Initial Rate Kinetic Analysis of the Mechanism of Initiation Complex Formation and the Role of Initiation Factor IF-3†

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ABSTRACT: Initial rate kinetics of the formation of ternary complexes of *Escherichia coli* 30S ribosomal subunits, poly(uridylic acid), and *N*-acetylphenylalanyl transfer ribonucleic acid in the presence and in the absence of IF-3 are consistent with the hypothesis that the ternary complex is formed through a random order of addition of polynucleotide and aminoacyl-tRNA to separate and independent binding sites on the 30S ribosome. The transformation of an intermediate into a stable ternary complex which probably entails a rearrangement of the ribosome structure leading to a codon–anticodon interaction represents the rate-limiting step in the formation of the ternary complex. The rate constant of this transformation, as well as the association constants for the formation of the 30S–poly(U) and 30S–*N*-AcPhe-tRNA binary complexes, are enhanced by the presence of IF-3 which acts as a kinetic effector on reactions which are intrinsic properties of the 30S ribosome. The IF-3-induced modification of these kinetic parameters of the 30S ribosomal subunit can per se explain the effect of IF-3 on protein synthesis without invoking a specific action at the level of the mRNA–ribosome interaction. This seems to be confirmed by the finding that IF-3 can stimulate several-fold the formation of a ternary complex even if one by-passes the ribosome–template binding step by starting with a covalent 30S–polynucleotide binary complex. Furthermore, the above-mentioned changes induced by IF-3 appear to be compatible with the previously proposed idea that the binding of the factor modifies the conformation of the 30S subunit. The random order of addition of substrates determined for the 30S–*N*-AcPhe-tRNA–poly(U) model system was found to be valid also for the more physiological 30S initiation complex containing poly(A,U,G) and fMet-tRNA formed at low Mg^{2+} concentration in the presence of GTP and all three initiation factors.

Initiation factor IF-3 acts as a ribosomal anti-association factor (Noll and Noll, 1971; Kaempfer, 1972; Godefroy-Colburn et al., 1975; Gottlieb et al., 1975) and stimulates the formation of physiological initiation complexes (Iwasaki et al., 1968; Revel et al., 1968; Noll and Noll, 1974; Jay and Kaempfer, 1975). The latter activity is usually attributed to the stimulation of the ribosomal binding of natural mRNAs.[1] In addition to these activities, which are commonly regarded as the physiological ones, IF-3 can also influence both formation and stability of ternary complexes of 30S ribosomal subunits, aminoacyl-tRNAs, and polynucleotides or codons. Thus, it has been reported that the formation of ternary complexes containing 30S ribosomes, poly(AUG) (or poly(U)), and fMet-tRNA (or *N*-AcPhe-tRNA) is dependent upon or strongly stimulated by the presence of IF-3 (Suttle et al., 1973; Bernal et al., 1974; Dondon et al., 1974). Since no mechanistic details are available to date concerning the mode of formation of the ternary complexes, the precise step at which IF-3 exercises its stimulatory action is not known. On the other hand, the stability of preformed ternary complexes seems to be greatly reduced in the presence of the factor following dilution or temperature shifts (Gualerzi et al., 1971; Pon and Gualerzi, 1974; Risuleo et al., 1976). The only exception found so far is the complex containing the initiator fMet-tRNA which appears to be quantitatively more resistant to the action of IF-3 (Risuleo et al., 1976). This IF-3 activity has been attributed to a conformational change of the 30S ribosome induced by the binding of the factor (Pon and Gualerzi, 1974; Gualerzi et al., 1975; Ewald et al., 1976; Michalski et al., 1976), but also in this case, the exact mode of action of IF-3 remains obscure.

In order to clarify these points we have undertaken a kinetic analysis of the formation of the ternary complexes in the presence and in the absence of IF-3 and studied in more detail the mechanism by which IF-3 exercises its action. This now allows us to propose a model for the formation of ternary complexes and to identify the steps where the IF-3 activity is expressed.

Experimental Section

Materials. [^{14}C]Phenylalanine (488 Ci/mol) was purchased from Amersham-Buchler and [^{3}H]methionine (8200 Ci/mol) was purchased from New England Nuclear Corp. The synthetic polyribonucleotides poly(U) and poly(A,U,G) were obtained from Boehringer-Mannheim and the manufacturer's specifications concerning their molecular weights were assumed to be correct. Unfractionated *Escherichia coli* MRE 600 tRNA was also purchased from Boehringer-Mannheim.

