# INHIBITION OF GTP HYDROLYSIS DEPENDING ON G FACTOR AND RIBOSOMES BY A FACTOR PREPARED FROM THE RIBOSOME WASH OF E. COLI Q13.

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SUMMARY: A protein fraction prepared from the ribosome wash of E. coli Q13 contained inhibitor of the GTPase reaction depending on G factor and ribosomes. The inhibitor which was called Cs factor did not inhibit the G factor dependent binding of GTP to ribosomes assumed as the first step of the GTPase reaction. Inhibition was observed on the steady state of the reaction.

INTRODUCTION: In a cell free system from E. coli, the elongation of peptide bonds requires at least two types of GTP hydrolysis; one is catalyzed by Tu(1) and the other by G factor(2) In the former reaction, one mole of GTP is consumed by one mole of aminoacyl-tRNA binding to ribosomes, the latter is required for the translocation of peptidyl-tRNA from the aminoacyl site to the peptidyl site of ribosomes. However, it is unknown how much GTP is hydrolyzed in each round of the translocation of peptidyl-tRNA, since high GTPase activity is observed in the absence of the translocation of peptidyl-tRNA(3,4). In fact, the amounts of hydrolyzed GTP are hundreds of times more than that of peptide bonds formed in a purified system of protein synthesis(5).

During the studies on the GTPase reaction, inhibitors of this reaction were detected in the ribosome wash. It is possible to suppress the GTP hydrolysis by these inhibitors. In this communication we will report on one of these inhibitors which is stable to heat treatment at 60°C for 20 minutes.

MATERIALS AND METHODS: G factor was purified by the procedure of



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Kaziro and Inoue(6). G factor-free ribosomes were prepared from E. coli Q13 by successive washing with a buffer containing 0.5 M $NH_4Cl$. An inhibitor of the GTPase reaction, which we call Cs factor, was prepared from the ribosome wash of 0.5 M $NH_4Cl$ in ribosome purification. To the ribosome wash solid ammonium sulfate was added to make 90 % ammonium sulfate saturation. The precipitate was collected by centrifugation and dissolved in a small volume of 20 mM Tris-HCl, pH 7.5, containing 5 mM 2-mercapto-ethanol (Buffer A). After dialysis against Buffer A overnight in the cold, insoluble material was removed by centrifugation. The supernatant was incubated at 60°C for 20 minutes. Denatured proteins were removed by centrifugation at 30,000 rpm for 3 hours (Spinco No.30 rotor). Approximately 10 ml of the supernatant (3.27 mg protein/ml) were obtained from 100 g of cells. Activities of G factor, T factors, RNase I(7), RNase II(8) and phosphatases were eliminated at the step of the purification. Solid ammonium sulfate was added to the supernatant. Material precipitating between 50 % and 70 % saturation was collected, dissolved in a small volume of Buffer A and dialyzed against Buffer A overnight in the cold. 1.2 ml of the preparation(8 mg protein/ml) were mixed with 0.3 ml of 1.5 M $NH_4Cl$ and then applied to a column (0.54 $cm^2$ x 80 cm) of Sephadex G-200 equilibrated with Buffer A containing 0.3 M $NH_4Cl$. Elution was carried out with Buffer A containing 0.3 M $NH_4Cl$. 0.6 ml fraction was collected in a tube. An inhibitory activity of the GTPase reaction was found in Fraction 55 to 75. These fractions were pooled, concentrated with ammonium sulfate(50 % to 70 % saturation) and then dialyzed against Buffer A overnight in the cold. This preparation revealed that the ratio of optical dencity at 280 to 260 mμ was about 1.0. The molecular weight of the inhibitor was tentatively caluculated

