-containing buffer, in which tRNA remains bound to the E site after translocation, we found no indication for tRNA release from the E site upon filling the A site with aminoacyl-tRNA. Thus, our results provide no indication for an allosteric coupling between E and A sites.

MATERIALS AND METHODS

Buffers and Reagents. Buffer A was 50 mM Tris·HCl, pH 7.5/70 mM NH_4Cl/30 mM KCl/7 mM $MgCl_2$/1 mM DTT; buffer B was 25 mM Hepes·HCl, pH 7.5/150 mM NH_4Cl/3 mM Mg Cl_2/0.6 mM spermine/0.4 mM spermidine/1 mM DTT; buffer C was the same as buffer B, except 6 mM $MgCl_2$ was used. Polymix buffer was prepared as described (24). In the experiments with Ni^{2+}-NTA-agarose (Qiagen, Düsseldorf) and His-tagged EF-G, DTT in buffer C was replaced with 2-mercaptoethanol. Biochemicals were from Boehringer Mannheim. ^{3}H-labeled amino acids were from Amersham; ^{14}C-labeled amino acids were from ICN.

tRNAs, Ribosomes, and Factors. Materials not mentioned were as described previously (23). tRNAs were from *E. coli*. [^{14}C]tRNA^{fMet} (850 dpm/pmol) and [^{14}C]tRNA^{Phe} (600 dpm/pmol) were labeled in the 3′-terminal adenosine (26) and chargeable to 1.6 nmol/A_{260} unit. fMet-[^{14}C]tRNA^{fMet} was prepared and purified by fast protein liquid chromatography (1.75 nmol/A_{260} unit; ref. 27). [^{3}H]Phe-tRNA^{Phe} was purified by HPLC (1850 dpm/pmol, 1.7 nmol/A_{260} unit). [^{3}H]Thr-tRNA^{Thr} (1260 dpm/pmol) was purified as ternary complex with EF-Tu and GTP (23).

Tight-couple ribosomes were prepared from *E. coli* MRE600 as described (23). The ribosomes were fully active in tRNA binding and peptide bond formation, and about 85% active in translocation, based on 23 pmol/A_{260} unit.

EF-G(His) carrying an extension of about 30 amino acids, including six histidines, at the N terminus was prepared by expression of the respective plasmid in *E. coli* and purified by affinity chromatography on Ni^{2+}-NTA-agarose; the His-tagged factor was as active as the wild-type protein (unpublished work). *E. coli* initiation factors were donated by C. Gualerzi (University of Camerino, Camerino, Italy).

mRNAs. Fast protein liquid chromatography-purified T7 RNA polymerase transcripts of 122 nucleotides length containing a Shine–Dalgarno sequence were used as mRNAs (28). Two different constructs were used [coding regions for Met-Phe-Thr (MFT-mRNA) and Met-Phe-Phe (MFF-mRNA)]; the respective plasmids were donated by C. Gualerzi and R. Spurio (University of Camerino).

Ribosome Complexes. Initiation complexes were prepared by incubating ribosomes (10 pmol) in 20 μl of buffer A (or polymix) with MFT-mRNA (40 pmol) (or, when indicated, MFF-mRNA), fMet-[^{14}C]tRNA^{fMet} (15 pmol), and initiation factors 1, 2, and 3 (15 pmol each) in the presence of GTP (1 mM) for 30 min at 37°C (23). For initiation in buffers B and C, the complexes were formed in the respective buffers without polyamines, which were added afterwards (10 μl); this way, unspecific initiation observed in the complete buffers B and C could be avoided.

