Effect of Sulfhydryl Reagents on the Ribosomes of Bacillus subtilis

RAJINDER SINGH RANU AND AKIRA KAJI

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication 29 March 1971

The effect of various sulfhydryl reagents on the ribosomes of Bacillus subtilis was studied. The 70S ribosomes were completely dissociated into 30S and 50S subunits by appropriate concentrations of p-chloromercuribenzoic acid (PCMB) and 5,5'-dithio-bis-(2-nitro-benzoic acid). The N-ethylmaleimide and iodoacetamide failed to dissociate the ribosomes even at relatively high concentrations. The rate of dissociation of ribosomes by PCMB varied with the concentration of ribosomes. A progressive decrease in the rate of dissociation was observed as the concentration of ribosomes in the reaction mixture was increased. The PCMB-induced ribosomal subunits were unable to reassociate into 70S monomers unless they were dialyzed against buffer containing β-mercaptoethanol. On the average, four molecules of PCMB per 70S ribosome and two molecules of PCMB per each 30S and 50S subunit were bound. The number of PCMB molecules bound per ribosome did not change with increasing concentrations of PCMB, even though higher concentrations of PCMB resulted in dissociation of ribosomes into subunits.

The knowledge of the interaction which links the 30S and 50S ribosomal subunits together is limited and based on a series of observations. It is generally accepted that magnesium ions or polyamines play an important role in the association of the ribosomal subunits (13). The divalent cations are believed to form ionic bridges between the phosphate groups of the ribosomal ribonucleic acid (rRNA) chains of the two subunits (2). The role of hydrogen bonds in the association of subunits has been suggested by Watson (15) and Moore (7). A number of workers (5, 8, 16) have suggested that ribosomal proteins may be involved in the interaction between two subunits.

Recently, Tamaoki and Miyazawa (10) observed that sulfhydryl reagents caused dissociation of 70S ribosomes of Escherichia coli into 30S and 50S subunits and suggested that sulfhydryl groups of the ribosomal proteins may have an important role in maintaining the integrity of the ribosomes of E. coli. More recently, Parish et al. (9) found that there is one thio-uridylic acid residue per 16S rRNA chain and two thio-uridylic acid residues per 23S rRNA chain of E. coli. In addition, they observed that ribosomes from those organisms (including Bacillus subtilis) which contained no detectable amount of thio-uridylic acid in their rRNA were not dissociated by p-chloromercuribenzoic acid (PCMB). From these observations, they suggested that a correlation exists between the presence of thiouridyl acid in the rRNA and the sensitivity of the 70S ribosomes toward the thiol reagent.

In this paper we describe the results of our study on the effect of various sulfhydryl reagents on the ribosomes of B. subtilis. It was found that, under appropriate conditions, the sulfhydryl reagents dissociated 70S ribosomes of B. subtilis into 30S and 50S subunits. On the average, four molecules of PCMB were bound per 70S ribosome, two molecules per 30S, and two molecules per 50S ribosomal subunit. The PCMB-induced ribosomal subunits from 70S ribosomes were unable to reassociate into 70S ribosomes unless they were dialyzed against buffer containing β-mercaptoethanol.

MATERIALS AND METHODS

The crystalline sodium salt of PCMB was obtained from Sigma Chemical Co., St. Louis, Mo. The β-mercaptoethanol and crystalline N-ethylmaleimide were from Eastman Organic Chemicals, Rochester, N.Y. The 5,5'-dithio-bis-(2-nitro-benzoic acid) (DTNB) and iodoacetamide were purchased from Aldrich Chemical Co., Milwaukee, Wis. The 3-C-PCMB, with a specific activity of 10 mCi/m mole, was from Calbiochem., Los Angeles, Calif. Ribonuclease-free sucrose (Mann Research Laboratories) was used to prepare sucrose density gradients. All other reagents used in this study were of Baker analytical reagent grade.

