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Cooperative Interactions in the System Ribosomes–Ribosomal Protein S1–Polynucleotide Triplets†

Dixie J. Goss, Lawrence J. Parkhurst,* Arkesh M. Mehta, and Albert J. Wahba

ABSTRACT: Association equilibria have been determined in the ternary system uridyl triplets (T)–ribosomal protein S1 (S)–ribosomes (Rb) depleted of S1 at 6 and 10 mM Mg^{2+}. For 1:1 stoichiometry of reactants, four thermodynamically independent equilibria characterize the ternary system. The binary interaction Rb + T was studied by following the fluorescence quenching of labeled ribosomes by added T. The Rb + T association constant for UpUpUp triplets was 10–20-fold greater than for ApUpG triplets. The interaction Rb + S was studied by following the changes in fluorescence anisotropy when labeled S1 reacted with ribosomes. The remaining two independent equilibrium constants (for S + T and RbT + S) were obtained from fits to observed anisotropy measurements when varying amounts of T were added to a solution of ribosomes and fluorescently labeled S1. This indirect procedure allows one to measure S + T binding, an association that is difficult to determine directly. Over the concentration interval 5–10 mM Mg^{2+}, the association constant for Rb + S increases with the sixth power of $[Mg^{2+}]$, whereas the association constant for S + T *decreases* approximately 2-fold as Mg^{2+} is increased from 6 to 10 mM Mg^{2+}. T binds to Rb more tightly at 10 mM than at 6 mM Mg^{2+}. When S1 is bound to Rb, however, at 10 mM Mg^{2+} the binding constant for T is *decreased* 10-fold and the Mg^{2+} dependence is reversed. These interactions can be described in terms of coupling free energies. For the ternary complex, three linearly independent coupling free energies can be written. These excess functions simply show by how much we err in estimating the overall free-energy change for formation of the RbST complex from free-energy changes for the formation of various binary fragments. One of the ternary coupling free energies, $\Delta G^\circ_{R,ST}$ ($=\Delta G^\circ_{RST} - \Delta G^\circ_{RT} - \Delta G^\circ_{RS}$), is positive (anticooperative interaction) at 10 mM Mg^{2+} but negative at 6 mM Mg^{2+}, primarily because of the sensitivity of ΔG°_{RS} to $[Mg^{2+}]$. Thus, at 6 mM Mg^{2+}, prior binding of S1 to the ribosome enhances binding of the triplet to form the ternary complex, but at 10 mM Mg^{2+}, S1 binding to Rb destabilizes subsequent binding of the triplet. More elaborate models that assumed multiple sites for T on S and Rb were used to fit the data; however, both the anisotropy data and the results for triplet quenching of ribosome fluorescence were in accord with a simple description where the binding of the triplets to the ribosome appeared to be to a single site. If there are multiple sites for triplet binding, the binding sites show little interaction or heterogeneity over the concentration ranges studied. Our results lend support to assigning primary importance to protein–protein interactions in the binding of S1 to the ribosome. Although S1 may well function as an unwinding protein, it can profoundly affect the binding of nonhelical trinucleotides to the ribosome.

Although there have been many studies of protein synthesis initiation, there is still not a clear understanding of this process on a molecular level. What are the control mechanisms for this process? Koshland et al. (1983) have pointed out that biological organisms must respond to their environment on a cellular level. Cells must "turn on" or "turn off" various processes in response to external signals. These biological systems require the amplification of some stimuli and the ability to adapt to background levels of other stimuli. The amplification and adaptation can be achieved in a variety of ways, among them are cooperative interactions of molecules, multistep input of regulators, allosteric effectors, and covalent modification of the proteins.

In order to determine possible control points for protein synthesis initiation, we have examined some of the simpler molecular interactions and the role of possible effectors. The simplest interactions are the binary interactions such as 70S ribosome dissociation and subunit association, the binding of IF3 to ribosomes, mRNA binding to ribosomes, and S1 binding to ribosomes. The interactions become more complex for ternary and higher order interactions. We describe here the equilibrium interactions for ribosomes (Rb), ribosomal protein S1 (S), and nucleotide triplets (T) at 6 and 10 mM Mg^{2+}. We have considered a number of different models for these interactions. The simplest model assumes (1) S1 has one nucleotide triplet binding site, (2) ribosomes have one S1 binding site, and (3) there is effectively one nucleotide triplet binding site on the ribosome. Because of a variety of sometimes conflicting evidence in the literature, we have examined more complex models as well.

Our data could be fit well with the simple model. However, just as simple models for IF3 interaction, such as the antiassociation model, were sufficient for early data (Dondon et al., 1974; Godefroy-Colburn et al., 1975; Gottlieb et al.,

† From the Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304 (D.J.G. and L.J.P.), and the Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216 (A.M.M. and A.J.W.). *Received October 13, 1983.* We thank the following agencies for support of this research: National Institutes of Health (Grants HL 15,284 and GM 25451), National Science Foundation (Grant PCM 8003655), and Research Council, University of Nebraska.

0006-2960/84/0423-6522$01.50/0

1975; Chaires et al., 1981), more precise data and other observations (Chaires et al., 1979, 1982; Goss et al., 1980a,b, 1982) required more complex models, and we regard this as a primitive model. Although they were not clearly superior to the simple model in fitting our data, the more complex models presented and discussed here provided useful limits for a number of parameters involving site heterogeneity and site–site binding interactions.

Materials and Methods

Preparation of Ribosomes and Components. Ribosomes were prepared from *Escherichia coli* MRE600 cells as described previously (Wahba & Miller, 1974). The 70S ribosomes contained about 0.3 copy of S1 per ribosome, which was removed by poly(C)–cellulose chromatography (Sobura et al., 1977). The ribosomes and subunits were heat activated at 37 °C for 30 min immediately prior to use. Ribosomal protein S1 was prepared by poly(C)–cellulose chromatography according to Sobura et al. (1977). The purified protein was stored at −80 °C in buffer containing 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.8 (at 20 °C), 10 mM magnesium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM dithiothreitol. At least eight different ribosome and five different S1 preparations were studied. Nucleotide triplets (ApUpG and UpUpUp) were purchased from Boehringer-Mannheim (Indianapolis, IN) and used without further purification.

