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Kinetic Evidence for the Obligatory Formation of a 30S Initiation Complex in Polyphenylalanine Synthesis Initiated with *N*-Acetylphenylalanyl-tRNA†

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ABSTRACT: The problem of whether the initiation of bacterial protein synthesis involves the obligatory formation of a 30S initiation complex intermediate was examined in a model system with *N*-acetylphenylalanyl-tRNA as initiator tRNA and poly(uridylic acid) as mRNA. The time courses of the formation of the 30S and 70S initiation complex with *Escherichia coli* ribosomes were measured simultaneously by stopping the reaction with dextran sulfate and differentiating the *N*-acetylphenylalanyl-tRNA bound to 30S ribosomal subunits from that bound to 70S ribosomes with RNase I, which hydrolyzes *N*-acetylphenylalanyl-tRNA bound to 30S subunits but not that bound to 70S ribosomes. A maximum in the 30S complex concentration was observed within the first 10–15 sec of the reaction, whereas 70S complex formed more slowly with a slight initial time lag. When an analog computer was programmed with rate constants determined separately for the formation of the 30S initiation complex and for the formation of the 70S complex from preformed 30S complex, kinetic curves very similar to the empirical curves were obtained for the entire time course of the reaction. The results show clearly that formation of the 70S complex obeys the kinetic laws for consecutive reactions, and the 30S complex is, therefore, an obligatory intermediate in the initiation of polyphenylalanine synthesis in the model system.

It is generally accepted that the initiation of bacterial protein synthesis occurs via the formation of a 30S initiation complex composed of the 30S ribosomal subunit, mRNA, and the initiator tRNA (Haselkorn and Rothman-Denes, 1973; Lucas-Lenard and Lipmann, 1971). The best evidence to date for the operation of this pathway is the work of Guthrie and Nomura (1968), which consists of the observation that 70S ribosomes of *Escherichia coli* equilibrate their 50S subunits with free 50S subunits in the course of binding *N*-formylmethionyl-tRNA (fMet-tRNA) but not in binding Val-tRNA. Their experiment, however, is open to an alternative interpretation (Klem and Nakamoto, 1968); moreover, according to studies carried out subsequently by Subramanian and Davis (1971), who took measures to avoid a potential artifact arising from hydrostatic pressure in the technique of sucrose density gradient centrifugation (Infante and Baierlein, 1971), mRNA-free 70S ribosomes readily exchange their subunits with free subunits, independently of protein synthesis.

We have, therefore, investigated the problem of whether there is an obligatory formation of a 30S initiation complex in the initiation of protein synthesis by using a model system with *N*-acetylphenylalanyl-tRNA (AcPhe-tRNA) as the initiator tRNA. This system has been shown to be similar in many essential respects to the natural system, even in its requirement for the three known initiation factors (Bernal et al., 1974a,b; Blumberg et al., 1974; Economou and Nakamoto, 1967; Lucas-Lenard and Lipmann, 1967). In the present study, we examined the problem by making kinetic measurements of the formation of 30S initiation complex simultaneously with that of 70S initiation complex, taking advantage of the observation of Pestka (1968) that RNase I hydrolyzes aminoacyl-tRNA bound to the 30S ribosomal subunit, but not that bound to the 70S ribosome. A

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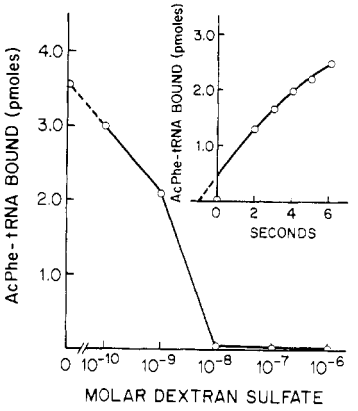

FIGURE 1: Inhibition of AcPhe-tRNA binding to 30S subunits by dextran sulfate. The reaction mixture for binding described in the Experimental Section was first diluted with 4 ml of stopping buffer at 15° containing the indicated concentrations of dextran sulfate. Binding was then initiated by addition of AcPhe-tRNA and poly(U), and was carried out for 10 min at 15°. Inset: Time course of AcPhe-tRNA binding to 30S subunits. Binding was conducted exactly as described in the Experimental Section.

