KINETIC STUDIES OF THE RATES AND MECHANISM OF ASSEMBLY OF THE PROTEIN SYNTHESIS INITIATION COMPLEX

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ABSTRACT Rate constants for a number of the assembly reactions involved in forming *Escherichia coli* ribosome initiation complexes have been measured. These reactions were monitored in a stopped-flow device in which Rayleigh scattering and fluorescence anisotropy were followed as a function of time. Fluorescence was induced by laser excitation modulated at 50 kHz. Aminoacyl-tRNA, initiation factor 3 (IF3), and 70S ribosomes were labeled with fluorescent probes. The light-scattering and fluorescence data show that the antiassociation model for IF3 function cannot be correct. IF3 can be considered to act as an effector in an allosteric model for ribosome function. Fluorescence anisotropy stopped-flow experiments provided rate constants for the binding of IF3 to both 30S subunits and to the intact 70S ribosome. Aminoacyl-tRNA's and nucleotide triplets appear to bind rapidly to 70S ribosomes and then a slow first-order conformational change occurs.

INTRODUCTION

The initiation of protein synthesis in prokaryotes is a complex process involving a number of components: mRNA[1], tRNA, ribosomes, GTP, initiation factors, and various ions (Mg^{2+}, NH_4^+, etc.). Many of the individual components of this system have been well characterized. Of the many complexes and intermediates which might result from interaction among these components, only a small number have so far been detected by ultracentrifugation, electrophoresis, and filtration. For even these few complexes, quantitative equilibrium and kinetic data are largely lacking. The above techniques isolate complexes which are present in quasi-equilibrium states, but such aggregates may be nonproductive complexes rather than intermediates on the reaction path. Protein synthesis in vivo is a rapid process and the detection of transient intermediates is necessary for the elucidation of the kinetic control of this process.

In a system of N interacting components, there are N^2 possible binary complexes, N^3 ternary complexes, etc. We have begun a quantitative study of protein synthesis by exploring properties of a limited number of the essential components and their binary interactions. The components studied include: 70S ribosomes, 30S and 50S ribosomal subunits, initiation factor 3 (IF3), tRNA, and mRNA. Determination of the mechanism of assembly of the protein

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; Dansyl, 1,5-dimethylaminonaphthalene sulfonyl-chloride; GTP, guanosine triphosphate; IAEDANS, *N*(iodoacetylaminoethyl)-5-naphthyl amino-1-sulfonic acid; IF3, initiation factor 3; Ile, isoleucine; mRNA, messenger ribonucleic acid; η, solvent viscosity; Phe, phenylalanine; τ, fluorescence lifetime; tRNA, transfer ribonucleic acid.

synthesis initiation complex requires a knowledge of the rate constants for the formation and dissociation of the various precursor complexes.

One of the primary binary reactions is the association of 30S and 50S subunits to form 70S ribosomal particles. The fact that changes in Mg^{2+} concentration can affect this process has been well established and recently a variety of physical techniques have been used to study the detailed kinetics and equilibria of this reaction. These techniques include: temperature jump relaxation methods (Chaires et al., 1977, Chaires et al., 1979), light-scattering stopped-flow (Wishnia et al., 1973, Godefroy-Colburn et al., 1975, Görisch et al., 1976), absorbance stopped-flow (Wolfe et al., 1973), static light-scattering (Zitomer and Flaks, 1972), and ultracentrifugation (Noll and Noll, 1976).

Equilibrium and kinetic studies have been hindered by complications arising from the structural heterogeneity of ribosomes. Subunits can combine to form two types of 70S ribosomes. These two types, A and B, or "tight" and "loose" couples, can be discriminated by their subunit association curves in the presence of Mg^{2+}. At 1 mM Mg^{2+}, both types of ribosomes are dissociated into subunits. Tight couples show a midpoint in the Mg^{2+} titration curve at about 2.2 mM Mg^{2+} and are completely associated at 8 mM Mg^{2+} (Debey et al., 1975). In contrast, the midpoint of the titration curve for loose couples is about 7–8 mM Mg^{2+} and complete association occurs at 18 mM Mg^{2+}. These two types of ribosomes have very different association-dissociation rates (Görisch et al., 1976). With special precautions, a homogeneous preparation of tight ribosomes can be obtained. Even when they are stored at −80°C, we find that within 10 d the association kinetics of tight couples will change to resemble a mixture of tight and loose couples. Chaires and Kegeles (1977) have found similar results from centrifugation data.