Growth of organisms. B. subtilis ATCC 15563 was cultured aerobically in (3%) Trypticase soy broth (BBL) at 37 C. When the culture approached 104 to 2
× 10⁴ bacteria/ml, the culture was chilled by mixing with ice, and the bacteria were harvested by use of a Sorvall Szent-Györgyi continuous centrifuge. The cells were washed three times with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6), 0.05 M NH₄Cl, 0.01 M magnesium acetate, and 0.001 M diethanolamine (buffer A) and were kept in the frozen state at −20°C until use.

Preparation of ribosomes. All isolation procedures were carried out at 2 to 5°C. The washed cells were disrupted by grinding in a porcelain mortar with pestle in the presence of two times their weight of Alumund 6C (Norton Company, Worcester, Mass.) for 30 to 45 min. A 1-ml amount of buffer A per g (wet weight) of bacteria was added to the paste, followed by 5 μg of crystalline DNAse I ( Worthington Biochemical Corp.) per ml. The extract was allowed to stand for 2 to 3 min with occasional stirring. The extract was centrifuged in a Sorvall SS-34 rotor at 12,000 × g (10,000 rev/min) for 30 min to remove unbroken cells and cell debris.

The cell membranes and debris were removed from the supernatant fluid by centrifugation at 41,000 × g (25,000 rev/min) for 25 to 30 min in a Beckman Spinco ultracentrifuge. The S25 supernatant fluid was centrifuged at 105,000 × g (40,000 rev/min) for 2 hr. The supernatant fluid (S100) was aspirated, and the walls of the tubes and surface of the ribosomal pellet were washed with buffer A. The ribosomes were suspended in buffer A, and any material which failed to go in solution was removed by centrifugation at 26,000 × g (20,000 rev/min) for 20 min. The ribosomal suspension was again centrifuged at 105,000 × g (40,000 rev/min) for a further 2 hr. The procedure was repeated three times, and larger particulate matter was removed by centrifugation at 15,000 × g (15,000 rev/min) for 20 min. The final suspension of ribosomes usually contained 10 to 18 mg of ribosomes per ml. The ribosome suspension was distributed into small volumes, quickly frozen in acetone and dry ice, and kept in a dry ice chest until use.

Treatment of ribosomes with the sulphydryl reagents. For routine studies on the effect of sulphydryl reagents, 0.2 ml of ribosomes in buffer containing 0.01 M Tris-hydrochloride (pH 7.6), 0.05 M NH₄Cl, and 0.01 M magnesium acetate (buffer B) were mixed with an equal volume of a solution of sulphydryl reagent in the same buffer. The final concentration of the ribosomes in the reaction mixture varied from 4 to 6 mg/ml. The reaction mixture was incubated at 5 or 30°C, or as described. At various times, a portion of the reaction mixture was removed and analyzed by sucrose density gradient centrifugation.

Sucrose density gradient centrifugation. The Beckman gradient former was used to prepare 4 to 20% linear sucrose gradients. The gradients were made in buffer B. However, the 30S and 50S ribosomal sub-units, routinely used as markers, were sedimented in gradients containing 0.01 M Tris-hydrochloride (pH 7.6), 0.05 M NH₄Cl, and 10⁻⁴ M magnesium acetate. A 0.040- to 0.075-ml sample (2.5 to 7 optical density units at 260 nm) was applied to the 4.8-ml sucrose gradient and centrifuged at 39,000 rev/min in an SW39 or SW50.1 rotor of a Spinco centrifuge for 110 min. The temperature of the chamber was maintained at 4°C during centrifugation. After centrifugation, the tubes were punctured from the bottom, and 10- or 15-drop fractions were collected. Each fraction (about 0.2 ml) was diluted with 0.5 ml of buffer B, and the absorbance at 260 nm was determined. Whenever it was necessary to determine the radioactivity, 0.5 ml of each diluted fraction was transferred to 10 ml of Bray's solution (1), and the radioactivity was measured in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Other methods. The concentration of the ribosomes was determined by measuring the absorbance at 260 nm (3, 13). The molecular weight of ribosomes was assumed to be 2.65 × 10⁴, 1.8 × 10⁴, and 0.85 × 10⁴ for 70S, 50S, and 30S ribosomes, respectively (3, 13).
sive decrease in the rate of dissociation was observed as the concentration of ribosomes in the reaction mixture was increased. At 4.3 mg/ml, nearly 95% of the ribosomes were dissociated in 5 hr, whereas, in the presence of 13 mg/ml of ribosomes, only 38% of the ribosomes dissociated under otherwise identical conditions. However, after a prolonged period of incubation, all of the ribosomes were dissociated even at high concentrations of ribosomes. These results suggested that high concentrations of ribosomes retard the rate of dissociation of the ribosomes, but the extent of dissociation was not changed.