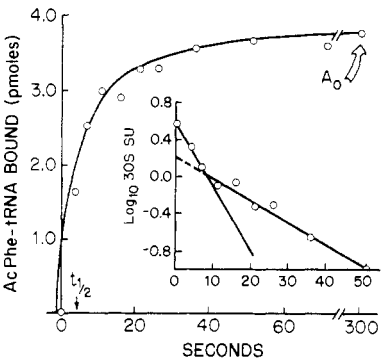

FIGURE 2: Time course of AcPhe-tRNA binding to 30S ribosomal subunits. Binding was conducted exactly as described in the Experimental Section. The curve was drawn by an analog computer programmed with the values for k_1^{fast} = 0.16 sec^{-1} (59%) and k_1^{slow} = 0.05 sec^{-1} (41%), determined from the inset plot of log of unreacted 30S subunits vs. time. The amount of active 30S subunits at zero time was assumed to be 3.8 pmol.

preliminary summary of this work has been presented (Blumberg and Nakamoto, 1972).

Experimental Section

Materials. Quarter-log *E. coli* B cells were purchased from the Grain Processing Company, and mid-log *E. coli* Q13 cells from General Biochemicals. Poly(U) was purchased from Miles Laboratories, dextran sulfate (mol wt 5 × 10^5) from Pharmacia, and crude *E. coli* B tRNA from Schwarz BioResearch. [^{14}C]Phenylalanine of specific activity 513 Ci/mol was purchased from New England Nuclear Corp., and ATP, GTP, and pyruvate kinase were from General Biochemicals. Bovine pancreatic ribonuclease (RNase I), Type A, was purchased from Worthington Biochemicals, and T factor was prepared by Dr. Ernest Hamel (Hamel et al., 1972).

Methods. [^{14}C]Phe-tRNA, Ac[^{14}C]Phe-tRNA, salt-washed ribosomes, protamine-treated supernatant, and initiation factors were prepared as described previously (Hamel et al., 1972; Blumberg et al., 1974). Ribosomal subunits were prepared from fresh S-30 extract by centrifugation in the Beckman Ti-14 zonal rotor at 5° for 2.5 hr at 48,000 rpm (172,000*g*) through a linear 5–20% sucrose gradient with a discrete change in the magnesium ion concentration. The buffer in the top one-third of the gradient contained 10 m*M* imidazole hydrochloride (pH 7.4), 0.1 m*M* $MgCl_2$, 20 m*M* KCl, and 5 m*M* 2-mercaptoethanol; in the bottom two-thirds of the gradient, the magnesium ion concentration was increased to 10 m*M*. When peak fractions were pooled, the procedure yielded 50S and 30S ribosomal subunits with less than 1% cross-contamination, as measured by incorporation of phenylalanine into polypeptide. The assay conditions for the incorporation of phenylalanine and of AcPhe-tRNA into polypeptides have been given by Hamel et al. (1972) and by Bernal et al. (1974a), respectively. Ribosome concentration was calculated by assuming 14.4 A_{260nm} units to be equivalent to 1 mg of ribosomes and the molecular weight of the 30S subunit to be 1 × 10^6 and that of the 50S subunit, 2 × 10^6.

In the assay for AcPhe-tRNA binding to 30S subunits, the complete reaction mixture contained, per 0.1 ml: 50 m*M* imidazole hydrochloride (pH 7.4), 6 m*M* $MgCl_2$, 80 m*M* KCl, 4 m*M* 2-mercaptoethanol, 1 m*M* GTP, 10 pmol of 30S ribosomal subunits preincubated for 20 min at 37° in a medium containing 0.5 *M* KCl and 10 m*M* $MgCl_2$ (Nakamoto and Hamel, 1968; Zamir et al., 1969), and sufficient initiation factors to give a maximal rate of binding. About 0.2 μg of IF-1, 1.0 μg of IF-2, and 0.5 μg of IF-3 were generally more than sufficient. The mixture was equilibrated at 15°, and binding was initiated by the addition of 24 pmol of *N*-Ac[^{14}C]Phe-tRNA and 6 μg of poly(U). The reaction was stopped by rapid addition of 4 ml of ice-cold "stopping buffer" containing 10 m*M* imidazole hydrochloride (pH 7.4), 8 m*M* $MgCl_2$, 80 m*M* NH_4Cl, and 1 × 10^{-6} *M* dextran sulfate which had been dialyzed at about 0.1 m*M* concentration against 0.1 *M* NH_4Cl to eliminate free sulfate and phosphate ions. The ribosomal complexes were then adsorbed onto Millipore filters, washed twice with 3 ml of binding buffer, dried, and counted in a liquid scintillation system with about 89% efficiency for ^{14}C and 40% for ^{3}H.