To form pretranslocation complexes, Phe-tRNA^{Phe} was bound to the A site of the initiation complexes using one of two different procedures, which follow. In procedure 1, [^{3}H]Phe-tRNA^{Phe} (11–15 pmol), EF-Tu·EF-Ts (10 pmol), 1 mM GTP, 3 mM phosphoenol pyruvate, and 0.5 g/liter pyruvate kinase in 10 μl of the respective buffer were incubated for 3 min at 37°C; after cooling to 20°C, the mixture was added to 30 μl of the respective initiation complex (10 pmol), and the mixture was maintained at 20°C until translocation was performed (A-site binding was complete within 10 s). Procedure 2 was used for the experiment mentioned in Table 1 and is as follows. The complexes of Phe-[^{14}C]tRNA^{Phe} and [^{3}H]Thr-tRNA^{Thr} with EF-Tu and GTP were prepared by incubating in 250 μl of buffer A 0.8 nmol [^{14}C]tRNA^{Phe} (600 dpm/pmol, 1.6 nmol/A_{260} unit) and 1.4 nmol tRNA^{Thr} (0.6 nmol/A_{260} unit) with 8.0 nmol EF-Tu, 1 mM GTP, 1 mM ATP, 30 μM phenylalanine, 30 μM [^{3}H]threonine (1260 dpm/pmol), about 1 unit each of partially purified phenylalanyl- and threonyl-tRNA synthetase, 3 mM phosphoenol pyruvate, and 0.5 g/liter pyruvate kinase for 30 min at 37°C. The EF-Tu·aa·tRNA complexes were purified by gel filtration as described previously (23); after purification, the concentration of the complexes was around 0.2 μM (Phe) and 0.6 μM (Thr). Eighty microliters of the mixture of ternary complexes were added to 30 μl of the initiation complex (10 pmol) at 20°C.

To induce translocation, usually 10 pmol (1 μl) of EF-G, preincubated with 1 mM GTP for 15 min at 37°C, was added to 10 pmol (40 μl) of pretranslocation complex at 20°C.

The amount of ribosome-bound tRNA was measured by rapidly filtrating aliquots through nitrocellulose filters (Sartorius) without prior dilution, followed by washing the filters twice with 1 ml of the buffer used for the respective experiment. For counting, the filters were dissolved in QS361 (Zinsser, Frankfurt). The extent of translocation of peptidyl-tRNA was assayed by incubating with puromycin (1 mM) for 10 s at 20°C; following the addition of 0.5 ml of 1.5 M sodium acetate saturated with magnesium sulfate (pH 5), peptidyl-puromycin was extracted into ethyl acetate and counted. Oligopeptides were analyzed by HPLC as described previously (23). Radioactivity was measured in a Packard 2500 TR scintillation counter using a double-label program.

RESULTS

Release of Deacylated tRNA from the P Site During Translocation. Fully occupied pretranslocation ribosomes contain-

Table 1. Release of [^{14}C]tRNA^{Phe} from the P site during translocation of fMet-Phe-Thr-tRNA^{Thr}

	Ribosome-bound		fMet-Phe[^{3}H]Thr	
EF-G	[^{14}C]tRNA^{Phe}	[^{3}H]Thr	Synthesized	Puromycin
−	0.90	<0.01	<0.01	<0.05
+	0.16	0.87	0.84	0.80

To initiation complexes on MFT-mRNA containing fMet-tRNA^{fMet} in buffer A (10 pmol), the purified EF-Tu·GTP complexes of Phe-[^{14}C]tRNA^{Phe} (15 pmol) and of [^{3}H]Thr-tRNA^{Thr} (50 pmol) were added as described (final vol 110 μl). Then, 10 pmol of EF-G·GTP was added, and ribosome-bound [^{3}H]Thr and [^{14}C]tRNA^{Phe} were measured by nitrocellulose filtration. After 5-min incubation, the efficiency of fMet-Phe[^{3}H]Thr formation was verified by HPLC analysis, and the extent of P-site location of fMet-Phe[^{3}H]Thr-tRNA^{Thr} was determined by the puromycin assay.