Buffers used included: (A) Tris-HCl (pH 7.1), 10 mM; $Mg(OAc)_2$, 8 mM; NH_4Cl, 40 mM; 2-mercaptoethanol, 3 mM; (B) Tris-HCl (pH 7.7), 10 mM; $Mg(OAc)_2$, 10 mM; NH_4Cl, 60 mM; 2-mercaptoethanol, 6 mM; (C) Tris-HCl (pH 7.5), 20 mM; $Mg(OAc)_2$, 5 mM; NH_4Cl, 100 mM; dithiothreitol, 1 mM; GTP, 1 mM.

The preparation of *E. coli* MRE 600 30S ribosomal subunits, aminoacyl-tRNAs, and initiation factor IF-3 has been described (Gualerzi et al., 1971; Pon and Gualerzi, 1974; Ri-

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[1] Abbreviations used are: tRNA, mRNA, and fMet-tRNA, transfer, messenger, and formylmethionine transfer ribonucleic acid; poly(U), poly(uridylic acid); poly(S_4U), poly(thiouridylic acid); *N*-AcPhe-tRNA, *N*-acetylphenylalanyl tRNA; poly(A,U,G), poly(adenylate, uridylate, guanylate).

suleo et al., 1976). The purification of initiation factors 1 and 2 will be described elsewhere.

Ternary complexes were formed by incubating 0.45 A_{260} unit of 1 M NH_4Cl-washed, heat-reactivated (50 °C, 5 min) 30S ribosomal subunits with or without a stoichiometric amount of IF-3 in 0.23 mL of buffer A containing variable amounts of *N*-Ac[^{14}C]Phe-tRNA and poly(U) as specified in the figure legends. The reaction was started by the addition of the 30S ribosomes (with or without IF-3) to the buffer containing *N*-AcPhe-tRNA and poly(U), and was allowed to proceed for 10 s at 10 °C. The reaction was then stopped by addition of 2.5 mL of ice-cold buffer B and the reaction mixture was immediately passed through Millipore filters (HA 0.45 μm). The total time required for this process was approximately 1 s. Ice-cold buffer was added because the IF-3 dependent dissociation of the complex at this temperature is negligible. The formation of the 30S initiation complex was followed in a similar manner with the exception that purified initiation factors IF-1 (0.25 μg) and IF-2 (1.0 μg) were included in a 0.075-mL reaction mixture in buffer C containing the amounts of random poly(A,U,G) and f[^{3}H]Met-tRNA as indicated in the figure legends. In addition, the amount of 30S subunits used was reduced to 0.15 A_{260} unit. Poly(thiouridylic acid) (poly(S_4U)) was obtained through the courtesy of Dr. K. H. Scheit (Göttingen) to whom we are also grateful for the irradiation (320 nm) of the 30S–poly(S_4U) complex and of the control 30S ribosomal subunits.

Results

Mechanism of 30S–poly(U)–N-Ac-Phe-tRNA Ternary Complex Formation. Although the 30S–*N*-AcPhe-tRNA–poly(U) ternary complex is different from the physiological initiation complex, it seems legitimate to assume (and the data of this paper will show that this assumption is largely justified) that the formation of these two complexes follows similar pathways. The formation of a ternary complex starting with 30S, poly(U), and *N*-AcPhe-tRNA can be regarded as analogous to an enzymatic reaction involving one enzyme (30 S) and two substrates (poly(U) and *N*-AcPhe-tRNA). In these reactions different pathways can lead to the formation of the ternary complex and the rate equations for the various mechanisms have been derived (Laedler and Socquet, 1950; Segal et al., 1952; Ingraham and Makower, 1954; Alberty, 1956; Fromm, 1975). In the case of the 30S initiation complex two different pathways have been proposed (Noll and Noll, 1974; Jay and Kaempfer, 1975; Benne et al., 1973; Vermeer et al., 1973). These two models are analogous to each other in that an ordered (obligatory) pathway has been postulated for each, but they differ for the order in which the mRNA and the initiator tRNA are added to the 30S subunit. The existence of an obligatory order of addition of the substrates, however, does not seem to be compatible with the finding that the 30S subunits show a measurable affinity for both aminoacyl-tRNA (Noll and Noll, 1974; Blumberg et al., 1974; Jay and Kaempfer, 1975) and mRNA (Szer and Leffler, 1974). Therefore, an alternative possibility could involve a random (nonobligatory) order of addition of the two substrates (aminoacyl-tRNA and polynucleotide) to the 30S subunit.