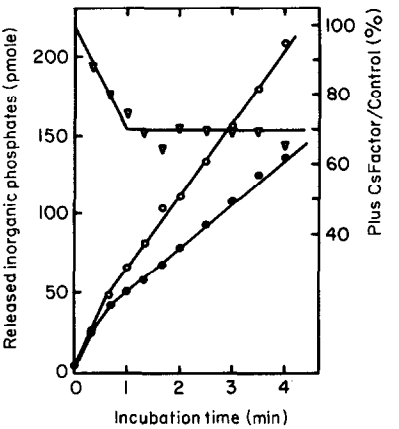


Fig. 1. Time course of the GTPase reaction at 0°C in the presence and the absence of Cs factor. The reaction mixture for the GTPase reaction contained the following in 0.5 ml of the final volume; 50 mM Tris-HCl, pH 7.5; 150 mM $NH_4Cl$; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 40 μM GTP-γ-$^{32}$P(2.28 x $10^7$ cpm/μmole); 1.17 nmoles of ribosomes and 332 μg of G factor. The reaction was started by the addition of GTP-γ-$^{32}$P after incubation of the reaction mixture at 30°C for 3 minutes and 0°C for 10 minutes, successively. The GTPase reaction was carried out at 0°C. 35 μl portions of the reaction mixture were taken into 0.25 ml of 20 mM silico-tungstic acid in 0.02 N $H_2SO_4$ at the interval of 20 seconds. The above procedure was carried out in the cold. Insoluble materials were removed by centrifugation after adding 0.715 ml of 10 mM $KH_2PO_4$. 0.25 ml of 5 % ammonium molybdate in 4 N $H_2SO_4$ was added to the supernatant, and then phosphomolybdate was extracted with isobutanol-benzene. Radioactivity extracted in isobutanol-benzene was measured in a Nuclear Chicago 2π gas flow counter. o———o, control; ●———●, 981 μg of Cs factor added; ▽———▽, plus Cs factor/control.

to be 13,000 from its migration on the Sephadex G-200 column chromatography, in which vitamin $B_{12}$, cytochrome C, RNase A, bovine albumin, bovine γ-globulin and blue dextran were used as standards.

GTP-γ-$^{32}$P was prepared by the method of Horiuti et al.(9). The assay of GTPase activity was carried out essentially by the same method as described by Conway and Lipmann(5). The formation of GTP(GDP)-G factor-ribosome complex was measured by the method described previously(10). Protein concentration was determined

by the method of Lowry et al. with bovine serum albumin as a standard(11).

RESULTS AND DISCUSSION: The GTPase reaction which depend on G factor and ribosomes was followed at 0°C. As shown in Fig. 1, the GTPase reaction proceeded in two phases. The first rapid reaction persisted for 40 seconds after the onset of the reaction and after that the second reaction proceeded at a constant rate which was about half of the initial one. The former reaction is referred to as the initial rapid reaction and the latter as the steady state reaction in this paper. Detailed studies of the relationship between the two reactions in the GTPase will be reported in another paper(12).

A protein fraction(Cs factor), which was prepared from the ribosome wash of E. coli Q13, inhibited the GTPase reaction. The degree of inhibition by Cs factor increased in proportion to the incubation time during the first 60 seconds of the reaction and then reached a plateau at 60 seconds as shown in Fig. 1. The increase of the degree of inhibition at the initial phase does not mean that the incubation for 60 seconds was required for the binding of Cs factor to G factor or(and) ribosomes to express the inhibition of the GTPase reaction, since the GTPase reaction was started by the addition of GTP-$\gamma$-$^{32}$P after incubation of G factor and ribosomes with Cs factor for 3 minutes at 30°C and 10 minutes at 0°C, successively. The result suggests that Cs factor may inhibit the steady state reaction, but not the initial rapid reaction. This point was further verified by the following experiment. As shown in Fig. 2, G factor and ribosomes were incubated with Cs factor at 30°C up to 10 minutes and then the GTPase reaction carried out one minute at 30°C. Neither enhancement nor depression of the inhibition were observed on the GTPase

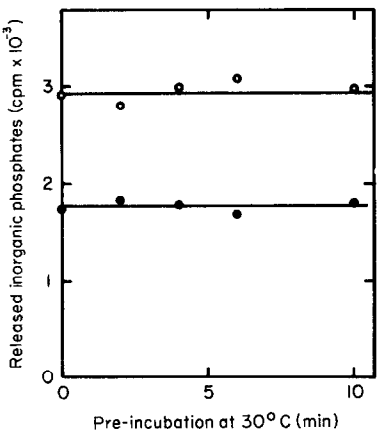


Fig. 2.

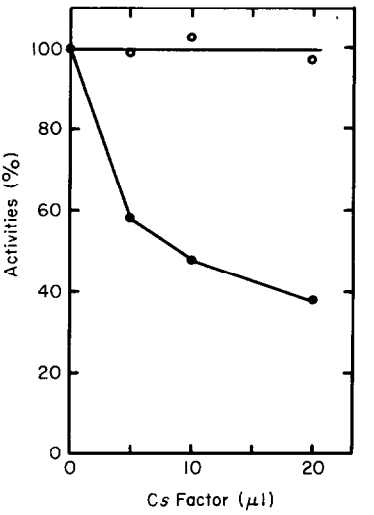


Fig. 3.

Fig. 2. Effects of preincubation of G factor and ribosomes with Cs factor. 13.3 μg of G factor and 51.5 pmoles of ribosomes in 0.1 ml of the reaction mixture described in the legend of Fig. 1 except for ommission of GTP-γ-$^{32}$P were incubated at 30°C. The GTPase reaction was started by the addition of 50 nmoles of GTP-γ-$^{32}$P(1.06 x $10^4$ cpm). $^{32}$Pi liberated for one minute at 30°C was measured. o———o, control; ●———●, 16.3 μg of Cs factor added.

