Kinetic and Thermodynamic Parameters for tRNA Binding to the Ribosome and for the Translocation Reaction*

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Susanne Schilling-Bartetzko, Andreas Bartetzko, and Knud H. Nierhaus‡

From the Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin-Dahlem, Germany

Kinetic analyses of tRNA binding to the ribosome and of the translocation reaction showed the following results. 1) The activation energy for the P site binding of AcPhe-tRNA to poly(U)-programmed ribosomes is relatively high ($E_a = 47$ kJ mol$^{-1}$; 15 mM Mg$^{2+}$). If only the P site is occupied with deacylated tRNA$^{Phe}_2$, then the E site can be filled more easily with tRNA$^{Phe}$(no activation energy measurable) than the A site with AcPhe-tRNA ($E_a = 47$ kJ mol$^{-1}$; 15 mM Mg$^{2+}$). 2) A ribosome with blocked P and E sites represents a standard state of the elongation cycle, in contrast to a ribosome with only a filled P site. The two states differ in that AcPhe-tRNA binding to the A site of a ribosome with prefilled P and E sites requires much higher activation energy (87 versus 47 kJ mol$^{-1}$). The latter reaction simulates the allosteric transition from the post- to the pretranslational state, whereby the tRNA$^{Phe}_2$ is released from the E site upon occupation of the A site (Rheinberger, H.-J., and Nierhaus, K. H. (1986) J. Biol. Chem. 261, 9133–9139). The reversed transition from the pre- to the post-translational state (translocation reaction) requires about the same activation energy (90 kJ mol$^{-1}$). 3) Both elongation factors EF-Tu and EF-G drastically reduce the respective activation energies. 4) The rate of the A site occupation rather than, as generally assumed, the translocation reaction is the rate-limiting step of the elongation cycle.

A complete set of binding constants for tRNA binding to the ribosomal A, P, and E sites has been presented (preceding paper, Ref. 1). The data were collected under the conditions with which the features of the allosteric three-site model for the ribosomal elongation cycle have been worked out (2, 3). In this paper we extend the analysis to kinetic and thermodynamic parameters of the elongation cycle under the same conditions (10 and 15 mM Mg$^{2+}$). We include some measurements at 6 and 3 mM Mg$^{2+}$ in the presence of polyamines. In this ionic milieu quantitative tRNA binding can be obtained, and the rate and accuracy of poly(U)-dependent poly(Phe) synthesis proceed with near in vivo values (4); the features of the allosteric three-site model could be confirmed with this system in an analysis with a heteropolymeric mRNA (5).

In this paper we show that programmed ribosomes with only one tRNA (at the P site) represent a state which is different from the pre- and posttranslational states of the elongation cycle where the ribosomes always carry two tRNAs. Both elongation factors EF-Tu and EF-G have a similar role, in that they reduce the activation energies of the corresponding allosteric transitions between pre- and post-translational states. The transition from the post- to the pretranslational state (A site occupation) is the rate-limiting step of the elongation cycle rather than the translocation reaction (transition from the pre- to the posttranslational state).

**MATERIALS AND METHODS**

The preparation of ribosomes, the acylation and acetylation of tRNA$^{Phe}_2$ and the assay conditions are described for 15 mM Mg$^{2+}$ in Ref. 6. For the 10 mM condition the Mg$^{2+}$ concentration was 10 mM at all steps, all other concentrations and parameters being kept constant. The assays performed at 6 and 3 mM Mg$^{2+}$ contained 2 mM spermidine and 0.05 mM spermine at all steps in addition to the compounds and components described in Ref. 6. This combination of polyamines is as effective as that described in Ref. 4 and approaches the respective values in vivo (7, 8). The measurements were performed using both substoichiometric amounts of AcPhe-tRNA (molar ratio AcPhe-tRNA: 70 S = 0.4:1) and excess amounts (AcPhe-tRNA: 70 S $= 1.5:1$).