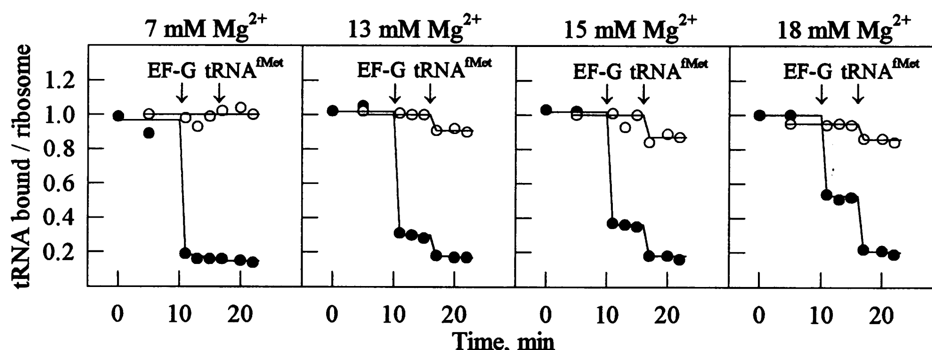

FIG. 1. Release of [^{14}C]$tRNA^{fMet}$ from the P site during translocation. Initiation complexes programmed with MFT-mRNA and carrying fMet-[^{14}C]$tRNA^{fMet}$ in the P site were prepared in buffer A; when required, higher $MgCl_2$ concentrations were adjusted subsequently. The A site was filled with [^{3}H]Phe-$tRNA^{Phe}$ using procedure 1. Where indicated (arrows), translocation was initiated by adding EF-G (1 per ribosome) and the chase by adding $tRNA^{fMet}$ (10 per ribosome). Ribosome-bound [^{14}C]$tRNA^{fMet}$ (●) and fMet[^{3}H]Phe-$tRNA^{Phe}$ (○) were determined by nitrocellulose filtration. According to HPLC analysis, the formation of fMet[^{3}H]Phe-$tRNA^{Phe}$ was quantitative, and it was 85% translocated according to the puromycin assay before and after adding EF-G.

ing [^{14}C]$tRNA^{fMet}$ in the P site and fMet[^{3}H]Phe-$tRNA^{Phe}$ in the A site were set up on MFT-mRNA by first forming the 70S initiation complex with fMet-[^{14}C]$tRNA^{fMet}$ in the P site and then binding [^{3}H]Phe-$tRNA^{Phe}$ to the A site in an EF-Tu-dependent manner (see *Materials and Methods*). To induce translocation, EF-G and GTP were added to the complex, and the amounts of [^{14}C]$tRNA^{fMet}$ and fMet[^{3}H]Phe-$tRNA^{Phe}$ remaining on the ribosome after translocation were determined by nitrocellulose filtration; subsequently, the amount of (chaseable) [^{14}C]$tRNA^{fMet}$ remaining in the E site was determined by adding unlabeled $tRNA^{fMet}$ in excess.

About 85% of the [^{14}C]$tRNA^{fMet}$ was released instantaneously when translocation was performed at 7 mM Mg^{2+}, while at higher concentrations of Mg^{2+} increasing amounts of [^{14}C]$tRNA^{fMet}$ remained bound to the E site (Fig. 1). However, after adding an excess of nonlabeled $tRNA^{fMet}$, the E site-bound [^{14}C]$tRNA^{fMet}$ was chased, and the same final level of ribosome-bound [^{14}C]$tRNA^{fMet}$, about 15% of the initial amount, was reached at all Mg^{2+} concentrations. The total amount of released [^{14}C]$tRNA^{fMet}$ was equivalent to the extent of translocation of fMet[^{3}H]Phe-$tRNA^{Phe}$, 85–90%, determined by the puromycin assay.