Since the reactions involved in AcPhe-tRNA binding followed a two-step pseudo-first:second-order kinetic scheme, the mathematical formulation resulted in three simultaneous, nonlinear differential equations with a complex solution (Chien, 1948). We, therefore, examined the problem on an analog computer, a Beckman EASE Model 2132 with an *X*–*Y* plotter, drawing curves by a procedure similar to that of Hommes (1962a). Details of the operation of the computer and a discussion of the curve-fitting technique will be presented elsewhere (B. M. Blumberg and I. S. Goldberg, manuscript in preparation).

Results

Dextran Sulfate Stops 30S Initiation Complex Formation. As seen in Figure 1, dextran sulfate is an effective agent for stopping the binding of AcPhe-tRNA to the 30S ribosomal subunit of *E. coli*. Inhibition of AcPhe-tRNA binding was essentially complete when dextran sulfate was present in the reaction mixture at a concentration as low as 1 × 10^{-8} *M* before binding was initiated with poly(U) and AcPhe-tRNA. When a reaction mixture in which AcPhe-tRNA binding was occurring was rapidly diluted 40-fold with ice-cold buffer containing 1 × 10^{-6} *M* dextran sulfate, binding was stopped in about 1 sec. This was demonstrated by back-extrapolation of the binding curve to the time axis at very short incubations (Figure 1, inset). Instead of NH_4Cl, which is actually more efficient, we used KCl in our reaction mixtures to obtain slower and more measur-

Table I: Summary of Kinetic Constants (k_1) for the Formation of the 30S Complex.

Expt	k_1^{fast} (sec^{-1})	k_1^{slow} (sec^{-1})	Ratio of Subunits (%) (Fast/Slow)
1	0.15	0.04	59/41
2	0.16	0.05	59/41
3	0.17	0.04	57/43
4	0.17	0.05	61/39
Optimated[a] value	0.17	0.045	59/41

[a] For a definition, see Garfinkel et al. (1970).

able rates of AcPhe-tRNA binding. On the other hand, NH_4Cl was used in the stopping buffer because K^+, at the concentrations required, precipitates dextran sulfate. The stopping action of dextran sulfate may be related to the observation of Miyazawa et al. (1967) that dextran sulfate competes with poly(U) for a binding site on the 30S subunit.

Kinetics of AcPhe-tRNA Binding to 30S Ribosomal Subunits. The time course of poly(U)-directed AcPhe-tRNA binding to isolated 30S ribosomal subunits is displayed in Figure 2. The experimental values for binding are plotted as individual points; the curve is computer drawn with an extrapolation to the time axis at −1 sec to allow for the delay in stopping the reaction with dextran sulfate. As can be seen, the reaction proceeds rapidly, with an overall $t_{1/2}$ of about 4 sec, and is more than 90% complete in 30 sec. A plot of log of unreacted 30S subunits vs. time shows that the binding can be resolved into two pseudo-first-order reactions (inset, Figure 2). From the slopes of the inset plot, which are equal to $-k/2.303$, we have $k_1^{fast} = 0.16$ sec^{-1} (59%), for the fast reaction, and $k_1^{slow} = 0.05$ sec^{-1} (41%), for the slow reaction. Rate constants determined in four separate experiments are summarized in Table I. It should be noted that, although 10 pmol of 30S subunits was added to each reaction, only about 3.8 pmol of AcPhe-tRNA was bound.