A single assay mixture contained ($n + 1$) aliquots if $n$ kinetic points were to be measured. At the beginning of each experiment the assay mixture was incubated for 5 min at the indicated temperature without the tRNA under observation, and the kinetic analysis was started by addition of the respective ice-cold tRNA solution. Each aliquot, withdrawn at a distinct time point, was diluted directly with 2 ml of the respective ice-cold binding medium and immediately filtered to prevent further reactions at 0 °C. Accordingly, the shortest practicable time interval between two measuring points was 1 min. All experiments were performed using a single large-scale preparation of the biological components and ionic mixtures as starting materials. Blanks (minus ribosomes) were subtracted from the binding data. Sometimes some binding occurred already at 0 °C during pipetting yielding significant values already at 0 min of the kinetics (see, for example, Figs. 1 or 5). This does not affect further processing, since for the assessment of the rates of reaction the value at 0 min is irrelevant. All kinetics were performed at least twice with equivalent results.

The activation energy $E_a$ of a reaction was determined assuming a first order reaction (i.e., a reaction with a conformational change as rate-limiting step). If the maximal activity is $c_{p\text{max}}$ and the activity at time $t$ is $c_{p_{t}}$, then $c_{p_{t}}$ corresponds to the concentration of the complex A before the conformational change ($= [A]_t$), and the difference ($c_{p\text{max}} - c_{p_{t}}$) to the complex concentration at the time $t$ ($= [A]_t$). The underlying assumption is that the tRNA binding follows a sequence of events according to tRNA + ribosome + mRNA $\rightarrow$ [tRNA-ribosome-mRNA]$^2$ $\rightarrow$ [tRNA-ribosome-mRNA] and that the transition [$]$$^2$ $\rightarrow$ [ ] is the rate-limiting step. The equation of a
first order reaction $\ln([A]/[A]_0) = -kt$ develops therefore to the following,

$$\ln([cpm_{\text{max}} - cpm_a]/cpm_{\text{max}}) = -kt$$

where $k$ is the rate constant. Equation 1 was successfully applied for the characterization of the rate-limiting steps in the course of the ribosomal assembly in vitro and for the assessment of the corresponding activation energies (9, 10). This method of data processing is justified if the plot $\ln([cpm_{\text{max}} - cpm_a]/cpm_{\text{max}})$ versus $t$ yields a straight line, the slope of which is $k$ (11). The activation energy is obtained from the following Arrhenius equation,

$$k = A \exp(-E_a/RT)$$ (2)

where $A$ is the frequency factor, $R$ the gas constant $[1.987 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}]$, $E_a$ or $8.3144 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, and $T$ the absolute temperature.

Since $\ln k = -E_a/RT + \ln A$, a plot of $\ln k$ versus $1/T$ should give a straight line with the slope $-E_a/R$ (12, 13).

The activation parameters $\Delta H^\circ$ (activation enthalpy), $\Delta G^\circ$ (free energy of activation), and $\Delta S^\circ$ (activation entropy) were calculated according to following equations

$$\Delta H^\circ = E_a - RT$$ (3)

$$\Delta G^\circ = -RT \ln k$$ (4)

where $h$ is the Planck constant ($3.2983 \cdot 10^{-34} \text{ ca1} \cdot \text{s}$ or $6.626.10^{-34} \text{ J} \cdot \text{s}$), $E_a$ or $8.3144 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, and $T$ the temperature.

The activation parameters were determined as follows: the standard enthalpy $\Delta H^\circ$ was derived from the van't Hoff reaction isochore,

$$\ln k = \ln k_a - \Delta H^\circ/(RT) \ln h/(k_B \cdot T)$$ (6)

where $k_a$ is the association constant at the temperature $T$. $K_a$ was derived from the binding values in the presence of substoichiometric amounts of AcPhe-tRNA (see above) after maximal incubation time. A plot of $\ln K_a$ versus $1/T$ yields a straight line with the slope $-\Delta H^\circ/ R$. The standard free energy $\Delta G^\circ$ at the temperature $T$ is obtained from

$$\Delta G^\circ = -RT \ln K_a$$ (7)

and the standard entropy $\Delta S^\circ$ from

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T.$$ (8)

For details see Refs. 11 and 12.