Fig. 3. Effects of Cs factor concentration on the GTPase reaction and the ternary complex formation between GTP, G factor and ribosomes. The reaction mixture for the GTPase reaction was the same as described in the legend of Fig. 1 except that 13.3 μg of G factor, 51.5 pmoles of ribosomes and 50 nmoles of GTP-γ-$^{32}$P (1.80 x $10^4$ cpm) in 0.1 ml of the final volume were used. $^{32}$Pi liberated for one minute at 30°C was measured. In the absence of Cs factor (3.27 mg protein/ml), 5832 cpm of $^{32}$Pi were liberated. The reaction mixture for the ternary complex formation was the same as that of the GTPase reaction except that 50 μM $^3$H-GTP (1.28 x $10^8$ cpm/μmole) and 0.5 mM fusidic acid were used. The reaction mixture was incubated at 30°C for one minute and then the reaction was terminated by the addition of 3 ml of a buffer containing 10 mM Tris-HCl, pH 7.5 and 10 mM $MgCl_2$. The diluted reaction minture was passed through a Millipore filter(HA0.45-μ pore size) and the filter was washed three times with 3 ml of the same buffer, dried and counted in a Beckman liquid scintillation spectrometer. In the absence of Cs factor, 3445 cpm of $^3$H-GTP were bound to ribosomes in the presence of G factor. ●———●, GTPase reaction; o———o, ternary complex formation.

reaction. This result eliminates the possibility that Cs factor inactivates G factor or(and) ribosomes in the manner of enzymatic digestion.

The inhibition of the GTPase reaction was depended on the amounts of Cs factor added to the reaction mixture(Fig. 3). Half inhibition was observed on the addition of 30 μg of Cs factor. With the purest preparation so far obtained, this amount was reduced to 8 μg protein.

It is unknown whether or not the translocation of peptidyl-tRNA on ribosomes is inhibited by Cs factor. However, polyphenylalanine formation directed by polyuridylic acid was not inhibited under the conditions in which the GTPase reaction was inhibited up to about 50 % by Cs factor(unpublished data) suggesting that the translocation of peptidyl-tRNA may not be inhibited by Cs factor. Further studies are required for the final conclusion on this point.

Previously, the mechanism of the GTPase reaction was postulated as that GTP and G factor bind ribosomes to form a GTP-G factor-ribosome complex, followed by the hydrolysis of GTP to form a GDP-G factor-ribosome complex, which presumably can be stabilized by fusidic acid(10). In order to determine whether or not Cs factor inhibits formation of $^{3}$H-GTP($^{3}$H-GDP)-G factor-ribosome complex, the G factor dependent binding of $^{3}$H-GTP to ribosomes was performed in the presence of fusidic acid. As shown in Fig. 3, the G factor dependent binding of $^{3}$H-GTP to ribosomes in the presence of fusidic acid was not inhibited by Cs factor. Cs factor itself did not stimulate the G factor dependent binding of $^{3}$H-GTP to ribosomes(Table 1), whereas 0.5 mM fusidic acid stimulated the $^{3}$H-GTP binding to ribosomes 4 to 5 times as reported previously(10). The guanosine nucleotides bound to ribosomes in the presence and the absence of Cs factor were analyzed by paper chromatography after extraction with trichloro-acetic acid. No significant differences in the ratio of GTP to

TABLE I

Effects of Cs factor on the formation of GTP(GDP)-G factor-ribosome complex.

| Addition | Bound nucleotides (cpm) |
|---|---|
| None | 904 |
| Cs factor | 738 |
| Fusidic acid | 4676 |
| Cs factor, fusidic acid | 4667 |

The composition of the reaction mixture and procedure of the assay were the same as described in the legend of Fig. 3 except for omission of fusidic acid. 65.4 μg of Cs factor and 50 pmoles fusidic acid were added to the reaction mixture.

GDP were observed. These results indicate that the mechanism of the inhibition by Cs factor is different from that by fusidic acid and also different from that by siomycine, which prevents the G factor dependent binding of GTP to ribosomes both in the presence and the absence of fusidic acid(13). Cs factor inhibited the GTPase reaction at the step after cleavage of the ester bond between β and γ phosphate of GTP. Therefore, it might be concluded that Cs factor is a new type of inhibitor on the GTPase reaction. Cs factor may offer a means for studying the GTPase reaction depended on the translocation of peptidyl-tRNA.

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