Differentiation of 70S from 30S Complexes by RNase. As shown in Figure 3, Ac[^{14}C]Phe-tRNA bound to isolated 30S ribosomal subunits is rapidly hydrolyzed at 0° by the addition of RNase I to the stopping buffer, whereas incubation of the binding mixture at 15° with 50S ribosomal subunits before addition of the RNase prevents hydrolysis of the Ac[^{14}C]Phe-tRNA. Similarly to the observation of Pestka (1968), the addition of 50S subunits to preformed 30S complexes presumably leads to formation of 70S complexes in which the AcPhe-tRNA is protected from RNase. A final wash of the Millipore filter with a buffer solution containing 20% ethanol is necessary to eliminate background radioactivity due to nonspecific binding of some hydrolyzed product of AcPhe-tRNA.

Dextran Sulfate Stops 70S Complex Formation. As shown in Figure 4, dextran sulfate at 1×10^{-7} *M* prevents the binding of 50S ribosomal subunits to preformed 30S complexes, blocking the formation of RNase-stable 70S complexes. Unlike the formation of the 30S complex, the formation of the 70S complex is stopped instantly by dilution of the reaction mixture with cold stopping buffer containing dextran sulfate (inset).

Titer of 50S Ribosomal Subunits. The fraction of active 50S ribosomal subunits was found to be less than the 38% of

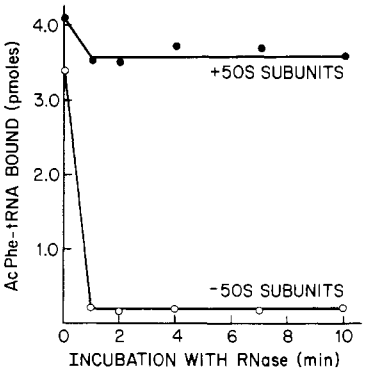

FIGURE 3: Differentiation of 70S from 30S ribosomal complexes by RNase. Duplicate binding reactions were carried out with 30S subunits for 5 min as described in the Experimental Section; 25 pmol of 50S ribosomal subunits was then added to one set of duplicates, and incubation was continued for 5 min at 15°. Binding was stopped by rapid dilution with 4 ml of ice-cold stopping buffer containing 0.5 μg/ml of RNase, and the reaction tubes were kept in ice for the indicated times before Millipore filtration. The filters were given a final wash with 3 ml of stopping buffer containing 20% ethanol (v/v).

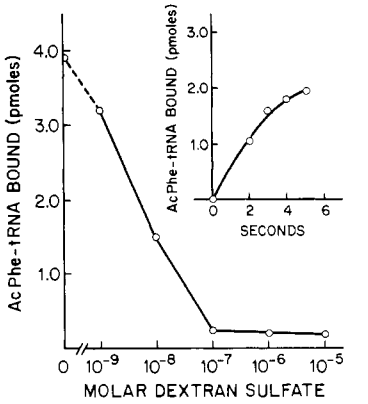

FIGURE 4: Inhibition of 70S complex formation by dextran sulfate. AcPhe-tRNA binding to 30S subunits was carried out for 5 min exactly as described in the Experimental Section. At this time, 4 ml of stopping buffer containing the indicated concentrations of dextran sulfate was added, together with 25 pmol of 50S subunits, and incubation was continued for 5 min at 15°. The reaction tubes were then chilled in ice, RNase was added at 0° to a final concentration of 0.5 μg/ml, and the tubes were kept in ice for 10 min before Millipore filtration was carried out as in Figure 3. Inset: Time course of 70S complex formation. Joining of the 50S subunits to the preformed 30S complexes was allowed to proceed for the indicated times, and was stopped by rapid dilution with stopping buffer containing 0.5 μg/ml of RNase. RNase-resistant 70S complexes were then collected on Millipore filters after 10 min, as described above.

active 30S ribosomal subunits noted in Figure 2 when the activity of 50S subunits was measured by the formation of RNase-resistant 70S complexes. As shown in Figure 5, more than 20 pmol of 50S ribosomal subunits must be added to 3.8 pmol of active 30S subunits (out of 10 pmol total 30S subunits) before RNase resistance is maximum. The amount of active 50S subunits can be estimated from the ratio of RNase-stable to total AcPhe-tRNA binding, after 70S complex formation has reached a plateau level (≥1 min). This particular preparation of 50S ribosomal subunits is thus estimated to be about 18% active.