**RESULTS**

Kinetic analyses of tRNA binding to P, E, and A sites were performed at various temperatures and at various Mg$^{2+}$ concentrations. The translocation reaction was analyzed in a similar manner.

**Occupation of the P Site (AcPhe-tRNA)—**Fig. 1A shows the kinetics of AcPhe-tRNA binding to the P site of poly(U)-programmed ribosomes at 15 mM Mg$^{2+}$ and various temperatures from 0 to 37 °C. Surprisingly, the rates of reaction have a maximum around 20 °C (Fig. 1D). The binding data were processed assuming a first-order law (Fig. 1B); the data obtained up to 5 min gave straight lines as expected only at temperatures below 20 °C. Accordingly, only the corresponding rates of reaction could be used for an estimate of the activation energy (Fig. 1C), for which a value of $E_a = 72 \text{ kJ mol}^{-1} (17.2 \text{ kcal mol}^{-1})$ was found. Clearly, a positive enthalpic term prevails at temperatures above 20 °C under the conditions used.

P site binding is a complicated process during which tRNA and mRNA bind to the ribosome in a cooperative manner involving codon-anticodon interaction. The cooperative character of the process can be easily observed when tRNA and mRNA are labeled with different isotopes in one and the same assay (e.g. Ref. 14).

Since the rate of AcPhe-tRNA binding at 20 °C is signifi-

![Fig. 1. Kinetics of binding of AcPhe-tRNA to the P site of poly(U)-programmed ribosomes at various temperatures (4) and the corresponding plots for the determination of the rates of reaction (B) and the activation energy (C; for details see "Materials and Methods"). (A) $\text{Ac}[^{14}C]\text{Phe-tRNA} (930 \text{ cpm/pmol; molar ratio AcPhe-tRNA: 70 S = 0.4:1); one aliquot of 100 \mu l contained} \ 8.6 \text{ pmol of 70 S. D, temperature dependence of the rates of AcPhe-tRNA binding to P sites. E, saturation curves of AcPhe-tRNA binding to P sites at 0, 20, and 30 °C. F, Arrhenius plot of the rates of AcPhe-tRNA binding to P sites at 6 \text{ mM Mg}^{2+} \text{ in the presence of polyamines. CPM, cpm}_{\text{max}}, \text{cpm, cpm; for explanation see "Materials and Methods."}}$]
Kinetic and Thermodynamic Parameters of the Ribosomal Elongation Cycle

The parameters are given in kJ mol\(^{-1}\) (kcal mol\(^{-1}\)) except the entropy values which are given in kJ mol\(^{-1}\) K\(^{-1}\). Experiment 6, only the spontaneous translocation (in the absence of EF-G) could be measured since in the presence of EF-G translocation was already observed at 0 °C, and at higher temperatures a precise measurement was not possible under our conditions. In order to prevent any translocation occurring during the incubation for the puromycin reaction (2 h at 0 °C), 5 μM thiostrepton was added to the reaction mixtures; this drug completely blocks both spontaneous and enzymatic translocation (18).

### Table I

The parameters of the ribosomal elongation cycle are given in kJ mol\(^{-1}\) (kcal mol\(^{-1}\)) except the entropy values which are given in kJ mol\(^{-1}\) K\(^{-1}\). Experiment 6, only the spontaneous translocation (in the absence of EF-G) could be measured since in the presence of EF-G translocation was already observed at 0 °C, and at higher temperatures a precise measurement was not possible under our conditions. In order to prevent any translocation occurring during the incubation for the puromycin reaction (2 h at 0 °C), 5 μM thiostrepton was added to the reaction mixtures; this drug completely blocks both spontaneous and enzymatic translocation (18).