Since the discrimination between different reaction models is sometimes possible by means of initial rate kinetic analysis, we sought to obtain some mechanistic information concerning the mode of formation of the ternary complex using a kinetic approach. Thus, we ran Michaelis–Menten kinetic experiments varying independently the concentration of either one of the two substrates (aminoacyl-tRNA and polynucleotide) while keeping a fixed concentration of the other (Ingraham and Makower, 1954). In this connection the first problem we had to solve was the selection of the most appropriate conditions to run initial rate kinetic experiments. The formation of the ternary complex in the presence of IF-3 proceeds too fast at 37 °C and at room temperature to be analyzed by Millipore filtration. The experiment of Figure 1 shows, however, that if the incubation temperature is lowered to 10 °C, the reaction proceeds linearly for at least 15 s in the presence of IF-3 and for at least 30 s in the absence of the factor. Therefore, we decided to stop our reactions at 10 s since incubations for this length of time, while still linear, yielded enough counts in the absence of IF-3 to allow the direct comparison of the reaction with and without the factor. In addition, the high experimental error inherent to the shorter incubation times in the presence of IF-3 was avoided.

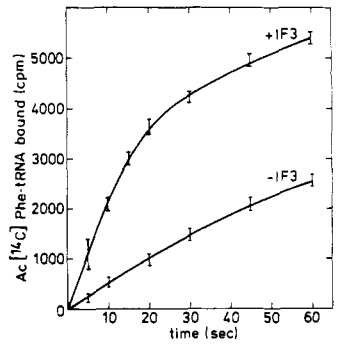

FIGURE 1: Time course of 30S–poly(U)–*N*-Ac[^{14}C]Phe-tRNA ternary complex formation in the presence and absence of IF-3. The ternary complex was formed as described in the Experimental Section. The reaction was started by the simultaneous addition of 46 pmol of *N*-Ac[^{14}C]Phe-tRNA and 5.6 pmol of poly(U) to 0.45 A_{260} unit of salt-washed, heat-reactivated 30S ribosomal subunits. All components were preequilibrated at 10 °C. The reaction was allowed to proceed for the indicated times at 10 °C and stopped by addition of 3 mL of ice-cold buffer B. The time required to stop the reaction and to complete the filtration never exceeded 1 s. Blank values obtained by determining the radioactivity remaining on the filters at 0 time when aminoacyl-tRNA and polynucleotide were added after the dilution with buffer B were subtracted from each point and three points for each time were measured.

In the next series of experiments (Figure 2) the initial rate of ternary complex formation in the presence of IF-3 was determined varying linearly the concentration of *N*-AcPhe-tRNA while keeping three fixed concentrations of poly(U). As seen in Figure 2a, three straight lines are obtained in a Lineweaver–Burk plot. Since the $1/v$ vs. $1/S$ plots all meet on the x axis and since the V_{max} increases as the concentration of the fixed substrate (poly(U)) is increased, the same K_m (K_A) and different V_{max} ($V_{max\,A} \rightarrow V_{max\,AP}$) are obtained. Similar (symmetrical) results are obtained when the concentration of poly(U) is varied for three fixed concentrations of *N*-AcPhe-tRNA with the three plots yielding a single K_m (K_P) and three V_{max} ($V_{max\,P} \rightarrow V_{max\,AP}$) (Figure 2b). These results, with the common intercept of the plots on the $1/S$ axis, and the influence of the concentration of one substrate on the V_{max} obtained by varying the concentration of the other represent the distinguishing kinetic features of a random or nonobligatory order of addition of the two substrates to two separate and independent sites (Bernhard, 1968). The rapid equilibrium rate equation for this mechanism is presented in the legend of Figure 7.