The association reaction for 30S and 50S subunits from *Escherichia coli* MRE 600 type A ribosomes is extremely fast, having a rate constant of 1.7×10^7 $M^{-1}s^{-1}$. The process is surprisingly complex. Chaires et al., (1977) have demonstrated from relaxation kinetics the existence of a transient 70S intermediate form of the intact ribosome. Recently we have proposed (Goss et al., 1980) an allosteric type model to account for a variety of kinetic data. We have examined and report here the effects of IF3 on the subunit association reaction, the rate of IF3 binding to ribosomes, the binding of aminoacyl-tRNA to ribosomes, and the kinetics of mRNA (nucleotide triplets) binding to ribosomes.

MATERIALS AND METHODS

Ribosomes and initiation factor 3 (IF3) were prepared as previously reported (Wahba and Miller, 1974; Schiff et al., 1974). All kinetic measurements were made within 72 h of isolation of the ribosomes. Kinetic experiments were performed in buffer A consisting of 0.01 M Tris, pH 7.8, 50 mM KCl, 6 mM β mercaptoethanol and $MgCl_2$ as indicated. The light-scattering stopped-flow apparatus has been previously described (Görisch et al., 1976). Fluorescence intensity and anisotropy changes were monitored in the light scattering stopped-flow device. A schematic diagram of the apparatus is shown in Fig. 1. The excitation is either by a He-Cd laser (Model 4110 Huv, Liconix, Mountain View, Calif.) operating at 325 nm or an Ar^+ laser (Lexel Model 75) with an output at 488 nm. The He-Cd laser excitation beam is passed through a half-wave plate (ESCO Products, Oak Ridge, N. J.), a Glan-Taylor polarizer (DKB Crystal Optics, Chicago, Ill.) and a photoelastic modulator (Morvue Electronics Systems, Tigard, Oregon) before entering the stopped-flow cuvet. The emission beam passes through a sodium nitrite liquid filter, a short wavelength cutoff filter ($\lambda < 520$ nm, Ditric Optics, Inc., Hudson, Mass.), and is then detected by the photomultiplier (9601B, EMI, Plainview, N. Y.).

For mutually perpendicular excitation and emission beams, and excitation polarization electric vector, $I_\parallel$ is the intensity of fluorescence with polarization parallel to the excitation polarization and $I_\perp$ is the fluorescence intensity with polarization perpendicular to the excitation polarization vector. The total

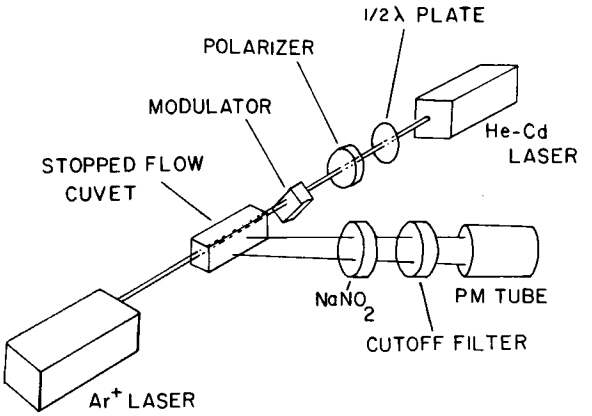

Figure 1 Schematic diagram of the apparatus. The Ar^+ laser was used for light scattering and the He-Cd laser was used for fluorescence intensity and anisotropy measurements.

fluorescence is defined as $I_F = I_\parallel + 2I_\perp$, and fluorescence anisotropy as $r = (I_\parallel - I_\perp)/I_F$. Fluorescence polarization, P, is related to the anisotropy by: $P = 3r/(2 + r)$. The photoelastic modulator operates at a frequency of 50 kHz and a depth of modulation appropriate for the excitation wavelength and instrumental geometry. Static polarization measurements were made in the stopped-flow apparatus using the method of Wampler and DeSa (1974). For our geometry and $\delta_0 = 1.22\pi$ however, their (Wampler and DeSa, 1974; Eq. 9) $V_m/V_0 = (4 - r + 3rC)/(4 + 2r)$. All polarizations and anisotropies were corrected for a small "grating" factor (Chen and Bowman, 1965). For kinetic measurements, the photomultiplier signal passed through a preamplifier and then through a demodulator (Giblin, 1978) from which were obtained signals proportional to I_F and r. The demodulator (I_F and r measurements) and photomultiplier (for light scattering) outputs were processed directly by an on-line Nova 2 minicomputer with 32 K of memory. The Perrin equation (Perrin, 1926),

$$(1/P - 1/3) = (1/P_0 - 1/3)[1 + RT\tau/(\eta V)], \quad (1)$$

was used to calculate expected values of P for IF3 and ribosomal complexes based on data for labeled BSA collected in our instrument, a value of P_0 for Dansyl = 0.376 (Chen, 1967), and molar volumes (V) of equivalent spheres calculated from molecular weights of the various complexes.