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<th>exp. no.</th>
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<th>reaction scheme</th>
<th>(k_{\text{cat}}^0) [μM]</th>
<th>activation parameters</th>
<th>standard thermodynamic parameters</th>
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<td></td>
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<td>A site free)</td>
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Table I continues on next page.
change significantly during the transition to the activated state. At 10 mM Mg^{2+} the order increases during the transition to the activated state (ΔS^* is significantly negative, -0.138 kJ mol^{-1}).

The standard thermodynamic parameters are also listed in Table I. At both 15 and 10 mM Mg^{2+} the standard free energy ΔG^* is negative (about -45 kJ mol^{-1}), which documents the exergonic nature of the reaction. The standard enthalpy ΔH^* is relatively large (about 115 kJ mol^{-1}), i.e. the reaction is endothermic. Therefore, the negativity of ΔG^* is determined by a large standard free entropy ΔS^* (about 0.55 kJ mol^{-1} K^{-1}) according to the equation ΔG^* = ΔH^* - TΔS^*, i.e. the reaction is driven by the entropy.

**Occupation of the E Site (Decacylated tRNA_phe)**—The P site of poly(U)-programmed ribosomes was blocked with nonlabeled tRNA_phe and then the binding of [14C]tRNA_phe to the E site was followed kinetically at various temperatures. The E site is filled as second site before the A site can be occupied.
Kinetic and Thermodynamic Parameters of the Ribosomal Elongation Cycle

Kinetics of the binding of \[^{14}\text{C}]\text{tRNA}^{\text{Phe}}\) to E sites (A) after prefilling the P sites with nonlabeled \(\text{tRNA}^{\text{Phe}}\) (tRNA: 70 S = 1.5:1) and the derived plots for the rates of reaction (B) and the activation energy (C). One aliquot contained 48 pmol of poly(U)-programmed 70 S.

Similar binding curves were observed at all temperatures (Fig. 2). It follows that no activation energy is required for filling the E site, and even a small negative value was found \((E_a = -6.3 \text{ kJ mol}^{-1}\) at 15 mM Mg\(^{2+}\), see Table I, experiment 2). When the P site is occupied with a deacylated tRNA, the ribosomal state can therefore be easily transformed to the posttranslational state, since the E site is ready to bind a deacylated tRNA without activation energy (see also the other activation parameters, e.g. \(\Delta G^o\) is negative, about \(-80 \text{ kJ mol}^{-1}\)). The standard free enthalpy is small and negative (i.e. the reaction is slightly exothermic), and the standard free entropy hardly changes.

**Occupation of the A Site (E site-free, i-type)**—AcPhe-tRNA becomes an A site ligand for a ribosome whose P site has been prefilled with deacylated tRNA\(^{\text{Phe}}\). This type of A site occupation of ribosomes with prefilled P sites but empty E sites occurs only once during the synthesis of a protein, namely as the first A site occupation just after filling the P site with the initiation tRNA, i.e. just after the formation of the initiation complex. Therefore, this type of A site occupation is referred to as "i-type," i for initiation. The next and all subsequent A
site occupations proceed from ribosomes with filled P and E sites ("e-type," e for elongation (18)). When the A site is occupied as second site the rates of binding clearly depend on the temperature (Fig. 3, A-C). An activation energy of 47 kJ mol$^{-1}$ (15 mM Mg$^{2+}$, Table I, experiment 3) is required for this A site binding. This finding contrasts to the situation where the E site is occupied as second site, in which case no activation energy is needed for the binding reaction (see preceding section). The enthalpic and entropic term of activation contribute equally (about 40 kJ mol$^{-1}$) to the free energy of activation (80 kJ mol$^{-1}$), i.e. the activation depends equally on enthalpic and entropic effects.

The temperature dependence of the association constants is shown in Fig. 3D (15 mM Mg$^{2+}$), and the resulting standard enthalpy $\Delta H^\circ$ amounts to 39.4 kJ mol$^{-1}$. The standard free energy $\Delta G^\circ$ is negative but has approximately the same value, i.e. the negativity is determined by the entropy. A site binding is thus an entropy-driven reaction.