The internal consistence of our kinetic data and their agreement with the random model can be further verified by looking at the replot of $1/S$ vs. the intercepts of the primary plots as presented in Figure 2c. As expected, two straight lines

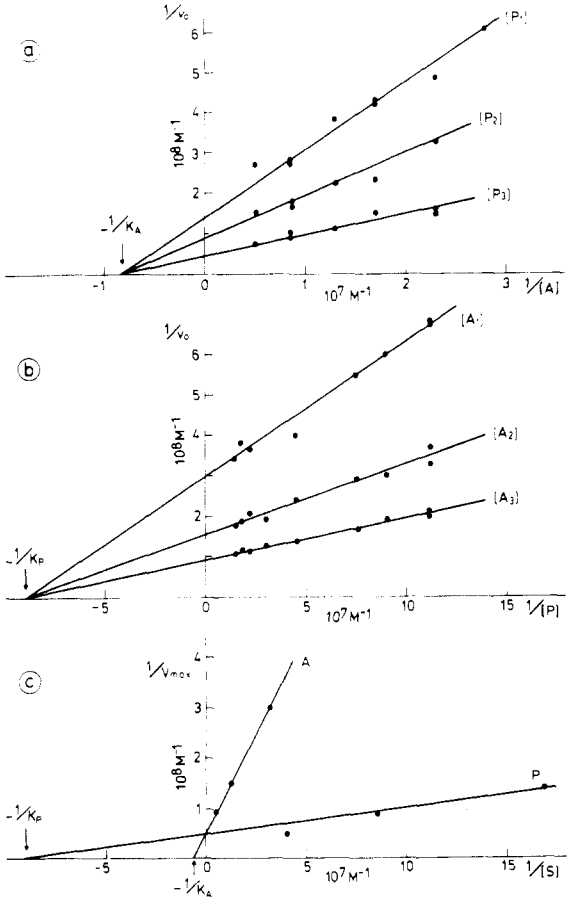

FIGURE 2: Kinetic analysis of the formation of 30S-poly(U)-*N*-AcPhe-tRNA ternary complex in the presence of IF-3. The determination of the initial velocity of ternary complex formation was performed as described in the Experimental Section and Figure 1. (a) Double reciprocal (Lineweaver-Burk) plot of $1/v_0$ vs. $1/[A]$ (A = *N*-Ac[^{14}C]Phe-tRNA) at three different poly(U) concentrations: $[P_1] = 0.59 \times 10^{-8}$ M, $[P_2] = 1.17 \times 10^{-8}$ M, $[P_3] = 2.46 \times 10^{-8}$ M. (b) $1/v_0$ vs. $1/[P]$ (P = poly(U)) at three different *N*-Ac[^{14}C]Phe-tRNA concentrations: $[A_1] = 0.31 \times 10^{-7}$ M, $[A_2] = 0.80 \times 10^{-7}$ M, $[A_3] = 2.0 \times 10^{-7}$ M. (c) Secondary plot of the data of a and b.

are obtained yielding the same V_{max} ($V_{max\ AP}$) and two K_m's (K_A and K_P) identical with those obtained in the primary plots. As seen from the legend of Figure 7, the $V_{max\ AP}$ is the maximum limiting rate when both substrates are present in saturating amounts while the K_m values obtained for poly(U) and *N*-AcPhe-tRNA represent the dissociation constants (K_P and K_A) of the two intermediate binary complexes, 30S-poly(U) and 30S-*N*-AcPhe-tRNA, respectively. These dissociation constants, in turn, are identical with the two dissociation constants of the pre-ternary complex (K_P^A and K_A^P).

In conclusion, the above kinetic data are inconsistent with an obligatory order of addition where the binding of a given substrate must precede the binding of the other. On the contrary, the data are consistent with the idea that, in the presence of IF-3, the 30S-poly(U)-*N*-AcPhe-tRNA complex is formed by the random addition of poly(U) and *N*-AcPhe-tRNA to separate and independent binding sites to form a pre-ternary complex. All these steps equilibrate rapidly with respect to the transformation of the pre-ternary complex into a stable ternary complex. In this transformation, which represents the rate-limiting step of the reaction, the 30S ribosomal subunit probably undergoes a structural rearrangement which brings the tRNA and the polynucleotide into mutual contact through the establishment of a codon-anticodon interaction.

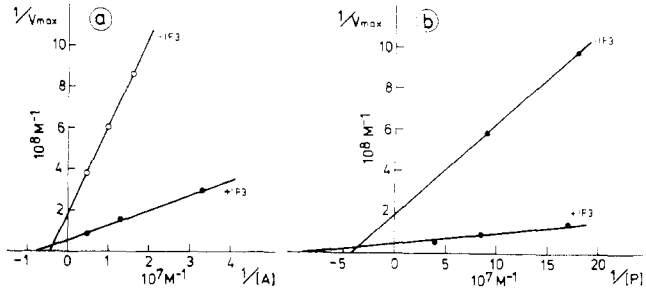

FIGURE 3: Effect of IF-3 on the kinetics of ternary complex formation. These are replots taken from the primary plots of Figure 2 (+IF-3) and from primary plots not shown (−IF-3).

