A STRINGENT FACTOR ACTIVITY ASSOCIATED WITH THE 50 S RIBOSOMAL SUBUNIT OF *BACILLUS STEAROTHERMOPHILUS**

Dietmar RICHTER** and Katsumi ISONO

Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Berlin-Dahlem, Germany

Received 24 May 1974

1. Introduction

The *Escherichia coli* stringent factor, activated by the ribosome–messenger RNA–tRNA complex, promotes the synthesis of guanosine tetra- (ppGpp) and pentaphosphates (pppGpp) by pyrophosphate transfer from ATP to GDP or GTP [1,2]. The stringent factor is closely associated with the ribosomes of the stringent strains of *E. coli* and can be extracted with NH_4Cl solutions [3]. It has also been reported, that, when 70 S ribosomes were converted into 30 S and 50 S particles by gradient centrifugation at low Mg^{2+}-concentrations, the stringent factor was released into the supernatant fraction [4]. The localization of the factor protein on the ribosomal particles has not yet been elucidated mainly because of the rapid dissociation of the ribosome–stringent factor complex at low Mg^{2+} concentrations.

In an attempt to localize the stringent factor on either of the two ribosomal subunits, we chose *Bacillus stearothermophilus* with the hope that the ribosome–stringent factor complex of this bacterium might be more stable. *B. stearothermophilus* has been reported to synthesize guanosine polyphosphates in vivo [5]. Upon separation of 70 S ribosomes of *B. stearothermophilus* into 30 S and 50 S subunits we found that stringent factor activity was associated with the large but not with the small subunit.

* Paper No. 94 on 'Ribosomal Proteins', preceding paper is by H. Paradies et al., manuscript submitted to Biochem. Biophys. Res. Commun.

** To whom reprint requests may be addressed: Abt. Zellbiochemie, Institut für Physiologische Chemie, Universität Hamburg, 2 Hamburg 20, Martinistr. 52, Germany.

2. Materials and methods

B. stearothermophilus strain 799 was grown at 60°C in a complete medium [6]. Cells were harvested at late logarithmic phase and washed once with buffer 1 (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol). Washed cells were ground with alumina powder and extracted with either buffer 1 to obtain 70 S ribosomes or buffer 2 (10 mM potassium phosphate, pH 7.2, 1 mM $MgCl_2$, 6 mM 2-mercaptoethanol) to obtain subunits. In both cases, DNase (Boehringer) was added at a concentration of 1 μg/ml. To obtain subunits, the low speed supernatant fraction was dialyzed against the extraction buffer for 4–5 hr, then applied to a 6 to 38% sucrose gradient (w/v; in buffer 2) in a Spinco B XV zonal rotor and was spun at 25 000 rpm for 18 hr. Subunits were pooled, recovered by centrifugation, resuspended in buffer 1 and kept at −70°C or in liquid N_2.

The assay for production of guanosine tetra- and pentaphosphates and the analysis of the product was the same as described (ref. [7] and legend to table 1). The stringent factor was isolated from *E. coli* strain CGSC 2834/a (a revertant of CGSC 2834 aroE$^-$) by zonal centrifugation of crude ribosomes and further purified by Sephadex G-150 gel filtration and hydroxyapatite chromatography [7]. *E. coli* ribosomal subunits were obtained by zonal centrifugation of 70 S ribosomes in 0.1 mM Mg-acetate [8]. [α-^{32}P]-GTP (specific activity 5–13 Ci/mmol) was obtained from New England Nuclear, Boston. Uncharged $tRNA^{Phe}$ (yeast) was purchased from Boehringer, Mannheim.

Table 1
Crude *B. stearothermophilus* ribosomes synthesize ppGpp and pppGpp

Conditions	Experiment									
	1	2	3	4	5	6	7	8	9	10
crude 70 S $_{B.\ stearo.}$	+	−	+	+	+	−	−	−	−	−
crude 70 S $_{E.\ coli}$	−	+	−	−	−	−	−	−	−	−
ATP	+	+	−	+	+	+	+	+	+	+
GTP	+	+	+	+	+	+	+	+	+	+
$tRNA^{Phe}$	+	+	+	−	+	+	+	+	+	+
poly U	+	+	+	+	−	+	+	+	+	+
stringent factor (*E. coli*)	−	−	−	−	−	+	+	+	+	+
50 S $_{E.\ coli}$	−	−	−	−	−	−	+	−	+	−
30 S $_{E.\ coli}$	−	−	−	−	−	−	+	−	−	+
50 S $_{B.\ stearo.}$	−	−	−	−	−	−	−	+	−	+
30 S $_{B.\ stearo.}$	−	−	−	−	−	−	−	+	+	−
% of ppGpp and pppGpp synthesized	31	78	0	2.3	0.8	0.7	68	63	69	73