**Occupation of the A Site (P and E Site Prefilled, e-type)—**
When P and E site are occupied with tRNAs, the ribosome is in the posttranslocational state. Filling of the A site triggers the allosteric transition from the post- to the pretranslocational state, whereby the E site loses its affinity for tRNA, resulting in the release of decylated tRNA (2, 5).

**AcPhe-tRNA binding depends strongly on the temperature** (Fig. 4.1), and the corresponding activation energy is relatively large (87 kJ mol$^{-1}$; Table I, experiment 4). The activation enthalpy $\Delta H^\circ$ and the free energy of activation $\Delta G^\circ$ are large and of the same size (about 85 kJ mol$^{-1}$ at 15 mM Mg$^{2+}$), and the activation entropy is negligibly small, i.e. the activation is a mere enthalpic process.

The standard free enthalpy $\Delta H^\circ$ is large (about 140 kJ mol$^{-1}$), and the negativity of $\Delta G^\circ$ is thus caused by a similarly large entropic effect (0.573 kJ mol$^{-1}$ K$^{-1}$).

The A site binding is certainly a complex reaction. The fact, however, that in all cases the processing of the data according to a first order reaction yields straight lines (B of Figs. 3 and 4) clearly indicates that the rate-limiting step of this process is a conformational change.

At 6 and 3 mM the activation energies for AcPhe-tRNA binding to the A sites are even larger (around 115 kJ mol$^{-1}$; Table I, experiment 4). The rates of the nonenzymatic binding of Phe-tRNA show a reduced temperature dependence at these Mg$^{2+}$ concentrations (see for example the 6 mM data, Fig. 4.2, A-C) with correspondingly lower values for the activation energies (about 85 kJ mol$^{-1}$; Table I, experiment 5). If Phe-tRNA is bound via the ternary complex Phe-tRNA-EF-Tu-GTP the rates increase significantly (compare Fig. 4.3A and 4.2C), and these rates were so fast that only the tRNA (△; 500 cpm/pmol; AcPhe-tRNA: 70 S = 0.4:1) to the A sites (A) and the corresponding plots for the determination of the rates of reaction (B) and the activation energy (C); one aliquot of 100 µl contained 48 pmol 70 S 4.2, the corresponding nonenzymatic binding of [H]Phe-tRNA (△; 530 cpm/pmol; Phe-tRNA: 70 S = 0.3; 4) and the derived plots (B and C); one aliquot of 65 µl contained 15.2 pmol of 70 S 4.3, Arrhenius plot of the rates of the binding of the ternary complex [H]Phe-tRNA-EF-Tu-GTP (530cpm/pmol; Phe-tRNA: 70 S = 0.3; EF-Tu:Phe-tRNA = 3:1; the ternary complex was preformed during an incubation at 37 °C for 5 min in the presence of 20 mM Tris-HCl (pH 7.8 at 0 °C), 7 mM Mg$^{2+}$, 45 mM NH$_4$Cl, 15 mM KCl, 0.4 mM GTP, 3 mM phosphoenolpyruvate in a volume of 15 µl containing 2.1 µg of pyruvate kinase); one aliquot of 65 µl contained 15.2 pmol of 70 S 4.4, pretranslocational state; 4.5, posttranslocational state.
data collected below 30 °C could be explored for the determination of the activation energies (Fig. 4.3A). The corresponding activation energies are strongly reduced (30–40 kJ mol⁻¹; Table I, experiment 6). The presence of the elongation factor EF-Tu thus lowers the activation energy for the binding of an aminoacyl-tRNA to the A site significantly.

The Translocation Reaction—As mentioned above A site occupation of a ribosome with prefilled P and E sites represents an allosteric transition from the post- to the pretranslational state. The translocation is the reverse reaction, namely the allosteric transition from the pre- to the posttranslational state. During this transition the tRNAs present in A and P sites are co-translocated to P and E sites, respectively (5, 19).