Kinetics of 70S Complex Formation. The time course of the formation of 70S complexes from preformed 30S complexes and 50S ribosomal subunits is displayed in Figure 6A. In curves a, b, and c, the amounts of 50S ribosomal subunits added were 8, 16, and 32 pmol, and the ratio of active 50S to active 30S subunits is estimated to be 0.58, 1.16,

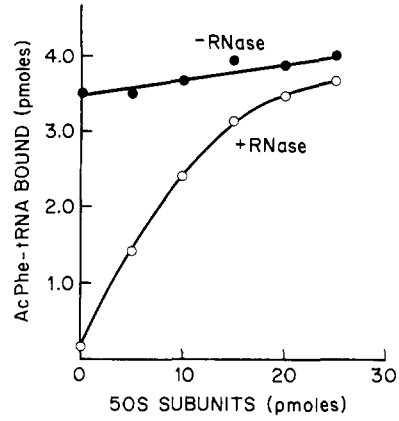

FIGURE 5: Titer of 50S subunits. Binding to 30S ribosomal subunits was carried out for 5 min, exactly as described in the Experimental Section; at this time, the indicated amounts of 50S ribosomal subunits were added to duplicate tubes, and incubation was continued for 5 min at 15°. Binding was then stopped and RNase-resistant 70S complex was measured as in Figure 4.

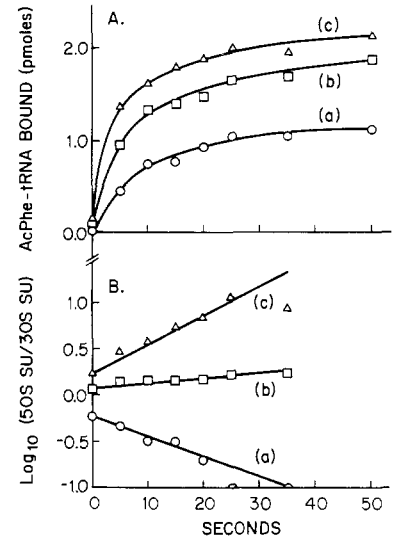

FIGURE 6: (A) Time course of 70S complex formation from 30S complex. 50S ribosomal subunits, 8 pmol in (a), 16 in (b), and 32 in (c), were added to 2.1 pmol of preformed 30S complexes, and 70S complex formation was measured as described for Figures 4 and 5. (B) Plots of log of the ratio of unreacted 50S subunits to unreacted 30S complexes vs. time.

and 1.74, respectively. The reaction is clearly first-order with respect to both 30S complex and 50S subunit concentration, or second-order overall since, as shown in Table II, the ratio of initial rates is very nearly the same, namely, 1:2:3, as the amounts of 50S subunits added. To determine the second-order rate constants, which are summarized in Table III, we plotted the logarithm of the ratio of the concentration of active 50S subunits to the concentration of unreacted 30S complexes as a function of time, as shown in Figure 6B.

Kinetic Evidence that the 30S Complex is an Obligatory Intermediate in the Initiation of Polyphenylalanine Synthesis. The foregoing results suggest that the overall reaction for the formation of the 70S initiation complex with an obligatory 30S complex intermediate may be represented by the following two consecutive reactions:

$$\underset{A}{\text{30S subunit}} + \text{AcPhe-tRNA} + \text{poly(U)} \xrightarrow{k_1} \underset{B}{\text{30S complex}} \quad (1)$$

$$\underset{B}{\text{30S complex}} + \underset{D}{\text{50S subunit}} \xrightarrow{k_2} \underset{C}{\text{70S complex}} \quad (2)$$

Since there are two species of 30S subunits with different k_1's in reaction 1, A = A^{fast} + A^{slow} and k_1 is either k_1^{fast} or k_1^{slow}. In mathematical terms:

$$-dA/dt = k_1^{fast}A^{fast} + k_1^{slow}A^{slow}$$

$$dB/dt = dA/dt - k_2BD$$

$$dC/dt = k_2BD$$

Providing evidence that the 30S complex is an obligatory intermediate, therefore, consists of showing that a 30S complex appears in the course of 70S complex formation, and that the rates and amounts of 30S and 70S complexes formed are adequately described by eq 1 and 2, above. In particular, a demonstration of a maximum in the concentration of the 30S complex and a time lag in the formation of the 70S complex would constitute the best evidence for a consecutive reaction. To provide such evidence, we have measured simultaneously the formation of 30S and 70S complexes with AcPhe-tRNA and have also compared the results with the theoretical curves for the reaction obtained with an analog computer.