Fluorescent Labeling of S1 and S1 Activity. Purified ribosomal protein S1 was labeled with IAEDANS [5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonate, Molecular Probes Inc., Junction City, OR] according to a modification of the procedure of Chu & Cantor (1979). The protein was first passed over a Sephadex G-25 medium column (0.5 × 15 cm) equilibrated with 10 mM Tris buffer, pH 7.6, and 30 mM KCl. A 100-fold excess of IAEDANS in the same buffer was added to the protein (20 μM), and the mixture was incubated in the dark at 37 °C for 1 h. After the incubation, 2-mercaptoethanol (1% v/v) was added to the mixture, and unreacted dye was removed on a Sephadex G-25 medium column equilibrated with 10 mM Tris buffer, pH 7.6, 20 mM $Mg(OAc)_2$, 250 mM NH_4Cl, and 6 mM 2-mercaptoethanol. Remaining noncovalently attached dye was removed by dialyzing the S1–AEDANS fraction into the same buffer containing 6 M urea for 3 h at 4 °C and then passing the protein over a Sephadex G-25 medium column (0.9 × 30 cm) equilibrated with the same buffer without urea. Some preparations of S1 that were extensively dialyzed after labeling appeared to have a molecular mass about 10 000 daltons smaller than that unlabeled S1 [see Goss et al. (1983) for details]. These samples were not used for the data reported here.

The dye to protein ratio determined from absorbance measurements was 1.1 ± 0.1 dyes per S1 [see Goss et al. (1983) for details]. The activity of labeled S1 was assayed by poly(U)-dependent synthesis of poly(Phe) (Sobura et al., 1977). The labeled S1 had at least 90% of the activity of unlabeled S1.

Labeling and Activity of Ribosomes. Ribosomes were fluorescently labeled with IAEDANS as described by Kang et al. (1979) with slight modifications. Salt-washed ribosomes were passed over a Sephadex G-25 medium column (0.5 × 20 cm) equilibrated with 20 mM Tris (pH 7.9), 10 mM magnesium acetate, and 30 mM ammonium chloride. The ribosomes were reacted with a 100-fold excess of IAEDANS in the same buffer at 25 °C for 10 min in the dark. The reaction was terminated by adding a 5-fold excess of 2-mercaptoethanol to IAEDANS. Excess unreacted IAEDANS was removed by passing the ribosomes over a Sephadex G-25 medium column (0.5 × 20 cm) equilibrated with the buffer described above containing 6 mM 2-mercaptoethanol. The ribosomes were then dialyzed overnight at 4 °C against buffer consisting of 10 mM Tris (pH 7.6), 10 mM magnesium acetate, 30 mM ammonium chloride, and 6 mM 2-mercaptoethanol. The dye to ribosome ratio was 1.2 ± 0.2 dyes per 70S ribosome. The ribosome activity was assayed by poly(U)-dependent synthesis of poly(Phe) (Sobura et al., 1977). The labeled ribosomes had 85% of the activity of unlabeled ribosomes.

Anisotropy Measurements. Steady-state fluorescence anisotropy was measured with a photoelastic modulator (Morvue Electronics Systems, Tigard, OR) similar to the apparatus previously described (Goss et al., 1980, 1983). The entire fluorescence anisotropy apparatus was controlled by an Apple II microcomputer. Data were acquired with a Nicolet 2090-III-206 12-bit digital oscilloscope (Nicolet, Madison, WI). The computer processed the data from the Nicolet oscilloscope to obtain anisotropies. Typically, three runs of 30 data points each were acquired and processed to obtain the anisotropy values for a given sample and set of experimental conditions. The final fitting was carried out on data from three separate preparations of both ribosomes and S1.

Determination of Equilibrium Constant for Triplet Binding to Ribosomes. Fluorescently labeled ribosomes stripped of S1 were heat activated at 37 °C for 30 min immediately prior to fluorescence measurements. Quenching measurements were made in the same apparatus as for the anisotropy measurements, except that the modulator was off and the Ditric filter was removed. The fluorescence intensity of the sample was measured before and after each addition of UpUpUp or ApUpG. Complete titration curves at varying concentrations of Mg^{2+} were obtained. At the end of the titrations, the fluorescence had decreased to 76 and 80% of the initial value for reaction with UpUpUp and ApUpG, respectively. The ribosome concentrations used ranged from 0.08 to 0.15 μM; triplet concentration was increased from 0 to 3.0 μM; buffer was 10 mM Tris, 30 mM NH_4Cl, and magnesium acetate as indicated (buffer A).

The binding of nucleotide triplets to AEDANS-labeled ribosomes was measured by fluorescence quenching. The equilibrium is assumed to be a simple equilibrium between two components, 70S ribosomes and nucleotide triplets, such that the equilibrium binding constant, K_3, is

$$K_3 = [RbT]/([Rb][T])$$

where [Rb], [T], and [RbT] represent the concentrations of ribosomes, triplets, and ribosomes with triplet bound, respectively. The measured fluorescence intensity, F_M, is the average fluorescence intensity from Rb (F_0) and RbT (F_B). Defining X as the mole fraction of ribosomes containing triplets that quench AEDANS fluorescence (Kang et al., 1979)

$$F_M = XF_B + (1 - X)F_0$$

and

$$\frac{F_0 - F_M}{F_0 - F_B} = X = \frac{[RbT]}{[Rb_T]}$$

Since total concentrations of ribosomes ($[Rb_T]$) and triplets ($[T_T]$) are known, the equation can be written as

$$K_3 = \frac{X}{(1 - X)([T_T] - X[Rb_T])}$$

where X is the observed experimental quantity and K_3 is the

only unknown. The titration curves of X vs. $[T_T]$ were fit by a Fletcher–Powell (Fletcher & Powell, 1963) sum of squares minimization for one parameter, K_3.

The data of anisotropy (r) vs. triplet concentration, shown in Figure 5, were fit to all models by a Newton–Raphson program embedded in a two-dimensional grid search. For each pair of equilibrium constant values (K_1 and K_4), a solution was found with a two-dimensional Newton–Raphson program that solved for the concentrations of free ribosomes and free triplets from which all other equilibrium concentrations could be readily obtained. A grid search was then carried out to minimize the variance in r for the data shown in Figure 5. Variance contours were plotted for variations in K_1 and K_4 (Figure 6).

