Studies on the kinetic sequence of *in vitro* ribosome assembly using cibacron blue F3GA as a general assembly inhibitor

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SUMMARY

We have found that all *E. coli* ribosomal proteins strongly bind to an agarose affinity column derivatized with the dye Cibacron Blue F3GA. We have also shown that the capacity to bind the dye is lost when the proteins are organized within the structure of the ribosome or are members of pre-formed protein-RNA complexes. We conclude that the binding of ribosomal proteins to this dye involves specific protein-RNA recognition sites. These observations led us to discover that Cibacron Blue can be used to inhibit *in vitro* ribosome assembly at any stage of the assembly process. This has allowed us to determine a kinetic order of ribosome assembly.

INTRODUCTION

The bacterial ribosome is the most complex organelle that has been successfully disassembled and reassembled *in vitro*. Although the methodology necessary to accomplish ribosome reconstitution has been available for many years (1), very little about the actual molecular mechanism of the reaction is known today. One reason the mechanism of ribosome self-assembly has received so little attention is the fact that transient intermediates in the reaction process have proven to be nearly impossible to isolate. Up to now the only particles successfully isolated that can be postulated to be intermediates in the pathway of ribosome assembly are the so called "reconstitution intermediates" or "RI particles" (2, 3). These particles, which accumulate at low temperatures due to the existence of a temperature dependent transition, have been studied extensively. However, the information obtained from these investigations is limited by the fact that only a single step, in a reaction involving a multitude of steps, is actually accessible to examination. It is essential that, if we are to

gain any further insight into the mechanism of ribosome assembly, we must first develop techniques which will permit the isolation of many more particles intermediate in the kinetic pathway of assembly.

One of our approaches to the isolation of kinetic intermediate particles has been to survey chemical reagents which potentially might have the capacity to block ribosome assembly at early stages in the reaction. We reasoned that, as the vast majority of the ribosomal proteins have very similar chemical and physical properties, there may exist a set of mechanistic rules used by all or most of the proteins when they enter into a stable association with the "growing" protein-RNA complex. Thus, our search for such an inhibitor centered on chemicals which might have a generalized effect either on protein-protein or protein-RNA interactions. This line of attack eventually led us to investigate the triazine dye, Cibacron Blue F3GA.

Cibacron Blue F3GA is a sulfonated polyaromatic dye that is routinely used in a form derivatized to dextran (dextran-blue) as a marker for the determination of the exclusion volume of gel filtration columns. By chance in 1968 Haeckel et al. (4), discovered that pyruvate kinase co-eluted with dextran blue due to the binding of the enzyme to the dye. Since this original observation, a large number of nucleotide-requiring enzymes have been found to form tight complexes with Cibacron Blue as well as several other dyes (5). Indeed, this phenomenon has led to a new methodology for the purification of enzymes of the oxidoreductase, kinase, transferase and synthetase classes, which utilizes Cibacron Blue and some other dyes bound to agarose as a chromatographic support medium. Most of the enzymes which bind to such a column can be eluted with a very low concentration of their respective effectors or with a high concentration of salt. It has been proposed, and supported by considerable evidence, that these dyes bind specifically to the structural domain of proteins referred to as the "dinucleotide fold" (6). This notion has been recently supported by X-ray crystallographic studies of a complex between Cibacron Blue and liver alcohol dehydrogenase (7). It appears that the high

flexibility of the dye molecule enables it to mimic different nucleotides and to bind to their respective protein binding sites with an "induced" specificity. With this background information, we investigated the possibility that Cibacron Blue could meet our requirements of a generalized inhibitor of ribosome assembly by binding to those ribosomal proteins capable of forming a specific interaction with ribosomal RNA.