Effect of magnesium ions on the dissociation of ribosomes by PCMB. It is well known that magnesium ions, as well as other divalent cations, play an important role in the dissociation and association of ribosomes and their subunits (2, 13). It was therefore of interest to examine the effect of magnesium ions on the dissociation of ribosomes by PCMB.

As shown in Fig. 5, there was no appreciable change in the effect of PCMB on the ribosomes between 10 and 20 mM magnesium ions. On the other hand, the effect of DTNB was considerably influenced by changes in magnesium ion concentration. It is clear (Fig. 5) that the dissociation of ribosomes by DTNB was inhibited by approximately 50% when the concentration of magnesium was raised from 10 mM to 20 mM.
ever, after prolonged incubation with DTNB, dissociation was complete even in the presence of a high magnesium ion concentration. These results indicated that a high concentration of magnesium ions decreased the rate but not the extent of dissociation.

**Reversibility of the effect of PCMB.** In order to study the reversibility of the effect of PCMB, ribosomes were treated with 2 mM PCMB at 5°C. A portion of these ribosomes was dialyzed against buffer B containing 10 mM β-mercaptoethanol and the other dialyzed against buffer B alone. After dialysis each sample was subjected to sucrose density gradient centrifugation. The effect of PCMB could be partially reversed in the presence of β-mercaptoethanol as shown in Fig. 6 (c). In the absence of β-mercaptoethanol, subunits failed to form 70S particles [Fig. 6 (b)]. Figure 6 (a) also shows that the treatment of control ribosomes with β-mercaptoethanol alone had no appreciable influence on their sedimentation behavior.

**Number of PCMB molecules bound to ribosomes and ribosomal subunits.** Since the effect of PCMB was observed at 2 mM and not at lower concentrations, it appeared possible that under these conditions more sulfhydryl groups on the ribosome would react with PCMB, resulting in the dissociation of ribosomes. It was therefore of interest to determine the number of PCMB molecules that would bind to a ribosome and its subunits in the presence of various concentrations of the reagent.

In the experiments illustrated in Fig. 7 and 8, the ribosomes were incubated with 2 mM and 0.5 mM 14C-PCMB. The unbound 14C-PCMB was removed by gel filtration through a Sephadex G25 column. The ribosomes were centrifuged in sucrose density gradients and analyzed both for absorbance at 260 nm and the distribution of radioactivity in the gradients. Figure 7 shows the distribution of radioactivity and the ribosomal subunits in the sucrose density gradient from the reaction mixture incubated in the presence of 2 mM 14C-PCMB. It is noted that the 30S and 50S subunits had corresponding peaks of radioactivity. In the presence of 0.5 mM 14C-PCMB, the radioactivity coincided with the position where 70S ribosomes sedimented (Fig. 8). The number of PCMB molecules bound to each ribosome and to 30S and 50S subunits were calculated from the reported values of the molecular weights of these particles (3, 13). Table 1 summarizes the results of experiments on the number of PCMB molecules bound to a ribosome and its subunits. On the average, four molecules of PCMB were bound per 70S monomer and two molecules for each 30S and 50S ribosomal subunit. In addition, the number of PCMB molecules bound per ribosome did not change to a great degree with increasing concentrations of PCMB, even though higher concentration of PCMB dissociated ribosomes into subunits (Table 1). These results suggested that the dissociation of ribosomes in the presence of high concentrations of PCMB was not the result of an increase in the number of molecules of PCMB bound to the ribosomes.