Labeling of IF3 with Dansyl

IF3 was fluorescently labeled by reacting with Dansyl chloride. 25 μl of 1.2 mM Dansyl chloride in 100% ethanol were added to 0.15 ml 70 μM IF3 in the following buffer: 0.05 M Tris, pH 7.5, 0.05 M KCl, 0.5M NH_4Cl, and 5% glycerol. After 15 min incubation at 4°C, the Dansyl-IF3 solution was passed over a Sephadex G-10 column (20 × 0.9 cm, plastic), 20°C, equilibrated with the above buffer. Two fluorescent bands were obtained from the column. The first band, labeled IF3, was collected, concentrated with an Amicon Model 3 concentrator with a PM-10 membrane, (Amicon Corp., Lexington, Mass.), and used for further experiments. The molar ratio of dye to IF3 determined from absorbance measurements was 0.87–1.05.

Labeling of tRNA

Transfer RNA (10–100 o.d. units) was aminoacylated with the desired amino acid as described previously (Goss and Parkhurst, 1974). The aminoacyl tRNA and deacyl tRNA were collected by precipitation with ethanol (2.5 vol) and centrifugation at 6,000 × g. The tRNA was dissolved in 2 ml of 0.05 M Tris buffer, pH 7.5. A fourfold excess of Dansyl over aminoacyl tRNA was added in 0.1 ml 100% ethanol. The tRNA was precipitated and collected by centrifugation as described above. The precipitate was dissolved in 0.05 M acetate buffer, pH 4.5, and then reprecipitated with ethanol. Several such cycles resulted in a supernate which had no detectable fluorescence and a fluorescently labeled tRNA precipitate. Previous studies (Goss and Parkhurst, 1978) indicate that under these conditions the amino acid NH_2 group of the aminoacyl tRNA is the labeling site.

RESULTS AND DISCUSSION

Effects of IF3 on Subunit Association

IF3 is a small protein (~22,000 daltons, Dondon et al., 1974, Schiff et al., 1974) found in loose association with the 30S subunits, from which it can be separated by washing with a high concentration of salt (1 M NH_4Cl). It is widely believed that an important function of IF3 is to act as an antiassociation factor (Sabol et al., 1973; Godefroy-Colburn et al., 1975; Gottlieb et al., 1975; and Grunberg-Manago and Gros, 1977) preventing combination of 30S and 50S subunits to form 70S particles. The antiassociation model is:

$$30S + IF3 \rightleftharpoons 30S\text{–}IF3$$

$$30S + 50S \rightleftharpoons 70S$$

Chaires et al., (1979) have concluded from relaxation amplitude data that the 30S-IF3 complex either reacts with 50S subunits or serves as a reactive pool of 30S particles. We have recently shown (Goss et al., 1979) that (*a*) the binding of IF3 at both low and high Mg^{2+} is slow, (*b*) the ribosomal subunit association kinetics in the presence of IF3 are second order, and (*c*) the Mg^{2+} induced subunit association reaction in the presence of IF3 goes to completion (i.e., the light scattering amplitudes of the association curves with and without IF3 are identical). These findings are inconsistent with the antiassociation model. We conclude from these and other data that the simplest model to accommodate all of our results is an allosteric type model as follows:

$$\begin{array}{ccccc} & & 6 & 5 & \\ P & \rightleftharpoons & PX_j & \rightleftharpoons & P^*X_n \\ \downarrow\uparrow & 7 & \Vert & 4 & \uparrow \quad 3 \\ S_0 & \rightleftharpoons & SX_j & \rightleftharpoons & S^*X_n \\ & & 1 & 2 & \end{array}$$

The notation suppresses free ligand (X) and the fact that there are two nonequivalent subunits. S denotes the nonidentical subunits (30S and 50S) that associate in a second-order step to give 70S particles (P). S_0 represents subunits which have only minimal ligand (Mg^{2+}) and do not react appreciably to form 70S particles. The rapidly reacting subunits observed at high Mg^{2+} are represented by S^* whereas SX_j denotes the slowly reacting form. The subscript j denotes an undetermined number of ligands, X, bound per subunit where $j < n$. We suggest the effect of IF3 is to shift the $S \rightarrow S^*$ equilibrium rather than to prevent association. This model is undoubtedly too simple to explain all details of the complex ribosomal kinetics, but appears useful for organizing a large amount of the existing kinetic data. The data could be correlated as follows: the Mg^{2+} jump association kinetics would follow the path through steps $1 \rightarrow 2 \rightarrow 3$. In the presence of IF3 and various amines the predominant steps would be $1 \rightarrow 4 \rightarrow 5$. Dissociation kinetics (Mg^{2+} drop) would follow steps $5 \rightarrow 6 \rightarrow 7$ and/or $5 \rightarrow 4 \rightarrow 1$.

