Association Factor of Ribosomal Subunits from *Bacillus stearothermophilus*

(ribosomal cycle/*E. coli*/dissociation factor/heat inactivation)

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ABSTRACT The isolation of a new factor, which can cause the *in vitro* association of 30S and 50S ribosomal subunits at low Mg^{++} concentration, is described. The association factor is eluted together with the dissociation protein when ribosomes of *Bacillus stearothermophilus* are washed with salt solutions of high concentration. The association activity is heat-stable, whereas dissociation factor is inactivated after 10 min at 80°C. This treatment allows the separation of both factors. Several properties rule out the possibility that uncharged, aminoacyl-, or peptidyl-tRNA are responsible for the association process described in this report. Digestion with trypsin shows that the association factor contains at least two components, one of which is a protein.

Bacterial ribosomes can be found as 70S particles, either free or complexed in monosomes and polysomes, and as 30S and 50S subunits (1). Several different conformations of each type of particle may also exist (2-8).

Much evidence has accumulated to show that a cycle of ribosome dissociation and association of 30S and 50S subunits to 70S monosomes occurs during the successive rounds of protein synthesis (1, 9-12). The conditions that favor dissociation and association have been thoroughly studied. 70S ribosomes tend to dissociate with increasing temperature (13), in the presence of sulfhydryl reagents (14), or when the ratio of monovalent- to divalent-ion concentrations is high (15, 16).

A protein factor that causes the dissociation of 70S particles into 30S and 50S subunits has been recently obtained from different bacteria (17-20). This factor (DF), necessary for cell survival, presumably dissociates ribosomes *in vivo* (12).

Ribosomal subunits tend to reassociate at high Mg^{++} concentration (21), in the presence of polyamines (22), or when the subparticles are bound to peptidyl-tRNA or aminoacyl-tRNA (21).

In a recent study on the effect of several antibiotics, we observed that neomycin was also able to reassociate subunits (23); this associating capacity of the antibiotic was markedly enhanced in the presence of a heated ribosomal wash fraction from *Bacillus stearothermophilus*. The possibility arose that an association factor (AF) could be extracted, along with initiation and dissociation factors, from ribosomes of the thermophilic bacteria by salt solutions of high concentration.

In this report we will describe the isolation and some properties of a factor that can produce the *in vitro* association of ribosomal subunits to form a monomer at a relatively low Mg^{++} concentration, similar to the physiological level.

Several attempts to detect the reversibility of ribosomal dissociation as mediated by DF have failed, except when the concentration of Mg^{++} was increased (1, 17).

MATERIALS AND METHODS

Escherichia coli D₁₀ and *B. stearothermophilus* 1503-4R were grown as indicated (18).

Trypsin (3-times crystallized) was purchased from Worthington; soybean trypsin-inhibitor (Worthington) was a generous gift from Dr. E. Cabib. RNase-free sucrose was obtained from Mann and tRNA from *B. stearothermophilus* was prepared by the method of von Ehrenstein (24).

Preparation of Ribosomal Subunits. *E. coli* and *B. stearothermophilus* cells were harvested from cultures in the logarithmic phase after slow cooling in order to allow the completion and release of nascent polypeptide chains. Under these conditions we have shown that most of the ribosomes are present as free 70S particles, which have been released from the mRNA and no longer carry peptidyl-tRNA (11).

Bacteria were broken and ribosomes were obtained as described (18).

The ribosomes, suspended in 10 mM Tris·HCl (pH 7.8)-2 mM magnesium acetate-5 mM 2-mercaptoethanol, were dissociated into their subunits either by dialysis for 12-16 hr against a large volume of 10 mM Tris·HCl (pH 7.8)-0.4 mM magnesium acetate-50 mM KCl-1 mM 2-mercaptoethanol or by suspension overnight in a solution containing 10 mM Tris·HCl (pH 7.8)-1 mM magnesium acetate-1 M KCl-2 mM 2-mercaptoethanol. The ribosomes washed with 1 M KCl, were sedimented by centrifugation at 165,000 × *g* for 5 hr. The pellet was resuspended in a small volume of buffer A (10 mM Tris·HCl (pH 7.8)-5mM magnesium acetate-50 mM KCl-1 mM 2-mercaptoethanol).

Purified 30S and 50S subunits from *E. coli* were isolated by centrifugation of 30-40 A₂₆₀ units of dissociated ribosomes in a 12-ml linear sucrose gradient (5-20%) in 20 mM Tris·HCl (pH 7.8)-10 mM magnesium acetate-50 mM KCl (buffer B). After centrifugation in a Spinco SW40 Ti rotor for 200 min at 35,000 rpm, the fractions corresponding to the 30S and 50S peaks were pooled and dialyzed against buffer A. All operations were performed at 0-4°C. The subunits were stored either in the refrigerator or at -70°C.