In this report we summarize our evidence that Cibacron Blue can bind to all ribosomal proteins when the proteins are free in solution, but not when the proteins are incorporated into a protein-RNA complex. These properties of the dye are precisely those we had hoped to find in our search for a generalized inhibitor of ribosome self-assembly. Further control experiments have shown that selection of a proper concentration range permits the expression of these properties without any detectable "stripping" of proteins already incorporated into the protein-RNA particle. We have therefore pursued the investigation of Cibacron Blue and have found it to behave as a potent inhibitor of both 30S and 50S ribosome assembly. Polyacrylamide gel electrophoresis analyses of the proteins bound to the protein-RNA complexes obtained after the addition of Cibacron Blue at different times during the assembly reaction, show a dramatic time dependent incorporation of all proteins into the RI particle. We suggest that the particles isolated after Cibacron Blue treatment may in fact be true kinetic intermediates in the pathway of ribosome assembly. We have analyzed the protein composition of many of these intermediates and propose a temporal sequence of assembly for both the 30S and 50S ribosomes.

MATERIALS AND METHODS

Ribosomes and their subunits were isolated from E. coli MRE600 as previously described (8). Ribosomal proteins were extracted from purified 30S and 50S subunits by acetic acid extraction (9). Ribosomal RNAs were prepared by phenol extraction and preparative sucrose gradient centrifugation (10).

Ribosome assembly reactions were performed essentially in

the manner described by Traub and Nomura (1). The protein:RNA ratio was 2:1.

The 30S reconstitution buffer consisted of 0.33M KCl, 0.03M Tris-HCl, pH 7.6 and 0.5 mM DTT or 2 mM 2-mercaptoethanol. The 50S reconstitution buffer consisted of 0.4M NH₄Cl, 0.004 M Mg-Acetate, 0.02 M Tris-HCl, pH 7.4, 0.02 mM EDTA, 0.5 mM DTT or 2mM 2-mercaptoethanol.

Cibacron Blue F3GA (Pierce) was modified with lysine by incubation at 44°C for 1 hr using a 10 fold molar excess of lysine to dye. Dioxane (final concentration 25%) was added to the reaction product to increase its solubility. The final concentrations of dioxane have no effect on the reconstitution reaction.

Polyacrylamide gel electrophoresis analyses (PAGE) in one dimension were performed using the systems described by Voynow

Figure 1. Elution profile of TP-70 ribosomal proteins bound to Cibacron Blue agarose. Two OD units (230 nm) of TP-70 ribosomal proteins were percolated through a column (1.4 cm i.d. x 6 cm) of Cibacron Blue Agarose. The proteins were in 1 ml of 30S reconstitution buffer). Nineteen ml of the same buffer was then passed through the column. The column was then eluted with 6M guanidine HCl in the same buffer. The flow rate was about 0.7 ml/min. One ml fractions were collected. The point at which guanidine HCl was added to the column is indicated. Absorption of the fractions was measured at 280 nm as guanidine HCl absorbs strongly at 230 nm.

and Kurland (11). Two dimensional gel analyses were done as previously reported (12) except that a capillary tube (2 mm I.D.) was used for the first dimension (13).

RESULTS

All Ribosomal Proteins Bind Cibacron Blue-agarose.

In an effort to determine the extent to which ribosomal proteins are capable of binding Cibacron Blue F3GA, we prepared a mixture of total ribosomal protein extracted from 70S ribosomes (TP-70). The protein mixture was percolated through a column of Cibacron Blue-Agarose (Sigma). As illustrated in figure 1, about a fifth of the total 280 nm absorbing material was not retained by the column. The remaining bound fraction could be quantitatively eluted by 6 M-guanidine HCl. Two dimensional polyacrylamide gel electrophoresis analyses of these fractions show that virtually all 70S proteins are bound to the column and

Figure 2. 2-Dimensional PAGE analysis of TP-70 ribosomal proteins bound to Cibacron Blue agarose (A) as shown in Figure 1, and TP-70 standard (B).