Effect of IF-3 on Ternary Complex Formation. In order to determine whether or not the presence of IF-3 modifies the mechanism by which the ternary complex is formed and to identify the step(s) at which IF-3 exercises its known stimulation of ternary complex formation, the experiments of Figure 2 were repeated in the absence of the factor. The results obtained in these experiments (not shown) were qualitatively identical with those of Figure 2 thus indicating that, in the absence of IF-3, the basic mechanism of 30S-poly(U)-*N*-AcPhe-tRNA ternary complex formation remains unchanged, although all kinetic parameters were otherwise modified. Thus, the dissociation constants for both intermediate binary complexes 30S-*N*-Ac-Phe-tRNA (K_A) and 30S-poly(U) (K_P) are approximately twofold higher while the limiting maximum velocity $V_{max\ AP}$ is approximately four times lower in the absence of IF-3.

The calculated dissociation constants K_A and K_P in the absence of IF-3 are 2.0×10^{-7} and 0.25×10^{-7} M, respectively. In the presence of IF-3 K_A and K_P become 1.2×10^{-7} and 0.11×10^{-7} M, respectively (Figure 3).

Mechanism of 30S Initiation Complex Formation. As mentioned above the 30S-poly(U)-*N*-AcPhe-tRNA ternary complex differs from the physiological 30S initiation complex. In fact, (a) a synthetic polynucleotide containing no initiation triplet and an analogue of the initiator tRNA are used in place of the natural mRNA and of the fMet-tRNA, respectively, (b) a higher Mg^{2+} concentration is used, and (c) initiation factors IF-1 and IF-2 as well as GTP are not included in the reaction mixture. In the following experiments, all conditions commonly regarded as physiological were used with the only exceptions of the temperature (10 °C) and the substitution of random poly(A,U,G) in place of the natural mRNA, the use of which would be difficult in this kind of experiment since the concentration of initiation triplets in natural mRNA is less than 0.01 of random poly(A,U,G). It should be noted, however, that the ribosomal binding sites for natural mRNA and synthetic polynucleotides must be largely equivalent since synthetic polynucleotides compete with natural mRNA for binding to ribosomes (Okuyama and Tanaka, 1973).

Preliminary experiments on the time course of formation of this initiation complex had shown that this reaction was also linear for at least 15 s at 10 °C. Figure 4 shows a kinetic analysis of the formation of such an initiation complex. Since the results are qualitatively identical with those of Figure 2, for the same reasons given above, it can be concluded that the initiation complex is also formed through a random pathway.

Further Evidence for a Random Mechanism. It has been pointed out recently that it is possible to obtain kinetic data which are characteristic of a random rapid equilibrium substrate addition mechanism which in reality reflect an ordered