The rate of the association of ribosomal subunits decreased fivefold when the subunits were incubated with IF3. This suggested that an indirect measure of the rate of IF3 binding to subunits could be obtained by measuring changes in the subunit association reaction with variations in IF3 concentration or incubation time. The IF3 binding reaction was much slower than the rate of subunit association, allowing us to measure the IF3 binding rate constant in a time-lapsed stopped-flow experiment. The experiment was performed as follows: Ribosomal

subunits, 1 mM Mg^{2+}, were mixed with IF3 (final concentrations: subunits, 0.066 μM (each); IF3, 0.67 μM), at time zero. Various known times were allowed to elapse (2.5, 4, and 7 min), then the subunits were flowed against high Mg^{2+} and the resulting association reaction monitored (see Fig. 2). The resulting curves were biphasic and could be fit using two rate constants and varying only the fraction of rapidly-reacting material present at the beginning of each elapsed time. The fast and slow rate constants used corresponded, respectively, to that obtained in the absence of IF3 and to that obtained after 40 min incubation with IF3. The fraction of rapidly reacting material was assumed to correlate with the fraction of 30S particles without IF3. A plot of the concentration of 30S ribosomal subunits unreacted with IF3 at the beginning of each elapsed time interval versus elapsed time gave a rate constant of 0.6×10^4 $M^{-1}s^{-1}$ for IF3 binding. This rate constant is $<1/1000^{th}$ that for the 30S + 50S association reaction, allowing us to neglect, to a first approximation, binding of IF3 during the subunit association reaction at high Mg^{2+}.

To study directly the binding of IF3 to ribosomes, we labeled IF3 with various fluorescent dyes and monitored fluorescence anisotropy and intensity changes when the labeled IF3 reacted with ribosomes. Fig. 3 shows a plot of the time dependence of the anisotropy change associated with the combination of labeled IF3 with 30S + 50S subunits at 1 mM Mg^{2+}. The 30S subunits have a molecular weight of 0.90×10^6; the 50S, 1.55×10^6 (Hill et al., 1969).

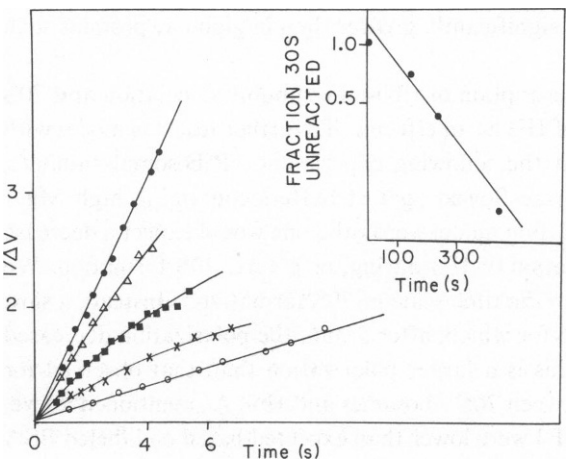

Figure 2

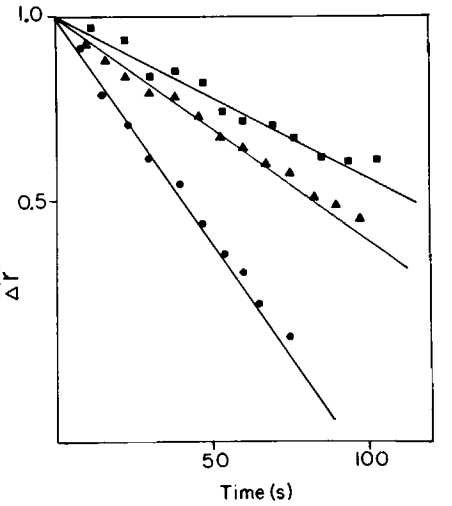

Figure 3

Figure 2 Time-lapse stopped-flow data. The lower portion of the figure represents the reaction of ribosomal subunits to form 70S ribosomes. The solid circles (●) depict the reaction of subunits (0.033 μM) to form 70S particles in the absence of IF3. The three intermediate curves (△, ■, and X) represent, respectively, the association reaction after 2.5, 4, and 7 min elapsed time after addition of IF3 (0.67 μM). The open circles depict the association reaction after 40 min elapsed time. In all experiments, the association reaction was induced by a rapid jump in (Mg^{2+}) from 1–18 mM. The inset shows a semilogarithmic plot of the fraction of 30S subunits unreacted with IF3 vs. time. The unreacted ribosome concentrations were calculated from the time-lapse stopped-flow data.

Figure 3 Normalized anisotropy change for the reaction of fluorescently labeled IF3 with ribosomal subunits. For a first order reaction, the change in anisotropy, Δr, (for negligible change in fluorescence intensity) should obey single exponential decay. The semilogarithmic plot of Δr versus time is linear for the three ribosome concentrations shown. The solid circles (●) represent the reaction of 1 μM ribosomes with 0.033 μM IF3. The triangles (▲) and squares (■) depict, respectively, concentrations that are 0.5 and 0.33 μM in ribosomal subunits.