Results

The simple stoichiometry, one triplet per ribosome, was not used for the model described below. This model assumes two noninteracting triplet binding sites (α and β) on each ribosome with only binding to the α site resulting in fluorescence quenching so that

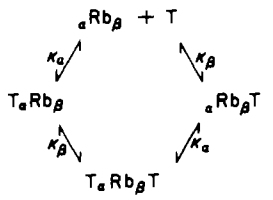
$$X = ([T_\alpha Rb_\beta] + [T_\alpha Rb_\beta T])/[Rb_T]$$

and

$$K_\alpha = [T_\alpha Rb_\beta]/([Rb][T])$$

$$K_\beta = [{}_\alpha Rb_\beta T]/([Rb][T])$$

Schematically, the model can be written as



The total ribosome and triplet concentrations are known. By use of conservation and the above equilibrium constants, the following can be written:

$$[Rb_T] = [T_\alpha Rb_\beta] + [{}_\alpha Rb_\beta T] + [T_\alpha Rb_\beta T] + [Rb] = (K_\alpha[T] + K_\beta[T] + 1 + K_\alpha K_\beta[T]^2)[Rb]$$

and

$$[T_T] = [T_\alpha Rb_\beta] + [{}_\alpha Rb_\beta T] + [T_\alpha Rb_\beta T] + [T] = ([T][Rb]K_\alpha K_\beta + [Rb](K_\alpha + K_\beta) + 1)[T]$$

With these two equations, [Rb] and [T] can be written as functions of K_α and K_β and

$$X = \frac{[T]^2 K_\alpha K_\beta [Rb] + [T][Rb]K_\alpha}{[Rb_T]}$$

The data fitting showed that values of K_β more than 3-fold larger than K_α lead to significantly worse fits to the experimental data. The calculated curves of X vs. $[T_T]$ were sigmoidal, rather than the observed hyperbolic binding curve. From these results, we excluded a second nonquenching site of much higher affinity from further consideration.

The quenching curves were fit well by a model with one ribosomal binding site. Figures 1 and 2 show plots of the normalized fluorescence quenching at 6 and 10 mM Mg^{2+} for AUG and UUU binding, respectively. The equilibrium constant obtained for triplet binding to ribosomes was the same within experimental error for ribosome concentrations of 0.08–0.15 μM. A plot of the concentration ratio of ribosomes with triplet bound to free ribosomes $[X/(1-X)]$ vs. free triplet concentration gave a slope of 1.0 ± 0.2 showing no evidence

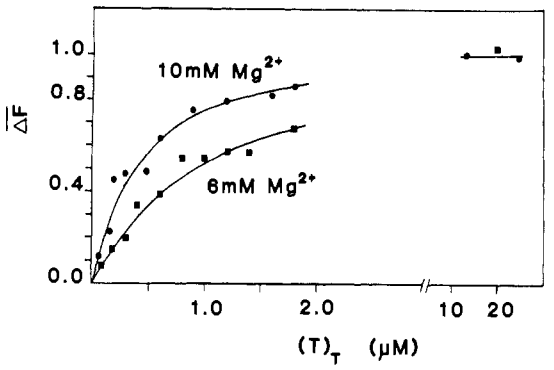


FIGURE 1: Titration of AEDANS-labeled ribosomes with ApUpG triplets. The left axis is the normalized change in fluorescence intensity, $(F_0 - F_{obsd})/(F_0 - F_\infty)$, where F_0 is the fluorescence intensity of the AEDANS-labeled ribosomes amd F_∞ is the intensity of the final quenched sample at the end of the titration. The x axis is the total AUG concentration. The squares and circles depict the data points at 6 and 10 mM Mg^{2+}, respectively. The ribosome concentration was 0.15 μM; T = 20 °C. The solid lines are the calculated curves for the equilibrium constants at 6 and 10 mM Mg^{2+}; K = 1.2 and 3.5 μM^{-1}, respectively.

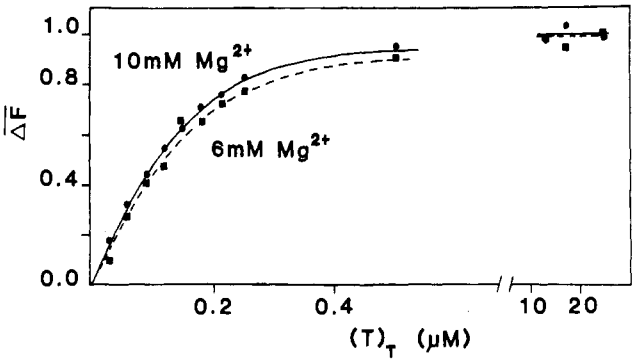
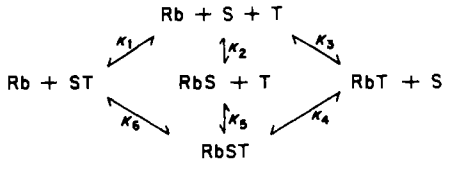


FIGURE 2: Titration of AEDANS-labeled ribosomes with UpUpUp triplets. The axes are the same as those in Figure 1. The ribosome concentration was 0.15 μM; T = 20 °C. The dashed and solid lines are the calculated curves for the best fit to the data according to model I where K_3 = 25 and 35 μM^{-1} at 6 and 10 mM Mg^{2+}, respectively. The squares and circles represent the data points at 6 and 10 mM Mg^{2+}, respectively.

for cooperative binding. We cannot rule out much weaker binding sites or multiple binding sites that are equivalent and statistically related. Figure 3 depicts the experimental curve for UpUpUp binding at 10 mM Mg^{2+} shown in Figure 2 and curves calculated for the two-site model where $K_\alpha = K_\beta$. The dotted line shows the curve for the case when either site binding results in maximal fluorescence quenching, and the dashed line shows the curve for no quenching by the second site binding.

A simple interaction scheme (model I) for triplets, ribosomes, and S1 can be written as follows:



where all K's are written as association constants and $K_5 = K_4K_3/K_2$ and $K_6 = K_4K_3/K_1$ were selected as the two thermodynamically dependent equilibrium constants.