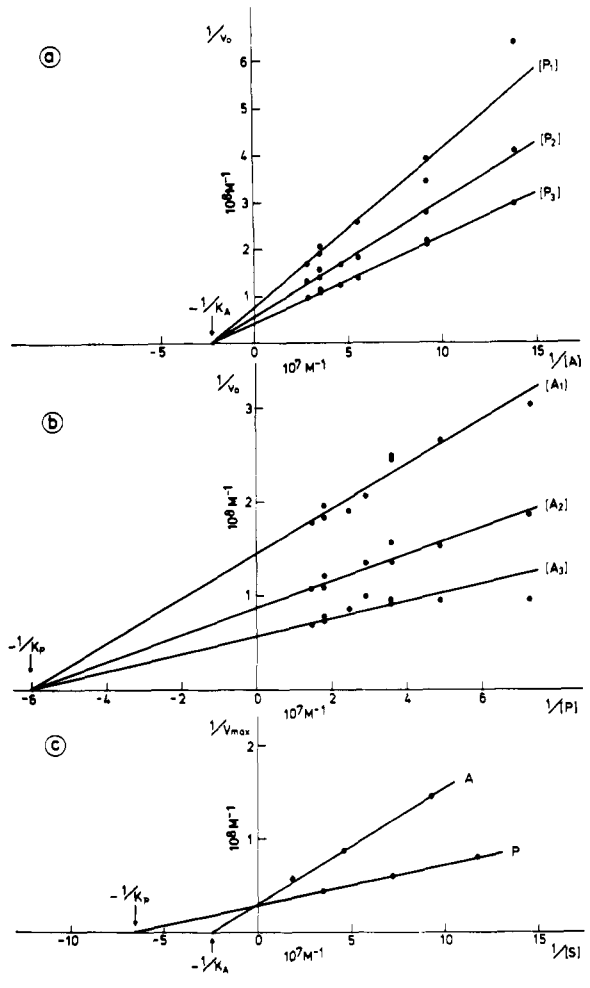

FIGURE 4: Kinetic analysis of the formation of the 30S-poly(A,U,G)-f[^{3}H]Met-tRNA ternary complex in the presence of initiation factors and GTP. The determination of the initial velocity of ternary complex formation was performed as described in the Experimental Section. The reaction was started by addition of 30S-IF-1-IF-3 to incubation mixtures containing IF-2, GTP, and poly(A,U,G) and f[^{3}H]Met-tRNA in variable amounts as specified. (a) Double reciprocal (Lineweaver-Burk) plot of $1/v_0$ vs. $1/[A]$ (A = f[^{3}H]Met-tRNA) at three different poly(A,U,G) concentrations: $[P_1] = 0.85 \times 10^{-8}$ M, $[P_2] = 1.39 \times 10^{-8}$ M, $[P_3] = 2.82 \times 10^{-8}$ M. (b) $1/v_0$ vs. $1/[P]$ (P = poly(A,U,G)) at three different f[^{3}H]Met-tRNA concentrations: $[A_1] = 1.08 \times 10^{-8}$ M, $[A_2] = 2.16 \times 10^{-8}$ M, $[A_3] = 5.4 \times 10^{-8}$ M. (c) Secondary plots of the data of a and b. The calculated dissociation constants K_A and K_P are 0.43×10^{-7} and 0.16×10^{-7} M, respectively.

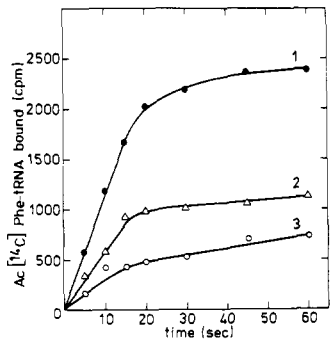

FIGURE 5: Determining the order of substrate addition in ternary complex formation. Pulse-chase experiment with *N*-AcPhe-tRNA. The ternary complex was formed as described in the Experimental Section and in the legend of Figure 1 with the exception that the 30S-IF-3 complex was preincubated at 10 °C for 10 min with 46 pmol of *N*-Ac[^{14}C]Phe-tRNA (curve 1), 46 pmol of *N*-Ac[^{14}C]Phe-tRNA, and 90 pmol of nonradioactive *N*-AcPhe-tRNA (curve 2) or 90 pmol of nonradioactive *N*-AcPhe-tRNA (curve 3). All reactions were started by addition of 6 pmol of poly(U) and (curve 1) 90 pmol of nonradioactive *N*-AcPhe-tRNA; (curve 2) no further addition; (curve 3) 46 pmol of *N*-Ac[^{14}C]Phe-tRNA.

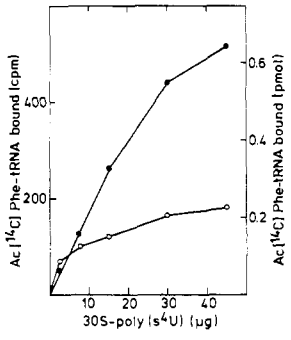

FIGURE 6: Determining the order of substrate addition. Formation of ternary complex starting with 30S-poly(S_4U) covalently bound complexes. 30S-poly(S_4U) covalent binary complexes were purified through sucrose density gradient centrifugation and incubated for 5 min at 37 °C in the presence and in the absence of IF-3 (12 μg for 350 μg of binary complex). After incubation aliquots of the binary complex (with or without IF-3) were incubated for 15 min at 37 °C with 90 pmol of *N*-Ac[^{14}C]-Phe-tRNA in 0.23 mL of buffer A as indicated in the abscissa: (●) binary complexes with IF-3; (○) binary complexes without IF-3.

binding of substrates. This occurs in the special situation where the obligatory binding is in rapid equilibrium and where the additional formation of a nonproductive complex between the enzyme and the second substrate takes place (Frieden, 1976).