One would therefore expect a large anisotropy change upon binding of labeled IF3 to the subunits. Ribosomes (0.33–1.0μM) were in large excess (IF3, 0.01 μM) and the reaction appeared pseudo-first order. We assume the binding was to the 30S subunit since this subunit preferentially binds IF3 (Sabol et al., 1970; Sabol and Ochoa, 1971; Thibault et al., 1972). The reactions were monophasic, giving no evidence for more than one type of binding site. The rate constant obtained from this measurement is 1.7×10^4 $M^{-1}s^{-1}$, in reasonable agreement with the indirect value obtained from analysis of the time-lapse experiments.

Further support for the assignment of this anisotropy change to the IF3 binding reaction is provided by several control experiments. Both labeled and unlabeled IF3 retard the subunit association reaction by a factor of five. Several freeze-thaw cycles inactivated IF3 so that it did not alter the subunit association reaction. Inactive labeled IF3 neither modified the subunit association rate nor showed a time dependent change in anisotropy when flowed against ribosomes in 1 mM Mg^{2+}.

The polarizations of labeled IF3, and of the 30S-IF3 and 70S-IF3 complexes were all less than expected, from Eq. 1, based on polarizations for Dansyl-labeled BSA and the molecular weights of IF3, 30S-IF3, and 70S-IF3. At low Mg^{2+}, there does not appear to be significant free labeled IF3 in equilibrium with 30S ribosomes since apparent IF3 binding rates increase linearly with ribosome concentration and the final P values do not increase with a doubling of the ribosome concentrations. Both results are expected for a K_{eq} at low Mg^{2+} of ~0.05 μM (Godefroy-Colburn et al., 1975)[2]. The low polarizations may derive from labeled IF3 which is inactive in binding to ribosomes, or from rotational mobility of the probe on the IF3 both free and in the IF3-ribosome complexes that is significantly greater than in globular proteins such as BSA.

The above allosteric model affords a description of ribosome subunit association and 70S particle dissociation kinetics and the role of IF3 as an effector. To further test this model with regard to the role of IF3, we carried out the following experiments: Ribosomal subunits, preincubated with IF3 at 1 mM Mg^{2+}, were flowed against buffers containing high Mg^{2+} (final Mg^{2+}, 8 or 18 mM). If the antiassociation model were valid one would expect a decrease in anisotropy corresponding to the dissociation of IF3 during, or before, 70S formation. We did not observe a decrease in anisotropy, on the time scale of 70S formation.[3] Instead, a slow first-order relaxation process was observed for which, after 5 min, the polarization decreased to a constant value of 0.120 ± 0.002[4]. This is a larger polarization than that observed for labeled IF3, suggesting an equilibrium between 70S ribosomes and IF3. As mentioned above, the polarizations for the various forms of IF3 were lower than expected based on labeled BSA measurements. Two approaches were followed in calculating an equilibrium constant for the IF3-70S $\rightleftharpoons$ IF3 + 70S reaction. In the first approach, it was assumed that all of the labeled IF3 was active for ribosome binding and that the low P values derived from a rotational mobility of the probe that was larger than that found in BSA. In the second approach, it was assumed that the correct values of P were those that could be calculated from Eq. 1 based on P = 0.224 for Dansyl-labeled BSA and the various molecular weights of the complexes. The low

[2]At 1 mM Mg^{2+}, the binding rate of IF3 to ribosomes is linear in ribosome concentration, 0.33–1 μM; thus, $K_{eq} < 0.1$ μM.

[3]No change in fluorescence anisotropy or intensity was observed when labeled IF3 in the absence of ribosomes (1 mM Mg^{2+}) was flowed against 18 mM Mg^{2+}.

[4]The polarization of the equilibrium mixture varies with ribosome concentration as would be expected. For P = 0.12, the ribosome concentration was 0.33 μM. The K_{eq} values calculated (Eq. 2) for different ribosome concentrations are within the range given below (4.6–5.3 μM). The K_{eq} calculated from the polarization of the final equilibrium mixture obtained when labeled IF3 was flowed against 70S ribosomes (see below) was also within this range.