The assay for 30S and 70S complex formation was carried out by running duplicate samples and treating one set with RNase. The data are displayed in Figure 7 as three sets of points; the curves in the figure are theoretical ones drawn by means of an analog computer programmed with the independently determined rate constants for the above consecutive reactions (Tables I and III). Binding of AcPhe-tRNA in the absence of RNase represents the sum of 30S and 70S complexes; binding in the presence of RNase, the amount of 70S complexes; and the difference between these two values, the amount of 30S complexes (triangles). No

Table II: Evidence that 70S Complex Formation from the 30S Complex is Second Order.

50S Subunits Added		AcPhe-tRNA Bound in 5 sec	
pmoles	Rel Amount	pmoles	Rel Amount
8	1.0	0.44	1.0
16	2.0	0.85	1.9
32	3.0	1.22	2.8

Table III: Summary of Kinetic Constants (k_2) for the Formation of 70S Complex from 30S Complex.[a]

Expt	50S Subunit/30S Complex	k_2 ($\times 10^6$ M^{-1} sec^{-1})
1	0.58	6.6
2	1.16	4.6
3	1.74	5.1
4	1.00	5.0
5	1.00	4.3
Optimated[b] value		4.6

[a] The rate constants for experiments 1–3 were calculated from the slopes of the log plots of Figure 6B using the relationship, $k_2 = [2.303/(B_0 - A_0)]$slope, where A_0 and B_0 are initial concentrations of the 30S complex and 50S subunit, respectively. The constants for experiments 4 and 5 were calculated from the second-order equation, $k_2t = 1/A - 1/A_0$. [b] For a definition, see Garfinkel et al. (1970).

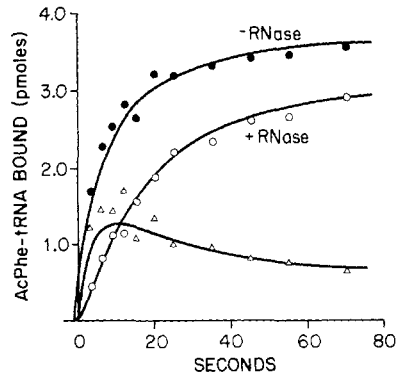

FIGURE 7: Kinetic evidence for the obligatory formation of a 30S initiation complex. The binding and RNase assays were conducted as described in the Experimental Section and in Figure 4, except that 20 pmol of 50S subunits were equilibrated in the reaction mixture at 15° before binding was initiated. (○) The amount of 70S complex (+RNase); (△) the amount of 30S complex (the difference between −RNase and +RNase). The theoretical curves were drawn by means of an analog computer (see Methods) with the following optimated values for the rate constants: $k_1^{fast} = 0.16$ sec^{-1} (59%), $k_1^{slow} = 0.045$ sec^{-1} (41%), and $k_2 = 4.3 \times 10^6$ M^{-1} sec^{-1}. A correction has been made for the 1-sec delay in stopping 30S complex formation with dextran sulfate.

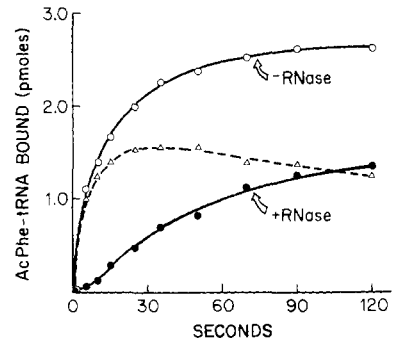

FIGURE 8: Time course of AcPhe-tRNA binding with an S-30 extract. Binding was conducted as described for Figure 7, except that the source of ribosomes was an extract of *E. coli* B, freshly prepared as described in the Experimental Section. (○) (−RNase) The sum of 70S and 30S complexes; (●) (+RNase) 70S complexes; and (△) (the difference between −RNase and +RNase) 30S complexes.