**DISCUSSION**

The data presented in this communication show that the ribosomes of *B. subtilis* are dissociated under appropriate conditions by some of
EFFECT OF -SH REAGENTS ON B. SUBTILIS RIBOSOMES

FIG. 3. Effect of temperature on the dissociation of ribosomes by PCMB. Ribosomes (5.38 mg/ml) were incubated with 2 mM PCMB at 20 to 21 C, 30 to 31 C, and 40 C. After incubation, a sample of the reaction mixture (4.1 optical density units at 260 nm) was analyzed by sucrose density gradient centrifugation to determine the extent of dissociation of ribosomes.

FIG. 4. Effect of the concentration of ribosomes on the dissociation of ribosomes by PCMB. Ribosomes (4.3, 7, 10, and 13 mg/ml) were incubated with 2 mM PCMB at 31 C for 5 hr along with control ribosomes, and 3.2 optical density units at 260 nm from each sample were centrifuged in a sucrose density gradient. The ribosomes which showed incomplete dissociation were incubated at 5 C for another 15 hr. The percentage of ribosomes dissociated was determined by sucrose density gradient centrifugation. Symbols: after 5 hr of incubation (○); after an additional 15 hr of incubation (□).
tion of ribosomes, whereas high concentrations of PCMB dissociate ribosomes in a manner not dependent on the presence of thiouridylic acid in rRNA.

The dissociation of E. coli ribosomes by PCMB has been reported to proceed through an intermediate which sediments at 60S (10). In our studies with B. subtilis no such intermediate was observed. It appears that in this case the 70S ribosomes do not undergo a structural change which results in a decrease in their sedimentation coefficient before they dissociate into 30S and 50S subunits. However, it is possible that such an intermediate exists only for a very short period and has escaped our detection. The fact that treatment with N-ethylmaleimide and iodoacetamide resulted in dissociation of E. coli ribosomes but not B. subtilis ribosomes may also have some relation to the absence of thiouridylic acid in the

![Graph showing percentage of ribosomes dissociated vs. mM of Mg++]

**Fig. 5.** Effect of Mg++] concentration on the dissociation of ribosomes by PCMB and DTNB. Ribosomes (4.83 mg/ml) were incubated with 2 mM PCMB or 10 mM DTNB in the presence of 10, 15, and 20 mM Mg++] concentrations at 30 C. After 5 hr, 3 to 4 optical density units of ribosomes, at 260 nm, from each sample were centrifuged in a 4 to 20% sucrose density gradient, and the percentage of ribosomes dissociated was determined. The ribosomes incubated with 10 mM DTNB in the presence of 15 and 20 mM Mg++] were partially dissociated. These ribosomes were incubated at 5 C for an additional 16 hr and analyzed by sucrose density gradient centrifugation. Symbols: incubated with 2 mM PCMB for 5 hr (Δ), incubated for 5 hr with 10 mM DTNB (○, dashed line), after 16 hr of incubation in the presence of 10 mM DTNB (●, solid line).

![Graph showing percentage of ribosomes dissociated vs. Fraction number]

**Fig. 6.** Reversal of dissociation caused by PCMB with β-mercaptoethanol. Ribosomes (4.75 mg/ml) were dissociated in the presence of 2 mM PCMB at 5 C for 14 hr. A portion (0.3 ml of 4.7-mg ribosomes/ml) was dialyzed against buffer B containing 10 mM β-mercaptoethanol, and another portion was dialyzed against buffer B alone for 16 hr at 5 C. A sample (containing 7 optical density units at 260 nm) of each sample was centrifuged through a sucrose density gradient: a, Control ribosomes; b, PCMB-treated ribosomes dialyzed against buffer B alone; c, PCMB-treated ribosomes dialyzed against buffer B containing 10 mM β-mercaptoethanol.
**Vol. 107, 1971**  
**EFFECT OF -SH REAGENTS ON B. SUBTILIS RIBOSOMES**

**Fig. 7.** Binding of $^{14}$C-PCMB to ribosomal subunits at 2 mM $^{14}$C-PCMB. Ribosomes (6 mg/ml) were incubated at 30 C for 5 hr, and 4 optical density units at 260 nm were centrifuged through a sucrose density gradient in buffer B. The absorbance at 260 nm and the radioactivity of each fraction were measured as described.