The first three equilibrium constants (K_1, K_2, K_3) represent the binary interactions. We have measured these independently wherever possible. The binding of S1 to triplets is difficult to measure independently, particularly in the presence of Mg^{2+}. Most attempts to measure this reaction have used

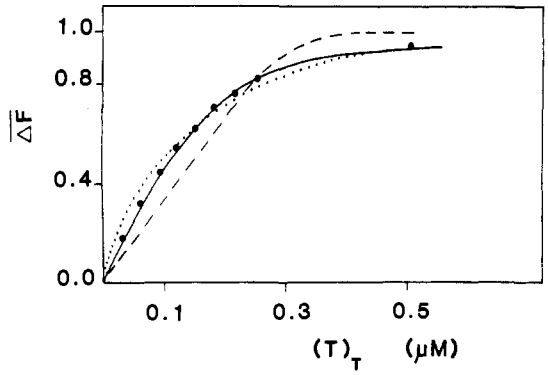


FIGURE 3: Model fitting for triplet (UpUpUp) binding to AEDANS-labeled ribosomes. The data from Figure 2 at 10 mM Mg^{2+} are shown. The solid line is the best fitting curve for model I, a single binding site; $K = 35\ \mu M^{-1}$. The dotted line is the best fit for a model where there are two triplet binding sites, K_α and K_β. The sites are assumed to have equal affinity, and binding to either site leads to maximal fluorescence quenching. The dashed line represents the best fitting calculated curve for a two-site model where $K_\alpha = K_\beta$, but binding to the β site does not result in fluorescence quenching.

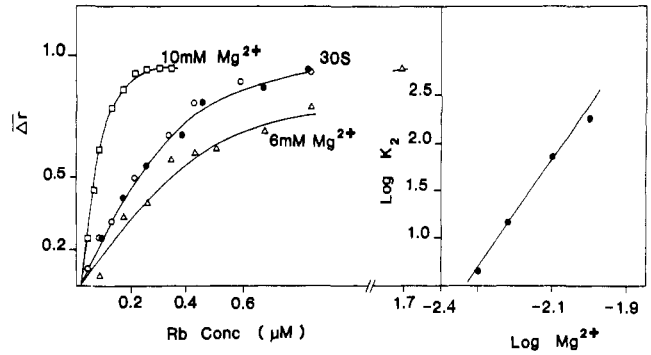


FIGURE 4: The Mg^{2+} dependence of AEDANS–S1 binding to ribosomes. The left panel shows the normalized anisotropy change for AEDANS–S1 (0.106 μM) when titrated with increasing concentration of S1-depleted ribosomes. The squares and triangles depict the reaction for 70S ribosomes at 10 and 6 mM Mg^{2+}, respectively. The open circles and solid circles show the reaction with S1-depleted 30S subunits at 10 and 6 mM Mg^{2+}, respectively. The lines represent the calculated binding curves. All reactions were at 20 °C. Experiments were performed in buffer A. The right panel shows a plot of the log K_2 (S1 binding to 70S) vs. log [Mg^{2+}]. The least-squares slope of the line is 6.0.

spectroscopic techniques. The binding of oligonucleotides by S1 results in a UV absorption change (Mülsch et al., 1981) and a quenching of the protein fluorescence (Draper & von Hippel, 1978a,b; Omar & Schleich, 1981). However, others have also observed significant precipitation of the protein nucleic acid complex (Mülsch et al., 1981). We were unable to obtain reliable equilibrium constants by UV absorption spectroscopy. The spectra gave neither stable isosbestic points nor well-defined end points for the titrations, probably due to some precipitation of the protein and/or S1–nucleic acid complex. The concentrations used in these experiments were >40-fold higher than those required for fluorescence measurements. At the lower concentrations used for fluorescence quenching and anisotropy titrations, well-defined end points were obtained. We were able, however, to obtain this equilibrium constant from model fitting to the fluorescence anisotropy data (see below).

The second binary equilibrium constant, K_2, the binding of S1 to ribosomes, was obtained from fluorescence anisotropy measurements of AEDANS-labeled S1. The details of these experiments are described elsewhere (Goss et al., 1983). For S1 binding to 70S ribosomes prepared as described above, there is a greater than 40-fold change in K_2 for a doubling of the [Mg^{2+}] from 5 to 10 mM. Values of 4 μM^{-1} at 5 mM and 184 μM^{-1} at 10 mM Mg^{2+} were obtained. This strong [Mg^{2+}] dependence (sixth power) shows that at this Mg^{2+} concentration the Rb–S1 complex has a net uptake equivalent to six Mg^{2+} ions compared to S1-depleted ribosomes. Figure 4 shows the fluorescence anisotropy change and [Mg^{2+}] dependence of S1 binding to 70S ribosomes.

For uridyl triplets, the values for K_3 (Rb + T $\rightleftharpoons$ RbT) increased slightly with increasing Mg^{2+}. At 6 and 10 mM Mg^{2+} the values were 25 ± 1 and 35 ± 1 μM^{-1}, respectively (Figure 2). Much lower values were found for the ApUpG triplet binding (Figure 1). These values ranged from 1.2 ± 0.4 μM^{-1} to 3.5 ± 0.5 μM^{-1} at 6 and 10 mM Mg^{2+}, respectively. At 25 mM Mg^{2+} the value of K_3 for AUG triplet binding to ribosomes was 15 ± 1 μM^{-1}.

As described, K_2 and K_3 have been determined independently. For the interaction scheme shown above, only two parameters (K_1 and K_4) are undetermined (since K_5 and K_6 were chosen as the two thermodynamically dependent constants). Ribosomes and AEDANS-labeled S1 were titrated with increasing amounts of uridyl triplets at constant (Mg^{2+}).