Influence of Polyamines on tRNA Retention in the E Site After Translocation. We have tested the influence of polyamines on the retention of $tRNA^{fMet}$ in the E site after translocation in polymix (24) as well as in two spermine/spermidine-containing buffers (buffers B and C) used by Nierhaus and colleagues (20, 22). Using pretranslocation ribosomes as above, we found that in polymix, compared with buffer A (cf. Fig. 1), somewhat less of [^{14}C]$tRNA^{fMet}$ was released concomitantly with translocation, but the same final level (about 20% of the initial amount) was reached after the chase (Fig. 2). As compared with buffer A, in buffers B and C much less [^{14}C]$tRNA^{fMet}$ was released immediately, i.e., the affinity of the E site was increased, but the amount of chased [^{14}C]$tRNA^{fMet}$ and the final level of [^{14}C]$tRNA^{fMet}$ remaining bound after the chase was similar, around 25% (Fig. 2).

The extent of translocation, based on the puromycin assay, was about 85% in buffers B and C; hence, about half of the [^{14}C]$tRNA^{fMet}$ that remained bound to the ribosome after the chase was still bound to the P site, while the other half probably was bound to the E site in an atypically stable fashion. We attribute the latter behavior to the formation of a subpopulation of (probably inactive) posttranslocation ribosomes induced by the presence of spermine/spermidine together with Mg^{2+} in these buffers.

tRNA Release During Translocation of Tripeptidyl-tRNA. To include an elongator tRNA, the study was extended to MFT-mRNA-programmed ribosomes that, after the incorporation of two amino acids and formation of tripeptidyl-tRNA, carried [^{14}C]$tRNA^{Phe}$ in the P site and fMet-Phe[^{3}H]Thr-$tRNA^{Thr}$ in the A site. The complex was set up in a way that a mixture of EF-Tu complexes with Phe-[^{14}C]$tRNA^{Phe}$ and with [^{3}H]Thr-$tRNA^{Thr}$ was added to the 70S initiation complex containing fMet-$tRNA^{fMet}$ in the P site to form pretranslocation ribosomes with $tRNA^{fMet}$ in the P site and fMet-Phe-[^{14}C]$tRNA^{Phe}$ in the A site (Table 1). Upon addition of EF-G, the latter was translocated into the P site, thereby allowing [^{3}H]Thr-$tRNA^{Thr}$ to enter the A site and forming fMet-Phe[^{3}H]Thr-$tRNA^{Thr}$. At this point, the P site contained [^{14}C]$tRNA^{Phe}$ that during the next round of translocation was displaced to the E site and released. Indeed, the extent of translocation of fMet-Phe[^{3}H]Thr-$tRNA^{Thr}$ (0.8 per ribosome, or more than 90% of the ribosome-bound peptidyl-tRNA) and the release of [^{14}C]$tRNA^{Phe}$ (0.74 per ribosome) were correlated quantitatively, as in the previous experiments.

In summary, the results of Figs. 1 and 2 and of Table 1 show that, by translocation, both initiator $tRNA^{fMet}$ and elongator $tRNA^{Phe}$ are shifted from the P site into the E site where they are bound in a kinetically labile fashion. The extent to which the E site-bound state is populated is determined by the concentrations of the binding partners as well as by the ionic conditions, in particular the concentration of Mg^{2+} or polyamines. Dissociation from the E site is spontaneous, and does not require and, as shown below, is not promoted by the occupancy of the A site.

Removal of EF-G from Posttranslocation Ribosomes. It has been claimed that the tRNA is released from the E site only