The reaction mixture (25 μl) contained: 20 mM Tris–HCl, pH 7.8; 20 mM Mg-acetate; 2 mM dithiothreitol; 0.02 A_{260} units poly U (1 mg corresponds to 25 A_{260} units); 0.01 A_{260} units $tRNA^{Phe}_{yeast}$ (Boehringer, Mannheim); 4 mM ATP; 0.177 mM [α-^{32}P]GTP (spec. act. 25 Ci/mol); 4 μg of *E. coli* stringent factor, 0.06 A_{260} units of 50 S from *B. stearothermophilus or E. coli* and 0.03 A_{260} units of 30 S from *B. stearothermophilus* or *E. coli* were used. The mixture was incubated at 37°C for 1 hr, the reaction was stopped with 1 μl 88% formic acid. ppGpp and pppGpp were analysed as reported [3]. The conversion of radioactive GTP into ppGpp and pppGpp is given in per cent.

3. Results and discussion

Crude 70 S ribosomes from *B. stearothermophilus* were active in synthesizing guanosine tetra- and pentaphosphates. The reaction was completely dependent on ATP as pyrophosphate donor and GTP as acceptor (table 1). This result is consistent with the findings by Lund et al. [5] that *B. stearothermophilus* produces guanosine polyphosphates in vivo. The in vitro stringent factor activity of *B. stearothermophilus* was apparently much lower than that of *E. coli*. This may be due either to conditions such as ionic strength and reaction temperature which are possible not optimal for the *Bacillus* system or to the presence of stronger ATPase and/or GTPase activity associated with the *Bacillus* ribosome preparation. As shown recently the isolated stringent factor of *E. coli* is not species specific and can react with other prokaryotic type of ribosomes [9,10]. *B. stearothermophilus* ribosomes depleted of the stringent factor activity by NH_4Cl extraction were active with the *E. coli* stringent factor (table 1). Also, ribosomal subunits from both strains were interchangeable.

In order to see to which ribosomal subunits the stringent factor activity is bound, 70 S ribosomes were separated into subunits at 1 mM $MgCl_2$ and the resultant 30 S and 50 S subunits were assayed for stringent factor activity. Fig. 1 shows that a significant fraction of activity was retained in 50 S subunits, whereas almost no activity was observed with 30 S particles. Repeated sucrose gradient centrifugation of the 50 S subunits caused a decrease in activity, although a complete loss was not observed. The possibility of a small contamination of the 50 S preparation by intact 70 S ribosomes cannot be completely excluded, although analysis of the 50 S particles by two-dimensional polyacrylamide gel electrophoresis yielded only the distinct 50 S protein pattern and therefore made this possibility rather unlikely. Based on the data of poly U-dependent synthesis of poly-

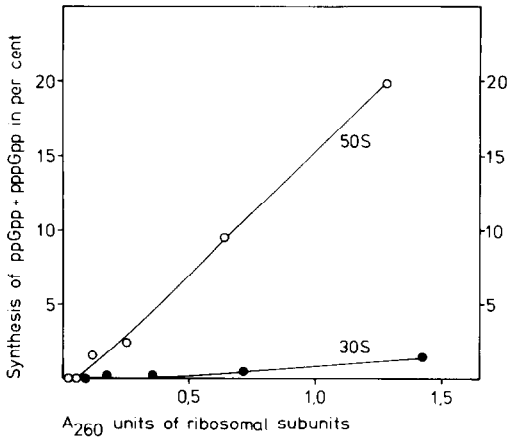

Fig. 1. Stringent factor activity associated with *B. stearothermophilus* 50 S ribosomal subunit. Conditions were the same as described in the legend to table 1. Conversion of radioactive GTP into ppGpp and pppGpp is given in per cent. o——o, 50 S Bac; •——•, 30 S Bac.