Figure 3. Binding of 30S (A), 50S (B), and 70S (C) ribosomes to Cibacron Blue agarose. The ribosomes were dialyzed against the 30S reconstitution buffer and 8-10 OD₂₆₀ units of 30S, 50S or 70S ribosomes in 1 ml of the same buffer were percolated through a column of Cibacron Blue Agarose under conditions similar to those of Figure 1. Fifteen ml of 30S reconstitution buffer was passed through the column. The column was then eluted with 6M guanidine HCl in the same buffer. 1 ml fractions were collected. The point of addition of guanidine HCl to the column is shown.

eluted by the guanidine wash (Figure 2). No proteins were detected in the fraction eluted before the guanidine washing of the column, following dialysis and lyophilization. We conclude that all ribosomal proteins have significant binding affinities for Cibacron Blue F3GA.

The Majority Of The Ribosome Populations Do Not Bind Cibacron Blue-agarose

We determined whether the ribosomal proteins bind Cibacron Blue F3GA when they are members of intact ribosomes. We examined the propensities of 70S, 50S and 30S ribosomes to bind Cibacron Blue-agarose columns. The resulting elution profiles are reproduced in figure 3. It is evident that the majority of all three ribosome populations fail to bind the column material and are washed off with the void volume of buffer. This is especially true for 50S ribosomal subunits which show a recovery in the buffer wash of 98% of the applied material. In the case of 70S and 30S ribosomes, a small, but significant fraction (about 10%) of the applied particles bound to the Cibacron Blue-

agarose and was eluted by 6 M-guanidine. We have analyzed these fractions of 70S and 30S ribosomes for their protein composition by two-dimensional polyacrylamide gelelectrophoresis. Both the fractions that failed to bind the column and the fractions that bound the Cibacron Blue-agarose showed a normal complement of all the ribosomal proteins (data not shown). In the case of the bound 70S and 30S ribosomes, however, we did observe a dramatic enrichment of high molecular weight, acidic proteins which often "contaminate" ribosome preparations. It is interesting to remember that so called "native" 30S ribosomes are associated with a similar complement of high molecular weight proteins (14). Such ribosomes have been found to contain large amounts of initiation factors. It is conceivable that the presence of a set of such proteins, capable of interacting with messenger RNA and transfer RNA, attached to the 70S and 30S ribosomes could interact with Cibacron Blue and account for the fraction of the ribosomes retarded on the column. In any event the conclusion we prefer to emphasize is that the large majority of either the 70S, 50S or 30S ribosome populations do not detectably interact with Cibacron Blue F3GA, despite the fact that they contain a full complement of proteins capable of strong binding to the dye when free in solution. We interpret this to mean that the sites involved in Cibacron Blue binding on the surfaces of the proteins must become inaccessible to the dye when the proteins become incorporated into the complex structure of the intact ribosome. It is precisely these properties of Cibacron Blue that fulfill our criteria for a chemical potentially capable of inhibiting in vitro ribosome assembly.

Cibacron Blue F3GA Pre-bound to Ribosomal Proteins Blocks Their Assembly Into Protein-RNA Complexes

We have shown above that Cibacron Blue F3GA has a strong binding affinity for all ribosomal proteins when the proteins are free in solution. We have also presented evidence that the Cibacron Blue binding sites become inaccessible when the proteins are components of the ribosome, suggesting that the formation of the intermolecular bonds involved in the ribosome complexes effectively blocks the sites recognized by the dye. With these results as background, we examined the corollary possibility that

Figure 4. 1-Dimensional PAGE analysis of 30S (A) and 50S (B) reconstitution intermediates formed in presence of Cibacron Blue. TP-30 or TP-50 proteins were treated with the dye at the dye:protein molar ratios shown, immediately followed by the addition of appropriate RNAs, all at 0° C. The mixtures were incubated for 1 hr at 0° C, cleared by a low speed centrifugation and centrifuged through a layer of 12% sucrose in respective reconstitution buffer. The pellet was analyzed for proteins by electrophoresis in the hard urea gel (A) or soft urea gel (B) (11).

pre-binding of the dye to the proteins would prevent effective protein-RNA interactions normally leading to protein-RNA complex formation.