Preparation of Association Factor. The pellet of ribosomes from *B. stearothermophilus* was suspended gently with a magnetic stirrer in 10 mM Tris·HCl (pH 7.8)-1 M KCl-1 mM magnesium acetate-2 mM mercaptoethanol. After

Abbreviations: AF, association factor. DF, dissociation factor.

FIG. 1. Association of ribosomal subunits from *B. stearothermophilus*. Conditions were those described for the standard assay, except that ribosomal subunits were from *B. stearothermophilus* and the incubation was performed at 65°C. (a) without AF; (b) with 80 µg of AF.

15 hr, the suspension was centrifuged at $165,000 \times g$ for 5 hr, and the upper 80% of the supernatant fluid was taken and stored at -15°C . This preparation contained AF, as well as initiation factors and DF, but the DF could be destroyed by heating for 10 min at 80°C (18), whereas AF was stable after this treatment. On the other hand, when the ribosomal extract was dialyzed for 5 hr against buffer A, part of AF precipitated free of DF and could be redissolved in 1 M KCl solution. This preparation of AF showed associating activity without being heated.

Ribosomes from *B. stearothermophilus* in the stationary phase gave good preparations of AF that contained only a small proportion of DF.

Standard Assay. The reaction mixture (0.2 ml) contained 10 mM Tris·HCl (pH 7.8), 5 mM magnesium acetate, 50 mM KCl, 2.5 mM dithiothreitol, 0.4–0.6 A_{260} units of 30S and 50S ribosomal subunits (ratio 1:2), and AF (2–100 µg of protein). Unless otherwise indicated, incubations were for 5 min at 55°C with particles from *E. coli*; the tubes were then chilled to 0°C . Samples of 0.16 ml were immediately layered on 12 ml of 5–20% linear sucrose gradients made up in buffer B. After centrifugation for 75 min at 35,000 rpm in a SW40 rotor at 5°C , gradients were analyzed by monitoring at 254 nm with an ISCO ultraviolet analyzer.

Association was calculated from the area under the absorbance tracing of the peaks or by use of an empirical formula (18) that gives the value $D = 100 - A$, where D and A

FIG. 2. Association of *E. coli* ribosomal subunits. Sedimentation patterns of standard incubation mixtures. (a) control without AF; (b) with 100 µg of AF.

FIG. 3. Time course of the association. Reaction mixtures and analysis by sucrose gradient centrifugation were as indicated in Methods.

mean the percentages of dissociation and association, respectively.

RESULTS

Properties of the association factor

The associating activity was eluted from *B. stearothermophilus* ribosomes together with DF. For this reason, AF was barely detectable before inactivation of DF by heating. When stationary-cell ribosomes were used as a source, the association effect prevailed because these ribosomes liberate little DF, as we have shown previously (18).

Figs. 1 and 2 demonstrate that AF was active with ribosomal subunits of both *B. stearothermophilus* and *E. coli* D₁₀. Control experiments demonstrated that under the conditions used neither isolated subparticle gave any detectable amount of dimers (either 50S–50S or 30S–30S) after incubation in the presence of AF.

Most of the experiments described in this work were done with purified ribosomes from *E. coli* because *B. stearothermophilus* subunits aggregate very easily *in vitro*, especially at low temperature.

The association of subunits increased linearly with respect to added factor, to about 50% association.

Fig. 3 shows that the reaction occurred very rapidly. It leveled off after 1–2 min, and reached a plateau at about 5 min.

Subunits produced by DF most likely contained this factor bound to the 30S subparticles. Nevertheless AF was still able to reassociate these subunits, as is indicated in Fig. 4.

FIG. 4. Association of subunits obtained by DF action. Dissociation of 70S ribosomes was at 45°C (28) in 10 identical reaction mixtures containing 50 µg of DF. After 1 min of incubation, 30 µg of AF was added to five of the tubes and the incubation was continued. At the times indicated, each pair of assays (with and without AF) was chilled and analyzed. (■) percentage of dissociation without AF; (●) percentage of dissociation after addition of AF.

FIG. 5. Association of subunits at different temperatures. Standard reaction mixtures contained 40 μ g of AF (●) or 40 μ g of tRNA (■). The broken line indicates spontaneous association without any additions.

On the other hand, DF could dissociate the monomer obtained by the action of AF on the ribosomal subunits (M. Garcia-Patrone, unpublished results).

Several studies (2, 25) have indicated the participation of uncharged tRNA, aminoacyl-, or peptidyl-tRNA in the association of 30S and 50S subparticles. Therefore, the possibility that the active component of our crude AF preparations was some kind of tRNA was investigated. The association activity did not change after 45 min of incubation at 37°C with NH_4OH (pH 10.5), a treatment that should deacylate the aminoacyl- or peptidyl-tRNA. Moreover, AF did not bind to DEAE-cellulose in a 0.3 M KCl buffer, whereas tRNA was quantitatively retained under the same conditions.