**B. subtilis** rRNA. Although it has been reported that mammalian ribosomes require high concentrations of PCMB for dissociation (4). This may be due in part to the general difficulty involved in the dissociation of mammalian ribosomes into 40S and 60S subunits (6, 11, 12).

It is conceivable that the binding of PCMB facilitates dissociation of ribosomes through steric hindrance or by impairing the S-S bond formation. The S-S bonds may stabilize the subunits as 70S ribosomes. However, the binding of PCMB to SH groups of ribosomes per se is probably not responsible for the dissociation of ribosomes. Although the actual mechanism for the dissociation of the ribosome-PCMB complex in the presence of 2 mM PCMB remains obscure, it is quite possible that higher concentrations of PCMB would force the unbound PCMB to associate with the PCMB bound to the ribosomes in such a manner that it works as a wedge resulting in the dissociation of ribosomes. This possibility is strengthened by the observation that the number of bound PCMB molecules remained unchanged whether or not the ribosomes had been dissociated. It has recently been suggested (14) that short-range attraction forces such as hydrophobic interactions may be partly responsible for the association of ribosomal subunits. It is conceivable that high concentrations of PCMB (a hydrophobic compound) may interfere with the hydrophobic interaction between the ribo-

**Fig. 8.** Binding of $^{14}$C-PCMB to 70S ribosomes. Ribosomes (6.0 mg/ml) were incubated with or without 0.5 mM $^{14}$C-PCMB at 30 C for 5 hr. A portion (containing 2.5 optical density units at 260 nm) of ribosomes from each sample was centrifuged in a sucrose density gradient, and the absorbance at 260 nm and the radioactivity of each fraction were measured.

**Table 1.** Number of $^{14}$C-PCMB molecules bound to a ribosome

<table>
<thead>
<tr>
<th>$^{14}$C-PCMB</th>
<th>Ribosome</th>
<th>$^{14}$C-PCMB/ribosome</th>
<th>SE of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM</td>
<td>70S</td>
<td>3.7</td>
<td>±0.145</td>
</tr>
<tr>
<td>2 mM</td>
<td>30S</td>
<td>2.31</td>
<td>±0.014</td>
</tr>
<tr>
<td></td>
<td>50S</td>
<td>1.61</td>
<td>±0.114</td>
</tr>
</tbody>
</table>

*The ribosomes (6 mg/ml) were incubated at 30 C for 5 hr. The unbound $^{14}$C-PCMB was removed by gel filtration through Sephadex G25 column. Ribosomes (2.5 to 6.5 optical density units at 260 nm) were analyzed by sucrose density gradient centrifugation. The optical density at 260 nm and radioactivity of peak fractions were used to calculate the molar ratio between PCMB and ribosomes. The standard error (SE) of the mean of the number of PCMB molecules bound to ribosomes was obtained from 10 observations with 70S ribosomes and 3 observations each with 30S and 50S subunits.

* Concentration at which ribosomes were treated.

* Molar ratio.
somal subunits resulting in the dissociation of the ribosomes.

ACKNOWLEDGMENTS

R. S. Ranu is a recipient of a teaching fellowship from the School of Veterinary Medicine and gratefully acknowledges this support. The material in this paper is taken in part from a dissertation to be presented to the Graduate School of Arts and Sciences of the University of Pennsylvania by R. S. Ranu in partial fulfillment of the requirements for the Ph.D. degree.

This investigation was supported by Public Health Service grant GM-12, 053 from the National Institute of General Medical Science, National Science Foundation grant GB-7454, and by grant DRG-799 from the Damon Runyon Memorial fund for Cancer Research. We thank T. Otaka for his contribution to the experiments shown in Table 1.

LITERATURE CITED