Our approach to test the effect of Cibacron Blue on protein-RNA complex formation, was to determine whether the dye can block in vitro ribosome self-assembly. In these experiments free Cibacron Blue F3GA was used rather than agarose-bound dye. The Cibacron Blue was first modified with lysine at alkaline pH to displace its very reactive chlorine atom. Early investigations with the unmodified dye indicated that it could readily form covalent adducts with free ribosomal proteins. A complete mixture of either 30S (TP-30) or 50S (TP-50) proteins was treated with increasing ratios of the modified Cibacron Blue. The dye-treated protein mixtures were then incubated with the appropriate ribosomal RNAs using the buffer conditions prescribed for the reconstitution of the corresponding subunit (1, 15). The reconstitution mixtures were centrifuged through a layer of 12% sucrose to pellet the protein-RNA complexes. The protein compositions of the protein-RNA particles were determined by one dimensional polyacrylamide gel electrophoresis. Photographs of the gel patterns obtained for both 30S and 50S ribosome reconstitution are shown in figures 4A and 4B respectively. In both reconstitution systems it was found that a dye to protein molar ratio of approximately 100:1 almost completely prevented all ribosomal protein-RNA complex formation. At a somewhat lower molar ratio of 40:1, the dye blocked all assembly except for several strong RNA binding proteins (S8, L1 and L24).

Cibacron Blue F3GA Inhibits Ribosome Self-assembly At All Stages

Having shown that a molar ratio of Cibacron Blue to ribosomal proteins of from 40:1 to 100:1 effectively prevents most of the proteins from associating with protein-RNA complexes, we investigated the possibility that the dye could also inhibit this process at later stages. We carried out the reconstitution reaction for both 30S and 50S using the conditions optimal for the respective subunits except that the incubation was done at 0°C. At this temperature the assembly of the two subunits proceeds normally until reaching the first reconstitution intermediate stage. It was hoped that this low temperature would

Figure 5. 2-Dimensional PAGE analysis of 30S reconstitution mixtures inhibited by Cibacron Blue at different times during assembly. For the 0 time experiment, the dye was added to the proteins immediately prior to addition of the RNA. A dye:protein molar ratio of 50 was used.

Figure 6. 2-Dimensional PAGE analysis of 50S reconstitution mixtures inhibited by Cibacron Blue at different times during assembly. Further details as in Figure 4.

slow down the formation of intermediate protein-RNA complexes substantially, thereby increasing the facility of their isolation.

A series of reconstitution mixtures were prepared and at various times the lysine modified Cibacron Blue was added at a molar ratio of dye:protein of 50:1. All reaction mixtures were allowed to incubate at 0°C for 1 hr followed by centrifugation through a layer of 12% sucrose as described above. The proteins associated with the ribosomal RNAs were analyzed by 1-dimensional and 2-dimensional polyacrylamide gel electrophoresis

Figure 7. 1-Dimensional PAGE analysis of 30S ribosomal proteins extracted from ribosomes treated with Cibacron Blue.

and the photographs of some of the 2-dimensional gel patterns are shown in figures 5 and 6. In both the 30S and the 50S reconstitution systems, we observe a time dependent appearance of proteins incorporated into a stable protein-RNA complex. In control experiments without the dye, only RI particles similar to the most mature RI particles shown were observed. Our interpretation of these results is that the Cibacron Blue effectively blocks ribosome self-assembly at virtually any stage up to the formation of the first RI particles. We suggest that this inhibition is a direct consequence of the dye's capacity to bind sites on the surface of the free proteins that are critical for incorporation of the proteins into assembly intermediate particles. If this interpretation is accurate, the judicious use of Cibacron Blue should permit the isolation of particles intermediate in the kinetic pathway of assembly.