The kinetics of the EF-G-dependent translocation of AcPhe-tRNA from A to P site (15 mM Mg²⁺) and the temperature dependence of the reaction rates are demonstrated in Fig. 5, A–C. The rate constant k (37 °C) is much faster than that for the corresponding A site binding (1.91 min⁻¹ versus 0.074 min⁻¹; Table I, experiments 7 and 4, respectively), whereas the corresponding activation energies are about the same (about 90 kJ mol⁻¹). The activation is an enthalpic process (the activation enthalpy and free energy of activation are similarly large, whereas the activation entropy is small; Table I, experiment 7). At 6 and 3 mM Mg²⁺ (and in the presence of polyamines) the rates are significantly slower and the activation energies higher (about 120 kJ mol⁻¹; Table I, experiment 7), indicating that the EF-G-dependent translocation of AcPhe-tRNA has to overcome a high activation energy barrier at 6 and 3 mM Mg²⁺.

In sharp contrast, the EF-G-dependent translocation of the dipeptidyl-tRNA AcPhe-Phe-tRNA is so fast and so efficient at low temperatures that we were not able to assess the activation energy for this reaction under our conditions. However, the spontaneous translocation in the absence of EF-G could be measured (Fig. 6). At 37 °C a significant fraction of the AcPhe-Phe-tRNA was already translocated at the first time point. However, we only took the translocation rate of the remaining AcPhe-Phe-tRNA into consideration (Fig. 6B). The respective activation energy derived from Fig. 6C was 85.4 kJ mol⁻¹. It is clear that the elongation factor EF-G drastically reduces the activation energy, since efficient translocation was already observed at low temperatures at significantly higher rates than those found in the absence of EF-G.

**DISCUSSION**

Functional States of Ribosomes—Essentially two systems were used in the experiments reported here, namely systems with either 10 or 15 mM Mg²⁺ but lacking polyamines, and systems with either 6 or 3 mM Mg²⁺ but containing the polyamines spermidine (2 mM) and spermine (0.05 mM). The results obtained under the various conditions do not differ qualitatively, i.e. the ribosomal binding sites can be occupied quantitatively and the features of the allosteric interactions of the A and E sites, respectively can be studied (2, 5, 15, 17). However, it is clear that the low Mg²⁺/polyamine systems are more physiologically, since the ionic conditions are similar to the corresponding in vivo states (see Ref. 1 for discussion), and also the rate and accuracy of protein synthesis show near in vivo perfection (4). However, quantitative differences can be seen. When one changes to the more physiological conditions, the affinity ratio E site/A site increases (1), i.e. the interplay between the A and E sites is better balanced. Furthermore, the activation energy barriers for the transitions between the two basic states of elongation, the pre- and the posttranslational states, are higher under physiological conditions, the activation energies for the post → pre-transition are about 85 and 115 kJ mol⁻¹ in the high and low Mg²⁺ systems, respectively (Table I, experiment 4). The corresponding values for the pre → post-transition are about 85 and 120 kJ mol⁻¹ (Table I, experiment 7). It follows that the pre- and posttranslational states are better defined under more physiological conditions. The fact that we observed a maximal rate of P site binding at 20 °C in the 15/10 mM Mg²⁺ systems (Fig. 1D), whereas the
corresponding rates increased up to 37 °C in the 6/3 mM Mg²⁺ systems, probably reflects the artificial character of the 15/10 mM Mg²⁺ systems.

An activation energy assessed via an analysis according to a first-order reaction (this paper) is a cogent indication of a conformational change taking place in the ribosome during the course of the respective reaction, whereas on the other hand the absence of significant activation energy does not necessarily mean that no conformational change occurs. The activation energies compiled in Table I allow four different functional states of the ribosome to be distinguished (Fig. 7, the activation energies in kJ mol⁻¹ given below the arrows were measured at 15 mM Mg²⁺).