To rule out this possibility, however, it would be sufficient to show that both substrates can productively be used as first as well as second substrate in the formation of the ternary complex. The two following experiments were designed to provide such evidence. In the first experiment (Figure 5) the 30S ribosomal subunits were mixed with IF-3 and with labeled *N*-AcPhe-tRNA (curve 1), unlabeled *N*-AcPhe-tRNA (curve 3), or a mixture of labeled and unlabeled *N*-AcPhe-tRNA (curve 2). After incubation for 10 min at 10 °C, the formation of the ternary complex was started by the addition of poly(U) and unlabeled *N*-Ac-Phe-tRNA, poly(U) and labeled *N*-AcPhe-tRNA, or poly(U) alone, respectively. The time course of ternary complex formation of Figure 5 shows that the amount of radioactivity associated with the ternary complex is much higher when the 30S ribosomal subunits are allowed to preequilibrate with the radioactive aminoacyl-tRNA (curve 1) than when they are preequilibrated with nonradioactive aminoacyl-tRNA (curve 3). When the ribosomes are preincubated with a mixture of labeled and unlabeled aminoacyl-tRNA, on the other hand, an intermediate amount of radioactivity is found in the ternary complex (curve 2). This experiment clearly shows that the 30S *N*-AcPhe-tRNA binary complex can be the starting point for the formation of the ternary complex (i.e., the *N*-AcPhe-tRNA can be the first binding substrate).

In the following experiment, the 30S ribosomal subunits were preincubated with poly(thiouridylic acid) (poly (S_4U)) and then irradiated at 330 nm to promote the covalent binding of the template to the ribosome (Frischauf and Scheit, 1973). The binary complex so obtained was then purified by sucrose gradient centrifugation to remove unbound poly(S_4U) and finally incubated with radioactive *N*-AcPhe-tRNA in the presence and absence of IF-3 to form a ternary complex. The results of Figure 6 show that both in the presence and absence of IF-3 the 30S-poly(S_4U) binary complex can be the starting

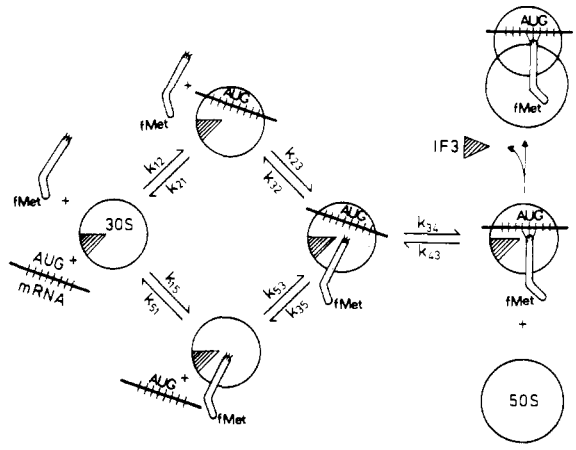

FIGURE 7: Schematic representation of the proposed mechanism for the formation of the 30S and 70S initiation complexes. The rapid equilibrium rate equation and the kinetic laws for a random mechanism of ternary complex formation with substrates having separate and independent binding sites are given: $K_A = k_{21}/k_{12}$; $K_A{}^P = k_{35}/k_{53}$; $K_A = K_A{}^P$; $K_P = k_{51}/k_{15}$; $K_P{}^A = k_{32}/k_{23}$; $K_P = K_P{}^A$; $K_AK_P{}^A = K_PK_A{}^P$, $v = k_{34}R_0/\{[1 + (K_A/[A])][1 + (K_P/[P])]\}$; $V_{max\ A} = k_{34}R_0/[1 + (K_P/[P])]$; $V_{max\ P} = k_{34}R_0/[1 + (K_A/[A])]$; $V_{max\ AP} = k_{34}R_0$, where A = aminoacyl-tRNA, P = polynucleotide, and R_0 = total concentration of 30S.

point for the formation of the ternary complex (i.e., the polynucleotide can be the first binding substrate). Furthermore, the results show that the formation of the ternary complex is stimulated several-fold by IF-3 in spite of the fact that the template is already ribosome bound. This finding strongly implies that the ribosomal binding of the mRNA per se is not the main step at which IF-3 exercises its stimulatory activity (cf. also Discussion).

Discussion

In the present study the formation of a 30S-poly(U)-*N*-AcPhe-tRNA ternary complex has been used as a model system to investigate the mechanism which leads to the formation of the physiological 30S initiation complex as well as the mechanism of action of initiation factor IF-3.