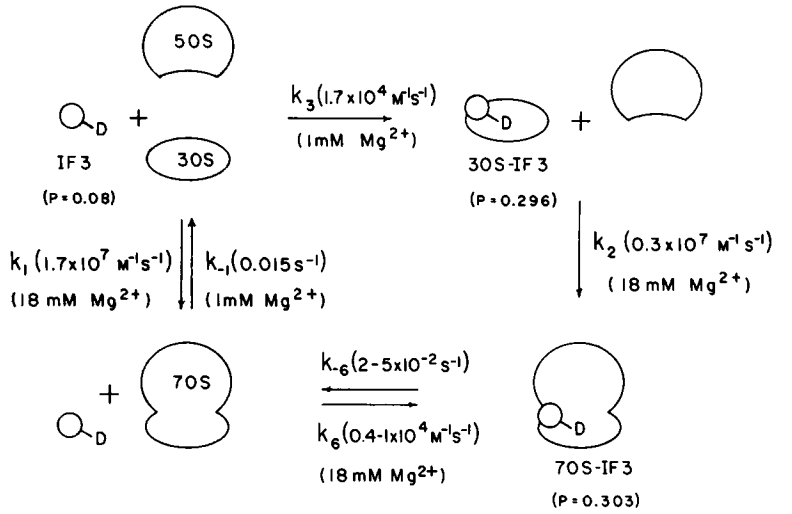

Figure 4 Schematic diagram of the interaction of Dansyl (*D*) labeled IF3 with ribosomal subunits and 70S particles. The measured polarizations are given and are consistent with ~21% labeled IF3 having a polarization of 0.05 and not in equilibrium with ribosomes. The rate constants are labeled to be consistent with previously published kinetic models (Goss et.al., 1980). All rate constants are for 20°. The errors (σ) in the static polarizations are ±0.005. The errors (σ) in the rate constants k_1, k_{-1}, k_2, and k_3 are, respectively, 6%, 13%, 17%, and 12%.

values of P were assumed to derive from an inactive form of IF3 having a polarization of 0.050, which was the value measured for labeled IF3 inactivated by freeze-thaw cycling. In both cases, the following equation was used (Weber, 1952):

$$\left(\frac{1}{P}-\frac{1}{3}\right)^{-1}=\sum_{i-1}^{n}\frac{f_i}{\left(\frac{1}{P_i}-\frac{1}{3}\right)}, \tag{2}$$

where P is the measured polarization, and f_i and P_i are, respectively, the fraction of the fluorescence from species i, and the polarization of species i.

For the first approach, $n = 2$. For the second, $n = 3$ and the fraction of inactive labeled IF3 was found to be 21% based on measurements on the IF3-30S system. If we denote the fraction of IF3 free in solution but active in ribosome binding as f_1, and that fraction bound to the ribosomes as f_2, then, since ribosomes are in considerable excess over IF3, K_{eq} = (70S)(IF3)/(70S-IF3) = $(f_1/f_2)(70S)_{Total}$.[5]

The two approaches lead, respectively, to values for K_{eq} of 4.6 and 5.3 μM. A value of 5 μM was assumed for the following relaxation calculations. This value for the dissociation constant in 18 mM Mg^{2+} is about 100 times greater than that at 2.5 mM Mg^{2+}. A mechanism that summarizes the reactions of IF3 and ribosomes with the values obtained for rate constants and polarizations is shown in Fig. 4.

Two relaxation experiments were carried out to calculate rate constants k_6 and k_{-6} in Fig. 4. Since a single slow equilibration was assumed, and $(70S)_{Total} \sim (70S)_{equil} >> (IF3)_{equil}$,

$$\tau^{-1} = k_6[K_{eq} + (70S)_{Total}]. \tag{3}$$

[5]By conservation, $\Sigma f_i = 1$. When $n = 3, f_3 = 0.21$ (from above), $f_2 = 0.79 - f_1$, and Eq. 2 can then be solved for f_1, since P_1, P_2, and P_3 are known.

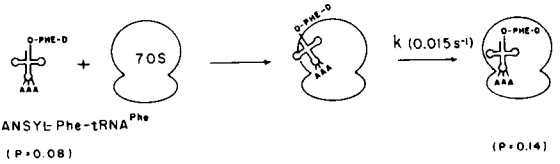

Figure 5 Diagramatic representation of the interaction of labeled tRNA with 70S ribosomes. The polarizations given are those measured in the stopped-flow device. The rate constant was obtained at 40 mM Mg^{2+}. The error in the rate constant (σ) was 15%.

In the first experiment, the time course of the slow decrease in anistropy after the reaction of IF3-30S + 50S at high Mg^{2+} was monitored. In the second experiment, labeled IF3 was flowed against 70S ribosomes at high (18 mM) Mg^{2+}. The two values for k_6 were, respectively, 0.43 and 1.1×10^4 $M^{-1}s^{-1}$. Ranges for k_{-6} were calculated from k_6 and K_{eq}. The value for k_3, the rate constant for the association of IF3 to 30S ribosomes[6] at low Mg^{2+} is 1.7×10^4 $M^{-1}s^{-1}$. Assuming a single step reaction, we conclude that the 100-fold variation in K_{eq} derives mainly from changes in the dissociation rate constant. These results provide direct evidence for the existence of the IF3-70S complex. Two entirely different methods have also provided evidence for an interaction of IF3 with 70S ribosomes. IF3 was covalently crosslinked to 70S particles using dimethylsuberimidate (Hawley et al., 1974). Binding of IF3 to 70S ribosomes was demonstrated by analyzing [^{14}C]IF3-70S mixtures on polyacrylamide gel electrophoresis (Sobura and Wahba, unpublished observations). Furthermore, the fact that IF3 is required for maximum translation at 12–14 mM Mg^{2+} (Sobura et al., 1977) strongly suggests an interaction between IF3 and 70S ribosomes.