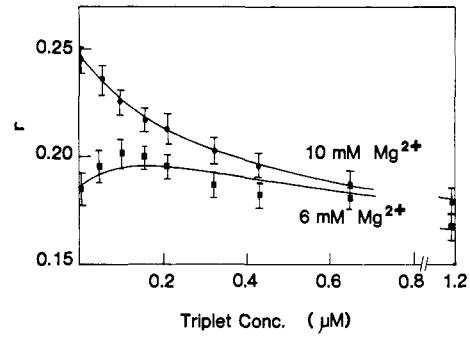


FIGURE 5: Anisotropy values obtained for AEDANS–S1 and 70S ribosomes at varying triplet (UpUpUp) concentrations. The circles and squares represent the data points at 10 and 6 mM Mg^{2+}, respectively, for a mixture of ribosomes (0.158 μM) and S1 (0.08 μM) titrated with increasing amounts of UpUpUp triplets. Experiments were preformed in buffer A. The reaction temperature was 20 °C. The error bars indicate the standard deviation for 10 readings (30 data points) on each of three preparations at each triplet concentration. The solid symbols represent grand means, i.e., an average over the three preparations and 10 readings on each preparation. The solid lines are the curves calculated for model I.

The anisotropy was measured at each point, and the data were fit to the above scheme with K_1 and K_4 as variable parameters. For these concentrations, the assumptions were (1) there is one ribosome binding site for S1, (2) S1 binds only one triplet, and (3) ribosomes with or without S1 bind only one triplet. The validity and consequences of these assumptions are discussed below. We do assume that the anisotropy of the RST complex is the same as that for RS. This is supported by the following: (1) the equilibrium constant, K_5, calculated from measuring the change in anisotropy of labeled S1 and that calculated by the quenching of fluorescence with labeled ribosomes are in close agreement, and (2) the observed effects of triplet binding at 6 and 10 mM Mg^{2+} described below are quite different. Qualitatively, Figure 5 shows very different effects for triplet binding at 6 and 10 mM Mg^{2+}. At 6 mM Mg^{2+}, there is an initial *increase* in anisotropy indicative of an enhancement by triplets of S binding to Rb. This is followed at higher concentrations of T by a decrease in r, which, in terms of the simple model I above, could only result from formation of an ST complex. The qualitative stabilization of the RbST complex at 6 mM Mg^{2+} is quantitated by calculations of $\Delta G°_{R,ST}$ (see below). At 10 mM Mg^{2+}, the picture

Table I: Association Equilibrium Constants for Ribosome (R), Nucleotide Triplet (T), and Ribosomal Protein S1 (S) Interaction[a]

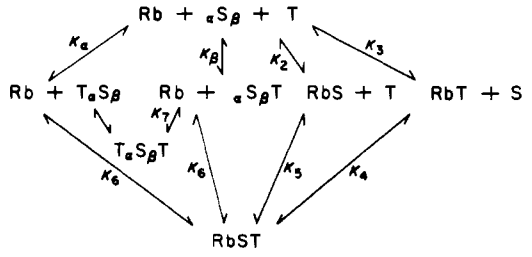
	equilibrium constants (μM^{-1})[b]							
$[Mg^{2+}]$ (mM)	K_1 (ST)	K_2 (RS)	K_3 (RT)	K_4 (RT, S)	K_5 (RS, T)	K_6 (ST, R)	K_7	SSQ[c]
model I								
10	0.9 ± 0.2	184 ± 1	35 ± 1	19 ± 3	3.6 ± 0.4	739 ± 30		6.1E − 5
6	1.6 ± 0.3	14 ± 1	25 ± 1	22 ± 3	39 ± 4	344 ± 20		2.1E − 4
model II								
10	0.5 ± 0.1	184 ± 1	35 ± 1	15 ± 2	2.9 ± 0.3	1050 ± 100	$K_1/4$	4.1E − 5
6	0.9 ± 0.2	14 ± 1	25 ± 1	20 ± 3	36 ± 4	555 ± 50	$K_1/4$	2.7E − 4
model III								
10	0.8 ± 0.2	184 ± 1	35 ± 1	18 ± 3	3.5 ± 0.4	788 ± 30	0.4 ± 0.1	4.9E − 5
6	1.0 ± 0.2	14 ± 1	25 ± 1	20 ± 3	36 ± 4	500 ± 40	0.5 ± 0.1	2.7 E − 4
model IV[d]								

[a] Experiments were performed in buffer A with magnesium acetate as indicated. T = 20 °C. Triplet is UpUpUp. [b] The errors in equilibrium constants K_2 and K_3 are calculated from the variance–covariance matrix in the least-squares data fitting. The errors in the other equilibrium constants are estimated from the variance contours (see Figure 3) and propagation of errors. [c] The sum of squares is calculated from the data in Figure 2. [d] Equilibrium constants are the same as those for model III. The fit is not improved by having $K_\alpha \neq K_7$.

is less clear. The anisotropy decreases for all additions of triplets. This could arise either from formation of the ST complex or by destabilization of RbST by addition of T—in effect, a displacement of S by T. Prior knowledge of the $\Delta G°$ for formation of RbT allows us to determine $\Delta G°$ both for formation of ST and for the destabilization, described by $\Delta G°_{R,ST}$ at 10 mM Mg^{2+}.

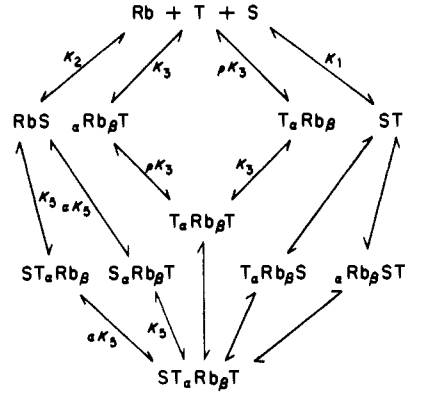
Models II–IV below relaxed assumption 2, allowing S1 to have two nucleotide triplet binding sites. There is conflicting evidence in the literature as to the number and affinity of binding sites. Mülsch et al. (1981) have concluded from UV difference spectra and temperature-jump studies that S1 has one nucleotide binding site. Yuan et al. (1979) found a 1:1 complex with S1 and the 49-nucleotide colicin fragment of rRNA. However, von Hippel and his colleagues have reported two different nucleic acid binding sites (Draper et al., 1977; Draper & von Hippel, 1978a,b). Lipecky et al. (1977) found that for oligonucleotides of chain length less than seven more than one molecule was bound, whereas for longer oligonucleotides the number bound was reduced to one. We have examined models, described below, that allow for two triplet binding sites and for different affinities at these sites.