The formation of the 30S initiation complex has so far been thought to proceed through an obligatory pathway in which the binding of the mRNA either precedes (Benne et al., 1973; Vermeer et al., 1973) or follows (Noll and Noll, 1974; Jay and Kaempfer, 1975) the binding of the fMet-tRNA. The kinetic data for the model system presented in this paper, however, are inconsistent with an obligatory pathway and suggest that the formation of the ternary complex proceeds through a random (nonobligatory) pathway in which the ribosomal binding of each component (aminoacyl-tRNA or template) is independent of the other and each component can freely and reversibly associate with the 30S ribosomes so that four equilibria are rapidly established (cf. Figure 7). The rate-limiting step in the formation of the ternary complex is the transformation of an intermediate into a stable ternary complex. While the exact nature of this transformation is probably very complicated we would like to propose that this transformation basically entails a rearrangement of the ribosomal structure which brings the aminoacyl-tRNA and the template into mutual contact through the establishment of a codon-anticodon base pairing. Although these conclusions were drawn mainly from our 30S-poly(U)-*N*-AcPhe-tRNA model system there is no a priori reason to believe that they cannot be extrapolated to the physiological situation since they have also been proven valid under conditions closely resembling the physiological ones (see Figure 4).

A scheme for the formation of the 30S initiation complex which summarizes our data is presented in Figure 7. The existence of two possible pathways for the formation of the 30S initiation complex with the initiator tRNA being either the first or the second binding substrate does not necessarily mean that both routes are taken in vivo. This is certainly the situation when both fMet-tRNA (and IF-2) and mRNA are present in saturating amounts. When the concentration of one of the two substrates is drastically reduced the other substrate present in excess will be bound first. It is possible that this kind of situation might provide a regulatory device based simply on the levels of fMet-tRNA, initiation factors, and concentration and nature of specific mRNAs.

As for the specific effect of IF-3, the data of this paper have shown that, in the presence of the factor, the basic mechanism leading to the formation of the ternary complex is not modified, but the dissociation constants of both 30S-poly(U) and 30S-*N*-AcPhe-tRNA binary complexes appear to be approximately two times lower probably as a result of an unequal increase in the forward and back rates of formation of these complexes, as will be shown elsewhere. Most important of all, the limiting maximum velocity for the formation of the ternary complex which reflects the rate constant of the rate-limiting step is increased approximately four times at 10 °C in the presence of the factor. These data, as well as the results of Figure 6 (which show that the formation of a ternary complex is stimulated several-fold by IF-3 even though the template is already ribosome bound), seem to cast serious doubts on the validity of the belief that the primary activity of IF-3 is to promote the interaction between the mRNA and the ribosomes. In fact, while the 30S ribosomal subunit alone seems to contain enough information for the recognition of the initiation site of the mRNA (Shine and Dalgarno, 1974; Shine and Dalgarno, 1975; Steitz and Jakes, 1975) and the 30S-mRNA interaction has also been observed in the absence of the factor (Szer and Leffler, 1974), IF-3 appears to stimulate a wide range of reactions including the net dissociation of 70S ribosomes (Noll and Noll, 1971; Kaempfer, 1972; Godefroy-Colburn et al., 1975; Gottlieb et al., 1975), the formation of 30S ternary complexes with both natural (Iwasaki et al., 1968; Revel et al., 1968; Noll and Noll, 1974; Jay and Kaempfer, 1975) and synthetic mRNAs (Suttle et al., 1973; Bernal et al., 1974; Dondon et al., 1974), the destabilization of these preformed complexes (Gualerzi et al., 1971; Dubnoff et al., 1972; Pon and Gualerzi, 1974; Risuleo et al., 1976), and the translation of natural mRNAs as well as synthetic polynucleotides such as poly(U) and poly(A) (Wahba et al., 1969; Schiff et al., 1974). Thus, the function of IF-3 appears to be much less specific than originally thought and probably can be ascribed to a general effect of IF-3 on the 30S subunit (conformational change) as previously reported (Pon and Gualerzi, 1974; Gualerzi et al., 1975; Ewald et al., 1976; Michalski et al., 1976). The finding that all kinetic parameters of the 30S ribosomal subunit are modified by IF-3 appears to further support this point of view. In addition, in a multistep reaction such as protein synthesis, which involves several enzyme-substrate type intermediates, the effect of modification of the rate constants at each step is additive (Knorre and Malyguine, 1972) and will ultimately determine the probability for a given product (a protein in this case) to be formed.

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