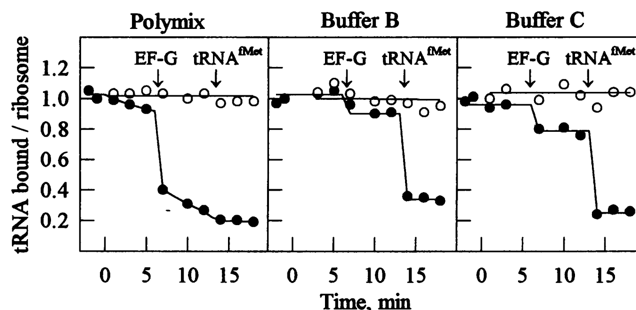

FIG. 2. Release of [^{14}C]$tRNA^{fMet}$ from the P site during translocation in polyamine containing buffers. Pretranslocation complexes on MFT-mRNA containing [^{14}C]$tRNA^{fMet}$ (●) in the P site and fMet[^{3}H]Phe-$tRNA^{Phe}$ (○) in the A site were prepared in polymix buffer or buffers B or C. Dipeptide formation was quantitative (HPLC) and translocation about 85% efficient (puromycin) at all buffer conditions.

when the A site becomes occupied, i.e., that the two sites are allosterically coupled in a negatively cooperative manner (19). In previous experiments with poly(U)-programmed ribosomes, we were unable to observe any coupling between the two sites. Neither binding of AcPhe-tRNA^{Phe} to the A site (25) nor EF-Tu-dependent binding of Phe-tRNA^{Phe} to the A site (29) would induce the release of tRNA^{Phe} from the E site or inhibit the binding of tRNA^{Phe} to the E site, respectively; the reciprocal effect of E-site occupancy on A-site binding we did not observe either. Rather, the effects observed by Nierhaus and colleagues were probably due to either deacylated tRNA contaminating the aminoacyl-tRNA added to fill the A site and chasing the labeled tRNA from the E site, or to second-round translocation by EF-G being present and active from the first translocation, or both (25).

To avoid the problem of second-round translocation when studying the effect of A-site binding of aminoacyl-tRNA on the occupancy of the E site, we have now developed a procedure for the removal of EF-G from posttranslocation ribosomes. It entails the use of EF-G modified by a histidine tag at the N terminus [EF-G(His)], which can be bound efficiently to Ni^{2+}-NTA-substituted agarose. When initiation complexes to which EF-G(His) was added in increasing amounts were treated with the affinity matrix once, the amount of EF-G(His) present in the sample was reduced to a very low level, even when the initial amount of EF-G(His) was equimolar to the ribosomes (Fig. 3). The treatment was sufficient to eliminate the problem of second-round translocation following A-site binding of Phe-tRNA^{Phe} in the experiment described below, in which 10 times less EF-G(His) was used for translocation and ternary complex binding was done within 1 min.

A-Site Binding and E-Site Occupancy. To establish conditions where [^{14}C]tRNA^{fMet} is retained in the E site of the posttranslocation ribosome, we have used buffer C (cf. Fig. 2 *Right*). A pretranslocation complex containing [^{14}C]tRNA^{fMet} in the P site and fMet[^{3}H]Phe-tRNA^{Phe} in the A site was formed on MFF-mRNA by adding a stoichiometric amount of ternary complex [^{3}H]Phe-tRNA^{Phe}·EF-Tu·GTP to the 70S initiation complex, and subsequently translocation was induced by adding EF-G(His) (Fig. 4). Then, EF-G(His) was removed by treating the sample with Ni^{2+}-NTA-agarose as described above. The posttranslocation complex was intact and functional after the treatment, as shown by both nitrocellulose filter binding and the puromycin assay. Finally, to refill the A site, a second equivalent of [^{3}H]Phe-tRNA^{Phe}·EF-Tu·GTP was added, and the amounts of ribosome-bound [^{3}H]Phe (i.e., fMet[^{3}H]Phe-[^{3}H]Phe-tRNA^{Phe} in the A site) as well as of E site-bound [^{14}C]tRNA^{fMet} were measured by nitrocellulose filtration. As evident from the data shown in Fig. 4, there was no significant release of [^{14}C]tRNA^{fMet} from the E site, whereas the A site was fully occupied.