tRNA Binding to Ribosomes

There are two sites, *P* (peptidyl), and *A* (aminoacyl), for tRNA binding on ribosomes. At high Mg^{2+} concentrations (40 mM) tRNA will bind to both *P* and *A* sites (nonspecific binding) (Pestka, 1974) in the absence of mRNA, initiation factors, etc. We have examined the kinetics of this nonspecific binding of fluorescently labeled amino-acyl-tRNA to ribosomes. In these experiments, tRNA was labeled with Dansyl chloride at the amino groups of the amino acid. The *N*-Dansyl-aminoacyl-tRNA was then flowed against 70S ribosomes in high Mg^{2+} buffer. This simplified system was studied to determine rates for nonspecific binding. The effects of other components in the more specific binding experiments can then be determined. For *N*-Dansyl-Ile-$tRNA^{Ile}$ and *N*-Dansyl-Phe-$tRNA^{Phe}$ binding, we observed both a fluorescence intensity decrease of 15% and a polarization increase from 0.08 to 0.14 when the tRNA's were flowed against ribosomes. The polarization does not increase further with increases in ribosome concentration, nor does the rate of the polarization change (0.015 s^{-1}) depend on ribosome concentration for concentrations > 0.5 μM. The observed reaction is a first-order transformation between two ribosome-tRNA complexes. The polarization of the labeled tRNA did not change after precipitation of the tRNA from ethanol, indicating that there were not significant amounts of free dye or deacylated tRNA present. We conclude that the low polarization values reflect considerable rotational mobility in the aminoacyl region for the tRNA free and in the tRNA-ribosome complex. Further studies will be directed toward specific *P* and *A* site binding kinetics.

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[6]If 21% of the IF3 were inactive in the time-lapse stopped-flow experiments, then the rate constant k_3 from those measurements would be 0.76×10^4 $M^{-1}s^{-1}$.

Triplet (mRNA) Binding to Ribosomes

Recently Kang et al. (1979) have reported the labeling of ribosomes with IAEDANS. They determined that under their conditions: (*a*) only one protein, S18, was labeled, (*b*) the ribosomes were biologically active, and (*c*) fluorescence quenching occurred upon binding of mRNA or triplets. We have labeled ribosomes by this method and measured the kinetics of triplet binding. Triplets of uridylic acid (UpUpU) were used in place of natural messenger because (*a*) the concentration of ligand can be determined, (*b*) the ligand sites are homogeneous and one does not have the possibility of multiphasic kinetics due to different association rates for different codons, and (*c*) the reactions are not complicated by possible cooperative binding of more than one ribosome per messenger.

Triplet binding appears to be biphasic with the first phase corresponding to the initial binding and the second phase corresponding to a conformational change.

The binary processes involved in assembly of the protein synthesis initiation complex are surprisingly complex. Future experiments will be directed toward providing information on specific binding of tRNA's and on the assembly of ternary and higher complexes.

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DISCUSSION

Session Chairman: Victor Bloomfield *Scribe*: Nanibhushan Dattagupta

BLOOMFIELD: Let us begin with an anonymous referee's question. Is dansylated *IF*3 active in protein synthesis?

GOSS: That has recently been assayed, and yes, the dansylated *IF*3 is active. The activity is the same as non-dansylated *IF*3. The stoichiometry of binding of dansyl to the *IF*3 is 1 : 1.

CHAIRES: I would like to comment on the interaction of *IF*3 with *E. coli* ribosomes, based on some recent experiments performed by Arnold Wishnia and me in the laboratory of Gerson Kegeles at the University of Connecticut.

We find that *IF*3 reacts rapidly with ribosomes, in contrast to the slow reactions you report, and that the association properties of ribosomes are rather quickly altered by exposure to low Mg^{++}, leading us to question the protocol you have used. All of our experiments were performed on ribosomes and *IF*3 provided to us by Albert Wahba of the University of Missisippi Medical Center. Thus we have used material identical to yours. This makes the apparent discrepancies even more puzzling. Further, two reports in the literature (Godefroy-Colburn et al., 1975, *J. Mol. Biol.*, **94**:461; Wooley and Box, 1980, *Febs. Lett.*, **108**:433) also show rapid *IF*3 binding, again in contrast to your observations.