At 0 °C an aminoacyl-tRNA (with or without EF-Tu-GTP) can bind to the A site (1), indicating that a programmed but otherwise empty ribosome is related to the pretranslocational state. The first conformational change of the programmed ribosome is induced by occupation of the P site with AcPhe-tRNA or deacylated tRNA⁹⁰, a process requiring a relatively high activation energy of 72 kJ mol⁻¹. This state does not occur during the elongation cycle; it is obviously related (but not identical, see next paragraph) to the postranslocational state, since the E site can be occupied without activation energy but not the A site (47 kJ mol⁻¹). It follows that a programmed ribosome with a deacylated tRNA at the P site can be easily transformed to the postranslocational state (P and E sites filled) rather than to the pretranslocational state (A and P sites filled). This observation explains the sequential filling of the ribosomal sites in saturation experiments with deacylated tRNA⁹⁰, i.e., after filling the P sites the E sites are occupied before the A sites (2, 15).

Ribosomes with only occupied P sites or with occupied P and E sites (postranslocational state) clearly represent different states, since the respective A site occupations of the i-type and e-type require distinctly different activation energies. When only the P sites are occupied the activation energy amounts to 47 kJ mol⁻¹, whereas when P and E sites are occupied 87 kJ mol⁻¹ are required. Both types of A site binding differ also functionally. A site occupation of the e-type (P and

Fig. 6. Kinetics of the translocation of Ac¹⁴C]Phe-[³H]Phe-tRNA in the absence of EF-G (spontaneous translocation) at 6 mM Mg²⁺ and in the presence of polyamines (A) and the plots for the rates of reaction (B) and the activation energy (C). Ac¹⁴C]Phe-tRNA (480 cpm/pmol; AcPhe-tRNA; 70 S = 1:1) was bound to P sites, the ternary complex [³H]Phe-tRNA-EF-Tu-GTP (530 cpm/pmol; Phe-tRNA; 70 S = 0.3:1; EF-Tu: Phe-tRNA = 8.3:1; preincubation of the ternary complex mixture for 5 min at 37 °C; see legend to Fig. 4) was added, and the sample was incubated for 3 min at 0 °C before the kinetics were started at the temperatures indicated. [↑], AcPhe-tRNA; [↓], Phe-tRNA.

Fig. 7. Four states of programmed ribosomes in the course of nonenzymatic tRNA binding. The four states can be distinguished by the activation energies involved. Ribosomes drawn one upon another are assumed to be in equivalent states. The numbers below the arrows represent the corresponding activation energies in kJ mol⁻¹ measured at 15 mM Mg²⁺ (Table I). Ribosomes carrying tRNAs in either P or P and E sites certainly represent different states, since the subsequent A site occupations markedly differ in their respective activation energies (47 and 87 kJ mol⁻¹, respectively); in addition, both reactions behave completely differently in the presence of various antibiotics (18).
which induce misreading, whereas these drugs barely affect the A site occupation of the i-type (only P site prefilled (18)).

Ribosomes carrying only a tRNA at the P site (E site-free) do not occur during the natural elongation cycle. This has the consequence that data from a structural analysis of a ribosome carrying only a tRNA at the P site cannot be directly extrapolated to the structure of the elongating ribosome as has been done at various times in the past (e.g. Ref. 20).