Model II included the steps shown for model I. Assumption 2 was changed so that protein S1 was now assumed to have two binding sites of equal affinity for triplets. It was assumed that S1 having either site (but not both) occupied could react with ribosomes with equal affinity. The steps for model II can be written as



where $_\alpha S_\beta$ denotes protein S1 with two (α and β) triplet binding sites and $K_1 = K_\alpha + K_\beta$ for equivalent sites. The sites are assumed to be noninteracting for this model, so that the statistical factors make $K_1 = 4K_7$. This model had two adjustable parameters: K_1 and K_4.

Model III also assumed two equivalent sites for triplet binding to S1, but in this model only one site, β, was the ribosomal binding site. The second vertical arrow from the left leading to RbST in model II is eliminated. For model fitting, $_\alpha S_\beta T$ was assumed not to form a complex with Rb, but the choice of reactive site was arbitrary and is not to be correlated with the binding sites I and II designated by Draper & von Hippel (1978a,b). Model IV was the same as model III except that the two S1 binding sites for triplets could interact and binding could show either positive or negative cooperativity ($K_1 \neq 4K_7$). This added one additional unknown (K_7) for the data fitting. Thus, model I has two unknowns, K_1 and K_4 (assuming K_2 and K_3 are known), model II has two unknowns ($K_1 = 4K_7$, K_1 and K_4 are unknown), model III has two unknowns (K_1 and K_4), and model IV has three unknowns (K_1, K_4, and K_7). Model V has four unknowns: K_1, K_4, α, and ρ. In this model, we sought to model the mRNA ribosome groove with two triplet binding sites. The ribosomal binding site for mRNA is known to be about 30–40 nucleotides long (Takanami & Zubay, 1964; Castles & Singer, 1969; Keuchler & Rich, 1970; Gupta et al., 1971; Pochon et al., 1977), and it seems reasonable that more than one triplet may be bound. A schematic representation of model V, with some paths deleted for clarity, is



In this case, besides K_1 and K_4 there are two additional unknowns (ρ, the ratio of the equilibrium constants for the two triplet binding sites on ribosomes, and α, the ratio of the equilibrium constants for the two triplet sites for S1Rb). From earlier quenching studies, ρ was found to be ~ 1. The value of ρ was varied ±10% about 1 in the actual data fitting. The data fitting showed that $2.5 > \alpha > 0.8$ for both 10 and 6 mM Mg^{2+}. The best fit at 6 mM was $\alpha = 1.2$ and at 10 mM $\alpha = 0.9$, suggestive of little heterogeneity for triplet binding on the ribosome.

Figure 5 shows the experimental data and the curves calculated for Model I for the Rb–S1–T system at 6 and 10 mM Mg^{2+}. Figure 6 shows 95% confidence regions for model I for data at 6 and 10 mM Mg^{2+}. The equilibrium constants and sums of squares obtained from the models are given in Table I. Although the more complicated models did not improve the overall fit (SSQ) to the experimental data, they did allow us to set limits on the heterogeneity of triplet binding

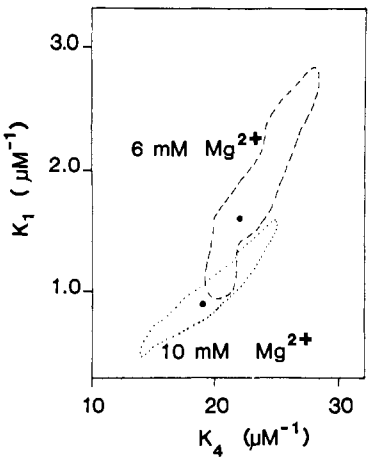


FIGURE 6: Variance contours for model I data fitting. The dotted lines indicate the 95% confidence region for K_1 and K_4 for fitting data at 10 mM Mg^{2+} to model I. The dashed lines show the 95% confidence region for K_1 and K_4 for 6 mM Mg^{2+} data. The solid circles are the minima at the two indicated [Mg^{2+}]. The data used in the fitting are shown in Figure 2.

sites for both S1 and the ribosome. At 6 mM Mg^{2+}, the presence of S1 on the ribosome enhances triplet binding ($K_5 > K_3$); however, at 10 mM this effect is reversed. This predicted effect from the fitting of the S1 anisotropy data led us to perform the following experiment to determine directly the binding of triplets to ribosomes containing S1. The equilibrium constant for S1 binding to ribosomes is large. The AEDANS-labeled ribosome concentration (0.25 μM) was sufficiently high to ensure binding of S1 to at least 90% of the ribosomes. A fluorescence titration was performed as described for triplet binding. The equilibrium constant measured, K_5, *decreased* with increasing Mg^{2+} concentration from a value of 30 ± 5 μM^{-1} at 6 mM Mg^{2+} to 4 ± 2 μM^{-1} at 10 mM Mg^{2+}. These values for K_5 from direct measurement are in quite close agreement with those found by model fitting of the anisotropy data where K_5 was determined from K_2 and K_3.

Discussion

Values for K_2 (S1 + Rb) as a function of Mg^{2+} have been discussed in detail elsewhere (Goss et al., 1983). We have examined the ribosome, triplet, and S1 interactions reported here at a [Mg^{2+}] of 6–10 mM. Above 6 mM Mg^{2+}, more than 90% of the ribosomal subunits ("tight couples") are associated to 70S particles. At Mg^{2+} concentrations greater than 10 mM, some aggregation of ribosomes can occur (Hocker et al., 1973). The binding of uridyl triplets showed little Mg^{2+} dependence (Figure 2). The equilibrium constant, K_3, changed less than a factor of 2 (25–35 μM^{-1}) in going from 6 to 10 mM Mg^{2+}. Kang et al. (1979) measured the binding of poly(U) and AUG triplets to ribosomes at 25 mM Mg^{2+}. They obtained values for K_3 of 1 and 20 M^{-1} for poly(U) and AUG, respectively. [Apparently they did not use a McGhee & von Hippel (1974) type analysis to relate the different nucleotide lengths.] The differences between their value for poly(U) binding and our results for uridyl triplet binding are unlikely to be due to the differences in Mg^{2+} concentration since we see slightly tighter binding at higher Mg^{2+}. The difference may be due to an intrinsic difference in binding affinity between triplets and poly(U), to statistical factors, or to differences in the ribosome preparations. Lipecky et al. (1977) reported association constant values of 0.049–40 μM^{-1} [uncorrected for statistical factors, see McGhee & von Hippel (1974)] for oligouridylate–S1 complexes where the nucleotide chain length varied from 5 to 80 nucleotides. Our measurements of AUG triplet binding were in agreement within a factor of 2 with those of Kang et al. (1979) at high (25 mM) Mg^{2+} concentrations.