At the Concentrations Used To Block Assembly, Cibacron Blue Does Not Remove Proteins From Intermediate Particles

A possible artifact in the use of Cibacron Blue to inhibit ribosome assembly is that the dye could strip proteins from

intermediate particles, thereby creating an incorrect picture of the temporal pathway. Unfortunately, this is a difficult proposition to disprove, but we have done several experiments which we believe argue against this possible pitfall. First, we have treated intact 30S and 50S ribosome preparations with Cibacron Blue, using conditions similar to those employed to inhibit in vitro assembly as described above. Specifically, the ribosome suspensions were treated with increasing ratios of the dye for 1 hr at 0°C and centrifuged through a layer of sucrose. The pelleted ribosomes were then analyzed by one dimensional polyacrylamide gel electrophoresis. A photograph of the resultant gel patterns obtained for 30S ribosomes is presented in figure 7. No stripping of any ribosomal proteins was detected up to a dye to protein ratio of 30:1. Beyond that point, mainly proteins S2, S3, and S21 were observed to be removed by the dye treatment. Although the rest of the proteins did diminish in amount with dye:protein ratios higher than 30:1, the relative amounts of these proteins remained the same. Similar experiments with 50S ribosome preparations showed absolutely no stripping even up to ratios of 270:1.

Although the results with 30S and 50S ribosomes were encouraging, it would be more significant to examine the effect of the dye on particles which are genuine intermediates in the assembly pathway. To this end we prepared RI particles (1, 3) for both 30S and 50S reconstitution systems, and treated them with increasing ratios of Cibacron Blue. Both the RI-30S and the RI-50S were generally resistant at the ratios of the dye to protein concentrations used to inhibit assembly and even at the higher ratios only two or three proteins showed any significant degree of loss in the presence of Cibacron Blue. Photographs of the gel patterns for the Cibacron Blue treatment of RI-30S particles are shown in figure 8. We conclude that dissociation of proteins does not take place at the concentrations of Cibacron Blue used to inhibit ribosome assembly.

DISCUSSION

Our results suggest that the dye, Cibacron Blue F3GA, can bind all ribosomal proteins when free in solution but that the

Figure 8. 1-Dimensional PAGE analysis of 30S RI particles treated with Cibacron Blue.

dye cannot bind the proteins when they themselves are members of a protein-RNA complex. These data have led us to believe that Cibacron Blue effectively competes with ribosomal RNA for the same recognition sites on most ribosomal proteins. This might mean that most ribosomal proteins have specific ribosomal RNA binding sites, which is an idea that has not been previously suggested by experimental evidence, although many workers have suspected that it might be the case simply because ribosomes consist of two-thirds RNA.

Another argument that all ribosomal proteins have specific sites capable of binding ribosomal RNA comes from our discovery that Cibacron Blue appears to be able to act as a general inhibitor of total ribosome assembly. It is evident that this dye can effectively stop in vitro assembly at any intermediate stage without stripping proteins from pre-assembled complexes. This observation indicates that the dye can out-compete all ribosomal proteins for their proper place in the ribosome structure, presumably by binding a specific site on the

Figure 9. Proposed temporal sequence of 30S and 50S ribosome assembly.

proteins. This has allowed us to isolate many particles which we suggest are kinetic intermediates in *in vitro* ribosome assembly. We have qualitatively analyzed some of these intermediates for protein composition. Our main conclusion is that the assembly of both the 30S and 50S particles can be broken down into a series of distinct steps. These stages in our proposed assembly pathways are summarized in figure 9.

Figure 10. Schematic illustration of the relationship of the proposed temporal sequence of 30S assembly to the protein topography determined by neutron scattering (16).

Recent results using neutron scattering to determine 30S ribosomal protein topography (16) can be related to the temporal sequence of assembly we propose. Figure 10 is a set of drawings which show a definite relationship between the in vitro kinetic assembly sequence and the three-dimensional arrangement of the 30S proteins. We conclude that proteins that assemble together in a temporal sequence, do so because they are physically close within the structure and probably have mutual sites for interaction. This idea is also supported by an examination of the known information about the 30S protein binding sites on 16S RNA (17). The 16S RNA binding regions can be divided into three sections. Our data indicate that the central region assembles first, followed by the 5' domain and then the 3' domain.

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