What do you think accounts for this discrepancy in the apparent rate of *IF*3 interactions? Will you describe in some detail your quality control measures for both ribosomes and *IF*3? What is your criterion for "tight" couples, i.e. how would you detect the inactivation described in your experiments?

PARKHURST: It's true that the source of ribosomes and *IF*3 is the same for both groups—from Dr. Wahba. The ribosomes are made on one day and shipped to us the next day. We do the experiments and finish them within 72 h of preparation of the ribosomes. Thereafter we see changes in the properties of the ribosomes. For these "juvenile" ribosomes, as they might be called, there are no observed changes in 1 mM Mg solutions for up to an hour, the conditions of the control experiment described for the time lapse stopped flow studies. If we try to do the same thing for week-old ribosomes (held by freezing at −80°C), we do see changes, but we don't use any data from those older ribosomes.

With regard to the *IF*3, we find that it is completely inactivated if it is refrozen; we find that we then get no change in fluorescence anisotropy when it's flowed against ribosomes. Inactive *IF*3, although it's not turbid or obviously denatured, will not give us fluorescence anisotropy changes.

These are two examples of quality control. We find no changes in the association kinetics between 10, 12, and 18 mM Mg, nor does the Mg^{++} at half-saturation shift. We have no evidence for loose couples in any of our experiments.

GOSS: Dr. Chaires doesn't exactly duplicate our experiments because we use ~10-fold lower ribosome concentration since we use a laser light source. We did repeat our own experiments first and then went to concentrations similar to those used by Dr Chaires in an attempt to resolve the inconsistencies mentioned.

*IF*3 is sent to us in 500 mM ammonium chloride. If we dilute without lowering the ammonium chloride we see an instantaneous slowing down of rate, by flowing against what is in effect an ammonium chloride jump. It is second order, as one would expect for a small ion rapidly binding to a ribosome. The rate of binding would be much faster than the association of the ribosomal subunits. This gives a rate constant ~10-fold lower than what one sees by flowing against Mg alone.

PARKHURST: I realize there are differences in our experimental results. I can only repeat an invitation we made a number of months ago, and that is for both Dr. Wishnia and Dr. Chaires to come to our laboratory and together we will do the experiments on the freshest possible ribosomes from Professor Wahba, who is in perfect accord with that invitation and with that agreement. The reason why I think this is an important experiment is because we can duplicate the optics carried out at the University of Connecticut, that is, with a xenon arc light source; we can also set our argon ion laser back up; finally, on the same batch of material, we can repeat the fluorescence anisotropy experiments. We can do all three types of experiments in the same laboratory. In that way we can hope to settle the issue.

A. SUBRAMANIAN: I have a comment regarding the form of *IF*3 used in these experiments. A few years ago I showed that in cell extracts there are two forms of *IF*3. Later sequencing has shown that these two differ in terms of one hexapeptide, and that different laboratories purified one or the other of these two forms. Since both of you have used *IF*3 from the same source, Dr. Wahba's laboratory, presumably it is the same form. It might be of interest for you to know that I have checked one sample of Dr. Wahba's *IF*3 and it was the long form.

LEHRER: I have a naive question. Why can't the discrepancy simply be due to the dansyl group? Is the dansyl perhaps affecting the association kinetics but not the equilibrium activity?

GOSS: For one thing our dansylated *IF*3 is completely active in protein synthesis. For another, our time lapse rates with non-dansylated *IF*3 are in good agreement with the fluorescence anisotropy measurements, and the time lapse experiments did not involve labeled *IF*3.

HANTGAN: Concerning the interpretation of your anisotropy data, have you in fact measured the lifetime of your dansylated *IF*3? Is it different from dansylated BSA? Are you not assuming in the interpretation of your anisotropy data that the lifetime was constant throughout all these binding reactions?

PARKHURST: Yes, we are assuming that the lifetime is the same but we see no change in fluorescence during the reaction so we do not detect a change in overall quantum yield. The stoichiometry of the binding is 1 to 1, so there should be little site heterogeneity.

HANTGAN: I ask because in some recent experiments we have done with fibrinogen we find the lifetime of dansylated fibrinogen to be significantly less than 12 ns.

PARKHURST: It may in fact be somewhat different from what it is in BSA. The lifetime studies are interesting for a number of reasons. If we use the molecular weights for the 30S particle, we get remarkable agreement, assuming a spherical particle with no rotational freedom of the dansyl. We haven't found such agreement with a simple model in human hemoglobin, for instance, or in hemoglobin or hemerythrin. This is really almost an ideal case for anisotropy measurements, We feel that the lifetime probably isn't very different. At any rate, during all those reactions that you saw we see no changes in fluorescence intensity, only anisotropy changes.