The remaining equilibrium constants were determined by model fitting and thermodynamic dependence. Consider the assumptions of model I: That there is only a single S1 binding site on the ribosome follows from the work of Laughrea & Moore (1977), who demonstrated that heat-activated subunits bind only one copy of S1. Laughrea & Moore (1977) suggested two binding sites for S1 on non-heat-activated ribosomes, with one binding site having much lower affinity. We see no evidence for such a low affinity site with these heat-activated ribosomes.

The second assumption (S1 binds one triplet) was made so that we could consider a simple model. Later models did not make this assumption and allowed two binding sites. There is evidence to suggest S1 does in fact have two triplet binding sites (Draper et al., 1977; Draper & von Hippel, 1978a,b); however, other workers (Mülsch et al., 1981) have maintained that this is an artifact caused by precipitation of the ribonucleic acid–S1 complex. We conclude that, with respect to UpUpUp binding, one binding site will adequately fit the observed data. If additional sites are present, low cooperativity for binding results and either there is little heterogeneity in binding or the affinities differ greatly so that at these concentrations binding to the second site is not observed.

The third assumption (ribosomes bind one triplet) was made because of the homogeneity of the triplet quenching curves. This does not rule out the possibility of having multiple binding sites of about equal affinity or a much weaker binding site that is not populated significantly in our experiments. The curves in Figure 3 show that a second nonquenching site of equal affinity leads to significantly worse fits to the data but that having two sites of equal affinity where either site binding leads to fluorescence quenching is very difficult to distinguish from single-site binding. The model fitting does lead to an interesting result for the equilibrium constant, K_5, for binding of T to RbS as a function of Mg^{2+}. The model predicts that the affinity of RS for triplets *decreases* with increasing Mg^{2+}. The experiment where labeled ribosomes were reconstituted with S1 and then fluorescence quenching was measured upon adding T gave values for K_5 within experimental error of those found by data fitting. This suggests that the site for triplet binding to the ribosome monitored by S1 anisotropy is the same as the site measured by quenching of ribosome fluorescence. The labeling site for the ribosome is protein S18. Cross-linking studies (Traut et al., 1979; Boileau et al., 1981) and immuno electron microscopy (Stoffler et al., 1979; Lake, 1979) have shown this protein to be very close to S1 in the intact ribosome, possibly within 5 Å. Affinity labeling studies with oligonucleotide reagents (small mRNA analogues) have labeled S1 and S18 (Cooperman, 1978). Data fitting for model V where there are two triplet binding sites on the ribosome shows that if more than one site is present and both are significantly populated with triplets in our experiments, these sites have about equal affinity. However, in the presence of S1 (Table I, K_5) binding of T is markedly altered at high Mg^{2+}, and the Mg^{2+} dependence is reversed. Triplet binding (Rb + T) in this domain thus appears to be such that binding is uniformly tighter at low Mg^{2+} and uniformly weaker at high Mg^{2+}.

For the binding of two different ligands X and Y to a macromolecule P, Weber (1975) has proposed a simple procedure for depicting the overall heterotropic free energy of interaction, $\Delta G^{\circ}_{P,XY}$, the "coupling free energy". This quantity

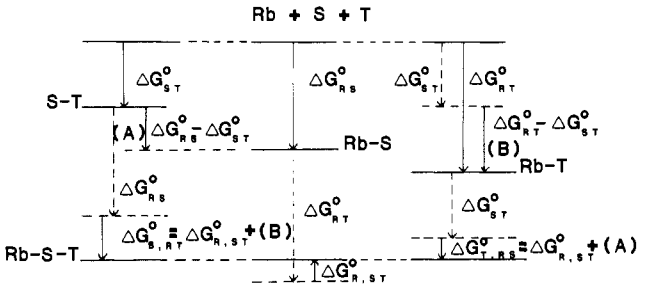
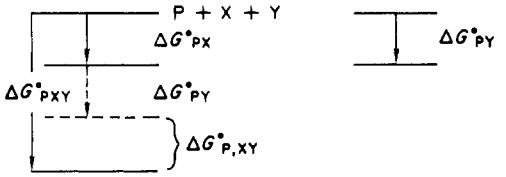


FIGURE 7: Qualitative representation of binding and coupling free energies for the ternary system ribosomes (R)–S1 (S)–triplets (T). The various interactions are not drawn to scale.

tells us by how much and in what direction we err in estimating the overall free energy of binding ($\Delta G°_{PXY}$) from knowledge of the separate binding free energies $\Delta G°_{PX}$ and $\Delta G°_{PY}$. Thus, $\Delta G°_{P,XY} = \Delta G°_{PXY} - \Delta G°_{PX} - \Delta G°_{PY}$ as shown:



A negative value of $\Delta G°_{P,XY}$ implies cooperative heterotropic interaction; i.e., the binding of X to P enhances the subsequent binding of Y. There is only one heterotropic or coupling interaction in this system, since $\Delta G°_{P,XY} = \Delta G°_{P,YX}$.