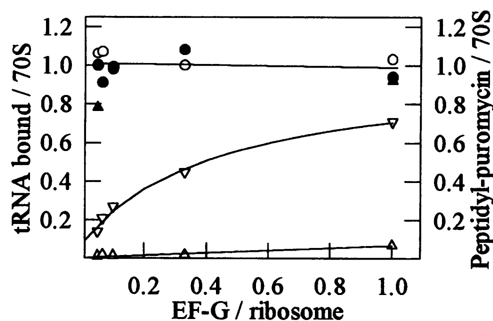

FIG. 3. Removal of His-tagged EF-G from ribosome mixtures by treatment with Ni^{2+}-NTA-agarose. To initiation complexes programmed with MFF-mRNA and carrying fMet-[^{14}C]tRNA^{fMet} in the P site (10 pmol in 30 μl buffer C) EF-G(His) (1.5 μl) was added at the indicated factor-to-ribosome ratios. Then 3 μl of a 50% suspension of Ni^{2+}-NTA-agarose in buffer C was added, the mixture was incubated for 15 min at 37°C under gentle mixing, and the agarose was removed by short centrifugation. Then, the complex of [^{3}H]Phe-tRNA^{Phe} with EF-Tu·GTP was added (1 per ribosome; final volume 40 μl) and the incubation continued. After 1 min, the amount of ribosome-bound [^{14}C]tRNA^{fMet} (●) and fMet[^{3}H]Phe-tRNA^{Phe} (○) was determined by nitrocellulose filtration, and the extent of translocation was determined by the amount of puromycin-reactive fMet[^{3}H]Phe-tRNA^{Phe} (△); the latter assay was also performed after 45 min (▽). Controls (▲) were treated in the same way (45 min incubation), except that buffer was added instead of Ni^{2+}-NTA-agarose.

DISCUSSION

The tRNA Leaving the Ribosome During Translocation Is Bound to the E Site only Transiently. The allosteric three-site model of elongation (19) is characterized by three main features: (*i*) codon-dependent and (*ii*) stable tRNA binding to the E site; (*iii*) negative cooperativity between E and A sites, that is, occupancy of one site excludes tRNA binding to the other. As discussed below, none of these features withstood experimental scrutiny in other laboratories.

With regard to the kinetic stability of the E-site complex, all groups studying the problem, except Nierhaus' group, agree that the tRNA is bound in a labile fashion (4, 5, 13, 14, 25, 30); the actual stability is strongly influenced by the concentration of Mg^{2+} and polyamines (20, 25, 31). The present results confirm this picture for posttranslocation complexes programmed with heteropolymeric mRNA. To keep a significant amount of tRNA^{fMet} in the E site after translocation, either the Mg^{2+} concentration had to be raised above 10 mM or spermine and spermidine had to be added to Mg^{2+}-containing buffers. However, even at the latter conditions, which stabilize binding the most, the majority of E site-bound tRNA remained readily chaseable.

Thus, programming the ribosomes with a heteropolymeric mRNA did not affect the stoichiometric coupling of tRNA release and peptidyl-tRNA displacement during translocation that has formed the basis for the classic two-site model of elongation (1), and was afterwards observed many times. As in previous experiments with poly(U)-programmed ribosomes (25), in the present experiments the deacylated tRNA was bound to the E site in a labile fashion, and the binding was