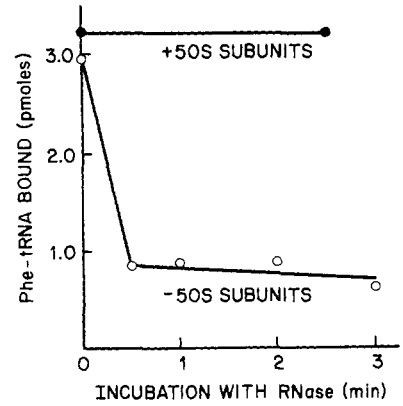

FIGURE 9: RNase differentiation of 70S from 30S complexes with bound Phe-tRNA. The method of binding was similar to that in Figure 3, except that [^{14}C]Phe-tRNA was used instead of Ac[^{14}C]Phe-tRNA, 4 μg of T factor was used instead of initiation factors, and the reaction volume was 0.25 ml.

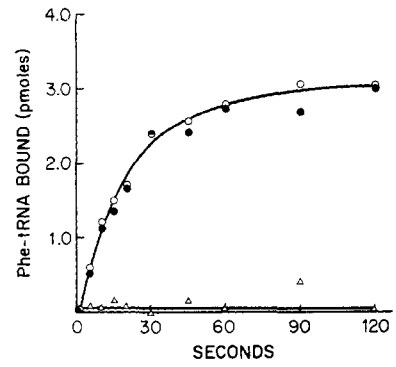

FIGURE 10: Time course of Phe-tRNA binding to 70S ribosomes. Binding was carried out by a method similar to that in Figure 7. Preincubation in duplicate tubes of 400 pmol of crude *E. coli* B tRNA, 10 μg of poly(U), and 60 μg of salt-washed ribosomes prepared as described in the Experimental Section was carried out for 30 min at 30°; 4 μg of peak 1 T factor was then added and preincubation was continued for 5 min. Binding was initiated with 100 pmol of [^{14}C]Phe-tRNA, and was stopped as described in Figure 4 with stopping buffer (○) or stopping buffer containing 0.5 μg/ml of RNase (●). (△) The difference between the −RNase and +RNase samples.

adjustment was made for any decrease in concentrations of free 30S and 50S subunits by their association into 70S ribosomes, since the rate of AcPhe-tRNA binding was essentially the same with 30S subunits alone (Figure 2) as with 30S and 50S subunits together (Figure 7, without RNase). It can readily be seen that the concentration of the 30S complex reaches a maximum and that a slight lag occurs in 70S complex formation. These observations, together with the close correspondence of the theoretical curves to the experimental values of AcPhe-tRNA binding, demonstrate that the 30S complex is an obligatory intermediate in the formation of the 70S initiation complex.

The absence of any significant amount of 70S ribosomes in this reaction left open the possibility that the 30S initiation complex was obligatory for poly(Phe) synthesis in our study because the ribosomal subunits might have been damaged during isolation and thus were unable to form 70S particles without first binding poly(U) and AcPhe-tRNA. To eliminate this possibility, we performed a similar experiment using a freshly prepared S-30 extract of *E. coli* treated only by rapid passage through a small column of Sephadex G-25 (Figure 8). Although the binding was considerably slower, the time course of the reaction reveals a maximum in the formation of the 30S complex and clearly shows a time lag in the formation of the 70S complex. The results thus indicate that the 30S complex is also an obligatory intermediate in the S-30 extract.

A Control for the RNase Assay. Phe-tRNA binds specifically to 70S ribosomes in a reaction catalyzed by T factor; it will also bind nonenzymatically to 30S ribosomal subunits. As was observed by Pestka (1968), [^{14}C]Phe-tRNA is hydrolyzed by RNase when bound to the 30S ribosomal subunit, but is resistant when bound to the 70S ribosome (Figure 9). An experiment similar to that in Figure 7 was performed, with Phe-tRNA and T factor instead of AcPhe-tRNA and initiation factors. The ribosomes were preincubated with poly(U) and uncharged tRNA to preform 70S ribosomes with uncharged tRNA^{Phe} in the P site (Revel et al., 1969). Since the Phe-tRNA can only bind directly to 70S ribosomes, our PNase assay, if reliable, should reveal no 30S complex formation in the course of Phe-tRNA binding. As seen in Figure 10, the binding proceeds rapidly, being complete in 1 min, as was observed with AcPhe-tRNA. In constrast, however, no 30S complex appeared throughout the course of the T factor-catalyzed binding of the Phe-tRNA to 70S ribosomes.