The situation is more complex, however, when all three binary interactions occur in a ternary system in addition to formation of the ternary complex. In this case, there are three linearly independent coupling interactions, so in the same ternary system, one can have anticooperative, noncooperative, and positive cooperative interactions. These interactions can be verbalized in various ways, some of which sound paradoxical. They are depicted in Figure 7 and can be written as follows (where R stands for Rb, ribosome devoid of S1):

$$\Delta G°_{R,ST} = \Delta G°_{RST} - \Delta G°_{RS} - \Delta G°_{RT}$$

$$\Delta G°_{S,RT} = \Delta G°_{RST} - \Delta G°_{ST} - \Delta G°_{RS} = \Delta G°_{R,ST} + \Delta G°_{RT} - \Delta G°_{ST}$$

$$\Delta G°_{T,RS} = \Delta G°_{RST} - \Delta G°_{ST} - \Delta G°_{RT} = \Delta G°_{R,ST} + \Delta G°_{RS} - \Delta G°_{ST}$$

We could consider the effects of Mg^{2+} on these interactions by developing a formalism for quaternary interactions or merely note the Mg^{2+} dependencies of the various coupling energies, in which case we may find that whether a given interaction is cooperative or anticooperative depends on the Mg^{2+} concentration. Owing to the linear independence of the three coupling interactions, we may find, for instance, that with respect to binding of S and then T to the ribosome the interaction is anticooperative; i.e., prior binding of S suppresses subsequent binding of T to Rb. On the other hand, we may shift our point of reference to S and consider the binding of Rb and subsequent binding of T and find that this interaction is *positively* cooperative: binding of Rb to S enhances the further binding of T to form the RbST ternary complex. The third vantage point is to consider sequential binding of Rb and S to the triplet.

Within the framework of model I, but not drawn to scale, Figure 7 shows a schematic diagram of the binding and interaction energies for ribosomes, S1, and triplets. The data are given in Table II. The binding of S1 and subsequent triplet binding to the ribosome are not independent. At 10 mM Mg^{2+} there is large negative or anticooperativity. The sum of the $\Delta G°$ values for Rb + S → RbS and Rb + T → RbT gives a total calculated $\Delta G°$ of −21.1 kcal/mol. The measured value for Rb + S → RbS and RbS + T → RbT is only −19.9 kcal/mol. The binding of S1 to ribosomes at 10 mM Mg^{2+} reduces the affinity for the binding of triplet to form the ternary complex by about 1.2 kcal/mol ($\Delta G°_{R,ST}$). At 6 mM Mg^{2+}, however, there is an *enhancement* of triplet binding, a positive cooperative interaction, of 0.3 kcal/mol.

Table II: Binding and Coupling Free Energies (kcal/mol) for Ribosomes (R), S1 (S), and Uridyl Triplets (T) according to Model I[a]

$[Mg^{2+}]$ (mM)	$\Delta G°_{ST}$	$\Delta G°_{RS}$	$\Delta G°_{RT}$	$\Delta G°_{S,RT}$	$\Delta G°_{T,RS}$	$\Delta G°_{R,ST}$
10	−8.0	−11.1	−10.1	−0.8	−1.8	1.3
6	−8.3	−9.6	−9.9	−1.8	−1.6	−0.3

[a] Experiments were performed in buffer A with magnesium acetate as indicated. T = 20 °C.

A similar analysis can be made for triplet and ribosome binding to S1. At 6 mM Mg^{2+} there is a positive cooperative interaction with $\Delta G°_{T,RS}$ = −1.8 kcal/mol. This is reduced at 10 mM Mg^{2+} to −0.8 kcal/mol. Clearly, Mg^{2+} has a strong effect on these interactions. The equilibrium constants most affected by $[Mg^{2+}]$ are K_2 and K_5. For K_2 (S1 binding to ribosomes, $\Delta G°_{RS}$), there is an enhancement of binding with increasing $[Mg^{2+}]$; for K_5 (triplet binding to RbS1), there is a decrease in affinity with increase in $[Mg^{2+}]$. Changing the Mg^{2+} concentration from 6 to 10 mM *decreases* the coupling free energy by about 1 kcal/mol for *both* the cooperative interaction of S1 and triplets with the ribosome and the cooperative triplet and ribosome interaction with S1. It is to be emphasized that this treatment, in terms of model I, makes no assignment as to fixed sites. That is, T may very well bind to S in the ST complex at a different location than in the ternary complex RbST, in which T might not even be in direct contact with S.

The above equations for the three heterotropic interactions are each written in terms of $\Delta G°_{RS}$, the binary interaction (R + S) with the largest sensitivity to Mg^{2+} concentration for binding on the 70S ribosome [there is very little Mg^{2+} sensitivity for S1 binding to the 30S subunit (Goss et al., 1983)]. The Mg^{2+} dependencies of the three heterotropic interactions are essentially all due to the sensitivity of $\Delta G°_{RS}$ to changes in Mg^{2+} concentration.

As benchmarks for these heterotropic interactions, the coupling free energy for oxygen and DPG with respect to hemoglobin is 1.3 kcal (Tyuma et al., 1971); that of NADH and oxalate with lactate dehydrogenase is −1.5 kcal/mol (Kolb, 1974).

It has been proposed that the main function of S1 may be to serve as an "unwinding" protein (Bear et al., 1976; Szer et al., 1976; Thomas et al., 1978). These results demonstrate that S1 also enhances binding of the nonhelical nucleotide triplet to ribosomes. The mechanism of the interaction is unknown but may be due to the conformational change S1 induces in the 30S subunit or unwinding of the 16S rRNA. The Mg^{2+} dependence of the binding of S1 to 30S and 70S ribosomes argues against assigning the unwinding process as the primary S1–ribosome interaction, however, since unwinding should be accompanied by a loss of Mg^{2+} [Gorenstein & Luxon, 1979; Rigler & Wintermeyer (1983) and references cited therein]. It is well-known that binding of divalent cations can further aggregation of multisubunit proteins (Chiancone et al., 1976, 1980; David & Daniel, 1974), and studies by Laughrea & Moore (1978) and Boni et al. (1982) point to the

importance of protein–protein interactions in the binding of S1 to ribosomes. The data fitting for model V suggests that one can think of the ribosomal triplet binding site as a domain where all triplets (if more than one are bound) have about equal affinity. At low Mg^{2+} (6 mM), the presence of S1 enhances binding. At higher Mg^{2+} (10 mM), there is a reduced affinity for triplets when S1 is present. The cooperative interactions described above quantitate the exquisite control exerted by S1 and Mg^{2+} on the binding of triplets, and presumably mRNA, to the ribosomes.

Registry No. UpUpUp, 3504-15-2; ApUpG, 3494-35-7; Mg, 7439-95-4.

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