The association process is temperature-dependent above 30°C (Fig. 5). Temperature curves obtained in the presence of AF or tRNA as associating agents were different. These experiments were performed with unwashed ribosomes dialyzed against 0.4 mM Mg^{++} solutions, because subunits washed with 1 M KCl associate very little with tRNA at 5 mM Mg^{++} .

Fig. 6 shows the association of ribosomal subunits produced at different Mg^{++} concentrations by AF or tRNA, compared with the corresponding spontaneous association. At low concentrations of Mg^{++} , the formation of monomer was strongly dependent on AF, which could not be replaced by tRNA.

Preliminary experiments have indicated that association

FIG. 6. Association of 30S and 50S particles at various Mg^{++} concentrations. Subunits were washed with 1 M KCl. Symbols and additions as in Fig. 5.

by AF of ribosomal subunits does not decrease their capacity to synthesize polyphenylalanine in the presence of poly(U).

Nature of AF

The association activity was partially destroyed by treatment with trypsin, and was protected by soybean trypsin-inhibitor against the proteolytic attack (Fig. 7). These results show that at least part of the AF is a protein. Further experiments (I. D. Algranati, unpublished results) indicate that after trypsin digestion AF still associates ribosomal subunits, but to an intermediate form of monomer that can be converted into the final product of the association process by a subsequent incubation with untreated AF.

DISCUSSION

Recent studies on the ribosome cycle during protein synthesis have established the existence of at least two steps that involve association of ribosomal subunits: (a) when the initiation complex and 50S subparticle bind to form the first monosome at the beginning of translation (9), and (b) when subunits can exchange with free 70S monomers (26, 27), simultaneously with or after the completion of polypeptide chains.

The presence of mRNA and fMet-tRNA in the initiation complex may be sufficient to explain its high affinity for the 50S subunit. On the other hand, the association of free ribosomal subparticles does not occur spontaneously at low concentrations of Mg^{++} ; when such an association of subunits takes place it is necessary to assume the existence of a stabilizing component for the 30S-50S couples. DF could perform this role if dissociation of 70S ribosomes were a reversible process. However, at 5 mM Mg^{++} concentration DF failed

FIG. 7. Effect of trypsin treatment on AF. 10 μ l of AF preparation containing 40 μ g of protein was incubated with 30 μ g of trypsin for 30 min at 30°C. The trypsin was then inactivated by the addition of 30 μ g of soybean trypsin-inhibitor and a subsequent incubation at 30°C for 30 min. The residual associating activity was measured at 45°C as described in *Methods*. (a) control with ribosomal subunits and inactivated trypsin; (b) association with untreated AF; (c) assay with trypsin-digested AF; (d) same as b with the addition of inactivated trypsin.

to reassociate 30S and 50S particles (1, 17) or to induce the exchange between free 70S monomers and radioactive subunits (C. A. Perazzolo, to be published).

Our results describe a new factor, which can associate ribosomal subunits that have been washed *in vitro*. The AF has been extracted from *B. stearothermophilus* ribosomes. Using a similar method, we could not show any associating activity in extracts from *E. coli* D₁₀ ribosomes.

The crude preparations obtained from exponentially-growing *B. stearothermophilus* contained AF as well as DF; therefore neither dissociating nor associating activity could be detected. After prolonged dialysis against salt solutions of low concentration most of the AF was lost, both as a precipitate and through the dialysis bag. The dialyzed extract then showed a high DF activity. On the other hand DF could be destroyed by heating at 80°C, whereas AF remained unaltered. The association factor seems to differ from the previously-reported inhibitor of DF present in the ribosome-free (S 100) supernatant fraction*.

Several facts argue against the possibility that some kind of tRNA is an active component in AF preparations: (a) the associating activity is neither destroyed by a 45-min incubation at pH 10.5 nor retained by DEAE-cellulose at 0.3 M KCl. (b) The temperature and Mg⁺⁺-concentration curves of subunit association with AF or tRNA are markedly different (Figs. 5 and 6). The trypsin treatment experiment, as well as other experiments presently underway in our laboratory, suggest that AF contains at least two components, one of which is a protein and the other one is a substance of low molecular weight. There is also some evidence that the association of 30S and 50S particles is a complex process occurring in several steps (González, N. S., M. García-Patrone, and I. D. Algranati, to be published).

Although it is tempting to speculate that AF participates *in vivo* during the association of ribosomal subunits and increases the efficiency of formation of (initiation) 70S ribosomes, more work is needed before any real physiological role may be ascribed to AF.

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