Discussion

The present study has shown that the formation of the 70S initiation complex with poly(U) and AcPhe-tRNA obeys the kinetic laws for consecutive reactions. In particular, the study has demonstrated the appearance of a 30S complex which rapidly reaches a maximum concentration, and the occurrence of a time lag in the formation of the 70S complex. The results of this study, therefore, constitute strong evidence for the participation of the 30S complex as an obligatory intermediate in the initiation of poly(Phe) synthesis with AcPhe-tRNA. Moreover, the close fit over the entire time course of the reaction between the experimental values for 30S and 70S complex formation and the theoretical curves obtained with the analog computer further supports this conclusion. The use of independently determined rate constants in the programming of the computer (Bates and Frieden, 1973) overcomes many of the pitfalls of a procedure in which rate constants of a complex reaction are determined by curve fitting (Hommes, 1962b).

The occurrence of a maximum in the concentration of the 30S complex and of a time lag in 70S complex formation in a minimally treated S-30 extract argues against the possibility that the 30S complex observed in our study was obligatory only because the ribosomes were damaged during isolation. A number of plausible explanations can be given for the slower rate of binding with the S-30 extract compared with the rate for the isolated subunits. Among them are the association of a significant portion of the subunits as 70S particles, inhibition of the binding by fMet-tRNA present in the S-30 extract (Blumberg et al., 1974), competition between poly(U) and endogenous mRNA for the ribosomes, and omission of the preincubation step to activate the ribosomes.

The absence of a 30S complex in the binding of Phe-tRNA to 70S ribosome catalyzed by T factor, which is known to bind directly to 70S ribosomes (Lucas-Lenard and Haenni, 1968), provided additional assurance that the RNase assay for 70S and 30S complexes is reliable. Although the possibility cannot be entirely excluded that AcPhe-tRNA binding might occur via a "loose" 70S complex which is susceptible to RNase hydrolysis, like a 30S complex, this is not considered likely in view of the close fit of the computer-drawn curves and the experimental values for binding. Attempts to correlate the formation of 30S initiation complexes, as measured by RNase sensitivity, with 30S complexes on sucrose gradients failed because such complexes are unstable on a gradient. Since fMet-tRNA forms a more stable 30S complex, it might be possible to show such a correlation with the natural initiator tRNA.

The resolution of fast- and slow-binding 30S ribosomal subunits, coupled with our observation that fMet-tRNA binds strongly to only half of the fast 30S subunits (Blumberg et al., 1974), suggests that there may be at least three species of 30S ribosomal subunits. The unexplained slow, linear GTP hydrolysis observed by Modolell et al. (1973) when they examined the kinetics of translocation may reflect ribosomal heterogeneity, as may the experiments of Moore (1973) and of Ginzburg et al. (1973) in which ribosomes were differentially inactivated by *N*-ethylmaleimide.

Many previous studies from this laboratory and elsewhere have pointed up the extensive analogies between the natural system and the poly(U)-AcPhe-tRNA model system in regard to their requirement of Mg^{2+}, GTP, and initiation factors (Bernal et al., 1974a,b; Blumberg et al., 1974; Economou and Nakamoto, 1967; Lucas-Lenard and Lipmann, 1967). Despite the fact that the components of the system include seven macromolecules, the model system obeys simple kinetic laws, so that rate constants for the initiation process could be determined. Although the analogous kinetic experiments with fMet-tRNA have not been performed as yet, the close parallelism between our model system and the natural system should firmly substantiate the assertion that the 30S complex is an obligatory intermediate in the initiation of protein synthesis in *E. coli*.

Acknowledgment

We gratefully acknowledge the many helpful discussions with Dr. Ferenc J. Kézdy.

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