**Two Main States of the Elongating Ribosome—**After the ternary complex has occupied the A site the two subsequent reactions are EF-Tu-dependent cleavage of GTP and peptide bond formation. Both reactions occur spontaneously at low temperatures in contrast to the subsequent translocation. The latter reaction is accelerated by the elongation factor EF-G by about 2 orders of magnitude (21, 22). The only high activation energy barriers during the elongation cycle concern the transitions between the pre- and posttranslocational states (about 80 kJ/mol in either directions at 10 mM Mg^{2+}, Table I), and these transitions are characterized by a reciprocal affinity change at the A and E sites (5). The important conclusion is that the activation energy barrier between the pre- and posttranslocational states separates two main states of the elongating ribosome and therefore that the various functional states of the elongating ribosome represent sub-states of either the pre- or posttranslocational state. Accordingly, tight binding of a ternary complex to the A site (e.g. in the presence of the noncleavable GTP analogue GMP-PNP), the situation after EF-Tu-dependent cleavage of GTP and that after peptide bond formation have to be considered as sub-states of the pretranslocational state. In support of this view, tRNA present at the A site in these sub-states shows overlapping (not identical) cross-linking patterns, which are clearly distinct from that of a P site-bound tRNA (23, 24).

The existence of two main states, where either the A site is fully active (pretranslocational state) or of low affinity (posttranslocational state), cannot easily be reconciled with the hybrid site model proposed by Moazed and Noller (25). This model assumes, for example, that the P site region on the large ribosomal subunit can combine with the A site region on the small subunit forming an A/P hybrid site. However, the protection pattern seen on the large subunit (23 S rRNA) in the presence of modifying reagents was solely dependent on the -CCA end of the tRNAs. It is likely that the flexible -CCA end is not a reliable reporter structure for the site location of the corresponding tRNA molecule.

The transitions between the post- and pretranslocational states are catalyzed by the elongation factors. EF-Tu promotes the post → pre transition, and EF-G the pre → post-transition. As expected, we find that both factors drastically reduce the activation energies of the corresponding reactions (see "Results"), as is known from the action of enzymes. However, the function of an elongation factor is more complex than the activity of an enzyme, since an elongation factor not only reduces the activation energy but also determines the direction of the reaction, i.e. EF-Tu establishes the pretranslational state and EF-G the posttranslational state.

In order to accomplish this, the interaction of an elongation factor with the ribosome probably proceeds in at least two steps.

Let us consider EF-Tu. After codon-anticodon interaction EF-Tu (within the ternary complex) is able to undergo its first ribosomal interaction (first step) with the posttranslational conformer (P and E sites filled). This interaction reduces the activation energy of the post → pre allosteric transition, and so the ribosome is converted to the pretranslational state which is fixed by a tight interaction (second step) of EF-Tu with the ribosome. The ribosome is now in the pretranslational conformation, where a tight binding of aminoacyl-tRNA to the high-affinity A site occurs. Only now may EF-Tu hydrolyze its GTP (possibly triggered by the second step interaction), causing the dissociation of EF-Tu-GDP. Correspondingly, EF-G-GTP has its first interaction with the pretranslational conformer after the dissociation of EF-Tu-GDP.

It is generally assumed that the rate-limiting step of the elongation cycle is the translocation reaction (pre → post transition; e.g. Refs. 22 and 26). This assumption is not supported by our rate measurements. AcPhe-tRNA binding to the A site (post → pre transition; Table I, experiment 4) is about 30 times slower than the translocational of AcPhe-tRNA (Table I, experiment 7) at 15 or 10 mM Mg^{2+}, whereas the rates are about the same at 6 and 3 mM Mg^{2+}. The most relevant comparison in this respect is a comparison of the rates between enzymatic A site binding and enzymatic translocation of AcPhe-Phe-tRNA. The rate of the enzymatic A site occupation can still be measured (Table I, experiment 6), whereas the enzymatic translocation is so fast that it cannot be measured under our conditions. Steady-state measurements during poly(Phe) synthesis also revealed that the A site binding is the most time-consuming process (27).

We conclude that the occupation of the A site, and not the translocation reaction, is the rate-limiting step of the elongation cycle. This observation is inconsistent with ribosomal models which assume a high-affinity A site throughout the elongation cycle, but it is in accord with the allosteric three-site model (28) where the A site oscillates between high (pretranslational state) and low affinities (posttranslational state).

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**REFERENCES**

Kinetic and Thermodynamic Parameters of the Ribosomal Elongation Cycle