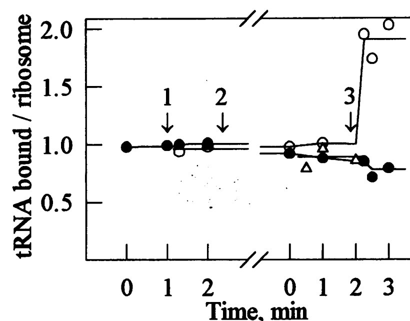

FIG. 4. Effect of A-site binding on the occupancy of the E site. The pretranslocation complex programmed with MFF-mRNA and carrying [^{14}C]tRNA^{fMet} in the P site and fMet[^{3}H]Phe-tRNA^{Phe} in the A site was prepared in buffer C as in Fig. 2 by adding the complex of [^{3}H]Phe-tRNA^{Phe} with EF-Tu and GTP to the initiation complex (1.1 per ribosome; arrow 1). Translocation was induced by adding EF-G(His) (0.1 per ribosome; arrow 2) and the incubation continued for 30 min. To remove EF-G(His), the sample was treated with Ni^{2+}-NTA agarose as in Fig. 3. Then another stoichiometric amount of ternary complex containing [^{3}H]Phe-tRNA^{Phe} was added (arrow 3). The amounts of ribosome-bound [^{14}C]tRNA^{fMet} (●) and [^{3}H]Phe (fMet[^{3}H]Phe-tRNA^{Phe} or fMet[^{3}H]Phe[^{3}H]Phe-tRNA^{Phe}) (○) were determined before and after the Ni^{2+}-NTA agarose treatment (break of time axis). Puromycin-reactive fMet[^{3}H]Phe-tRNA^{Phe} (△) was determined before adding the second ternary complex.

strongly influenced by Mg^{2+} and, more strongly, by spermine and spermidine.

The contribution of codon binding to E-site binding of tRNA has been studied extensively; results show that the contribution is not significant (5, 14) or small, increasing the average affinity 4- to 20-fold, depending on buffer condition (13, 15). Moreover, it was found that among various tRNAs there is an equally large variation of the intrinsic affinity of E-site binding apart from the effect of codon–anticodon interaction (13). Nierhaus' group has made different statements (12, 32, 33) regarding codon–anticodon interaction in the E site. In some papers, it was qualified as necessary or dominant (12, 32), and, in another paper, a 10-fold affinity difference between cognate and noncognate tRNA was stated (33). In more recent work from Nierhaus' group using heteropolymeric mRNAs, quantitative data are missing, although stable tRNA binding to the E site due to cognate codon–anticodon interaction remained a constitutive feature of the allosteric three-site model.

According to measurements performed by fluorescence resonance energy transfer, the distance between the anticodon loops of tRNAs in P and E sites is too large (34 ± 8 Å) to allow for simultaneous interaction of both anticodons with contiguous codons on the mRNA (34). Recently, Agrawal *et al.* (35) have reported the direct visualization by cryoelectron microscopy of three $tRNA^{Phe}$ molecules on poly(U)-programmed ribosomes. According to this model, which probably requires refinement, the anticodon region of the E site-bound tRNA is located such that codon–anticodon interaction is excluded. A different arrangement of the tRNA in the E site, but also no indication for codon–anticodon interaction, was obtained by cryoelectron microscopy by van Heel and colleagues (H. Stark, E. V. Orlova, J. Rinke-Appel, N. Jünke, F. Müller, M.V.R., W.W., R. Brimacombe & M. van Heel, unpublished work). Hence, it is unlikely that a large fraction of E site-bound tRNAs is in contact with the mRNA on the 30S ribosomal subunit. On the other hand, fluorescence stopped-flow experiments have revealed two states of the E site-bound tRNA, E′ and E, which are distinguished by their kinetic stabilities (36). In E′, the state that is reached immediately after the displacement, the anticodon is still in contact with the mRNA, and the codon–anticodon base pairs are disrupted during the subsequent rearrangement to E, which seems to be the predominant state (34).

Allosteric Coupling Between E and A Sites Cannot be Confirmed. At conditions prevailing *in vivo*, the E site-bound state of the tRNA probably is never highly populated, due to rapid aminoacylation of the tRNA dissociating from the ribosome during or after translocation (25); hence, there is no need to postulate the necessity of removing the tRNA from the E site by negative allosteric coupling with the A site. *In vitro*, it is possible to establish conditions of high E-site occupancy. However, working at such conditions, we were unable to observe any influence of A site binding of aminoacyl-tRNA on the occupancy of the E site of ribosomes programmed with heteropolymeric mRNA (Fig. 4) or with poly(U) (29), and vice versa.