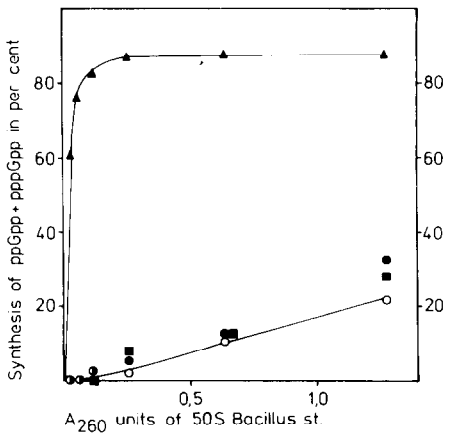

Fig. 2. Stringent factor activity in the presence of 50 $S_{Bac.}$, 30 S E. coli, and *E. coli* stringent factor. Where indicated 0.08 A_{260} units of 30 S E. coli and/or 5 μg of *E. coli* stringent factor were added. For other conditions see legend to table 1. Conversion of radioactive GTP into ppGpp and pppGpp is given in per cent. o——o, 50 $S_{Bac.}$; •——•, 50 $S_{Bac.}$ + *E. coli* stringent factor; ▪——▪, 50 $S_{Bac.}$ + 30 $S_{E. coli}$; ▲——▲, 50 $S_{Bac.}$ + 30 S E. coli + *E. coli* stringent factor.

phenylalanine, contamination of the 50 S preparation by 30 S subunits was less than 3%.

To test further whether the activity observed with the 50 S subunits was due to contaminating 30 S or not, a partially purified preparation of the stringent factor from *E. coli* was added to the reaction mixture, which contained particles from the *B. stearothermophilus* 50 S preparation only. Since the *E. coli* stringent factor is only active in the presence of both ribosomal subunits, and since the reaction proceeds maximally at extremely low concentrations of the subunits (0.06 A_{260} units of 50 S and 0.03 A_{260} units of 30 S per 25 μl reaction volume), 30 S ribosomes present in the 50 S preparation as slight contaminants should show a significant stringent factor activity. Fig. 2 demonstrates, however, that the *E. coli* stringent factor did not greatly enhance the reaction, even at a very high concentration of 50 S particles (1.28 A_{260} units/assay) where 1% contamination of 30 S should result in 60% activity. In the control experiment the 50 S subunits from *B. stearothermophilus* combined with 30 S and stringent factor from *E. coli* were found to be very active; even at very low concentrations of 50 S subunits (less than 0.1 A_{260} units/assay) synthesis of guanosine polyphosphates was almost optimal. From these results it can be concluded that the stringent factor activity is associated with 50 S subunits in *B. stearothermophilus* and the observed activity is not caused by the contaminating 30 S and/or 70 S particles.

The stringent factor activity of the 50 S subunits from *B. stearothermophilus* did not absolutely require poly U and $tRNA^{Phe}$ as shown in table 2, although omission of either of them caused a strong reduction. This may suggest that mRNA and tRNA are necessary for the binding of the stringent factor to ribosomes but not for its activity per se. The stimulation of the stringent factor activity by tRNA and poly U may be explained by a stabilization of the 50 S–stringent factor complex.

Note added in proof

Since completion of this work, a paper by Ramagopal and Davis [11] has appeared which describes experiments that stringent factor activity is retained in native 50 S ribosomal subunits and run off 70 S ribosomes from *E. coli*.

Table 2
Stringent factor activity associated with *B. stearothermophilus* 50 S subunits in the absence of mRNA and tRNA

Conditions	Experiment 1	2	3	4
50 S B. stearo.	+	+	+	+
poly U	–	+	–	+
$tRNA^{Phe}$	–	–	+	+
% of ppGpp and pppGpp synthesized	17	16	14	29

Conditions were the same as outlined in the legends to table 1 and fig. 1. 1.3 A_{260} units of 50 S ribosomal subunits from *B. stearothermophilus* were used. Conversion of radioactive GTP into ppGpp and pppGpp is given in per cent.

Acknowledgement

We wish to thank Dr. H. G. Wittmann for critical discussion of the paper.

References

[1] Pedersen, F. S., Lund, E. and Kjeldgaard, N. O. (1973) Nature New Biol. 243, 13.
[2] Haseltine, W. A. and Block, R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1564.
[3] Haseltine, W. A., Block, R., Gilbert, W. and Weber, K. (1972) Nature 238, 381.
[4] Sy, J., Ogawa, Y. and Lipmann, F. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2145.
[5] Lund, E., Pedersen, S. and Kneldgaard, N. O. (1973) in: First Symposium on Ribosomes and RNA Metabolism, Slovak Academy of Sciences, Bratislava, Czechoslovakia, page 307.
[6] Isono, K., submitted to J. Bacteriol.
[7] Nowak, P. and Richter, D., manuscript in preparation.
[8] Eikenberry, E. F., Bickle, T. A., Traut, R. R., and Price, C. A. (1970) Eur. J. Biochem. 12, 113.
[9] Richter, D. (1973) Febs Letters 34, 291.
[10] Sy, J., Chua, N.-H., Ogawa, Y., and Lipmann, F. (1974) Biochem. Biophys. Res. Commun. 56, 611.
[11] Ramagopal, S. and Dawis, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 820.