On the basis of our present and previous results, which have been obtained under carefully controlled conditions with well-defined ribosomes that were fully active in all partial reactions of elongation, we consider the allosteric three-site model of elongation untenable. There may be a number of reasons to explain the results of Nierhaus and colleagues. As mentioned above, one reason for observing E site release upon adding aminoacyl-tRNA is contamination with uncharged tRNA, competing for E site binding. To exclude this possibility of artifact, the absolute charging level of the aminoacyl-tRNA preparation has to be high and known, information that is lacking in the papers from Nierhaus' group. Another way to get tRNA release from the E site upon binding aminoacyl-tRNA to the A site is translocation catalyzed by EF-G present from the previous translocation (second-round translocation). In their experiments with posttranslocation complexes, Rheinberger and Nierhaus (21) attempted to inactivate EF-G after the first round of translocation by decreasing the temperature to 4°C before filling the A site. However, as shown previously (25), EF-G remains quite active at this temperature, and second-round translocation is not at all suppressed. In contrast, when the posttranslocation complex was carefully purified from EF-G, as described here with His-tagged factor (Fig. 3), there was no second-round translocation and no release of deacylated tRNA from the E site when the A site was filled with aminoacyl-tRNA (Fig. 4).

Functional Role of the E Site as Exit Site for Deacylated tRNA in Translocation. Previous experiments have shown that the deacylated tRNA leaving the P site during translocation is transferred to the E site before dissociating from the ribosome (25). Using the stopped-flow technique with fluorescence detection, we could demonstrate that deacylated tRNA and peptidyl-tRNA move together to reach their immediate posttranslocation positions, i.e., E′ and P site, respectively, and that subsequently the deacylated tRNA rearranges from the E′ state into the E state (34), as discussed above. The equilibrium between free and E site-bound tRNA then is determined by the conditions, in particular by the concentrations of Mg^{2+} and polyamines (Figs. 1 and 2).

The functional significance of intermediate E-site binding during translocation has been discussed earlier. The specific interaction with the E site of the 3′ terminus of the leaving tRNA (11) was shown to promote translocation (15). On the basis of footprinting data, Moazed and Noller (17) have suggested that, after peptide bond formation, the 3′ domains of P site-bound deacylated tRNA and A site-bound peptidyl-tRNA are reaching into their respective posttranslocation positions on the 50S subunit, thus establishing P/E and A/P hybrid states, respectively. In recent work with mutant EF-G lacking the G domain, a ribosomal complex was obtained in which a puromycin-reactive intermediate translocation state of peptidyl-tRNA similar to the A/P state was frozen (18).

Thus, for the functional role of the E site, a model is emerging in which the interaction of the 3′ end of the P site-bound tRNA with the E site on the 50S subunit (P/E state) is an early step of translocation that, after the binding of EF-G to the pretranslocation complex, initiates and thereby catalyzes the movement of the tRNAs on the 30S subunit. The immediate product of 30S translocation is a transient posttranslocation state of the ribosome with peptidyl-tRNA in the P site (P/P state) and deacylated tRNA in the E site (E′/E state); the latter rapidly rearranges to the E/E state by disrupting the codon–anticodon interaction on the 30S subunit. In the E/E state, the tRNA is bound in a labile fashion, predominantly via the interaction of the 3′ terminus with the 50S subunit (5, 11, 37), and spontaneously dissociates from the ribosome to form the posttranslocation state of the ribosome with only peptidyl-tRNA in the P site and an open A site.

The various transient states of E-site binding, as well as intermediate states of A-site binding or of translocation, are features of the mechanism of elongation that have been unraveled in recent years and are to fill the schematic two-site model that is meant to describe the initial and final states of the system. It depends on the desired level of resolution to which extent these mechanistic features should enter the textbooks.

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