SELECTIVE INHIBITION OF THE REACTIONS CATALYZED BY RIBOSOME-SPECIFIC TRANSFER FACTORS G

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1. Introduction

It is well known that one of the transfer factors required for peptide chain elongation, transfer factor G (translocase) catalyzes the translocation of peptidyl-tRNA onto the ribosome [1]. The translocation requires GTP and it has been calculated that one GTP is hydrolyzed to GDP and inorganic phosphate for every peptide bond formed [2]. In addition the factor, whether isolated from prokaryotic or eukaryotic organisms, is endowed with GTP-ase activity dependent on the presence of ribosomes [2–5]. Albeit evident also under conditions in which there is no peptide chain elongation, it has been assumed that the hydrolytic activity is related to the synthetic activity [4, 6] since the two activities are purified together and all the antibiotics so far tested that inhibit translocation also inhibit ribosome dependent GTP-ase. Indeed fusidic acid appears to inhibit both activities in the case of the factors G from prokaryotic and eukaryotic organisms [7, 8], while siomycin exerts a similar effect on *Escherichia coli* transfer factor G and *E. coli* ribosomes [6] and diphtheria toxin and factors G (transfer factors II) * and ribosomes from mammalian cells and organs [9–11].

We have previously shown that the achloric alga *Prototheca zopfii* is endowed with two separated transfer factors G, one specific for 70 S ribosomes (such as are those present in prokaryotic organisms and in cellular organelles) and the other specific for ribosomes of the 80 S type as are those present in the cytoplasm of eukaryotic organisms [12]. The availability of the two transfer factors G at a degree of considerable purification (fig. 1) has prompted an investigation on the sensitivity of such factors to specific inhibitors. The reported data show that the different activities catalyzed by the ribosome-specific transfer factors G_{70} and G_{80} from *P. zopfii* may be specificially and selectively inhibited. More interestingly, evidence will be presented showing that at least in the case of *P. zopfii* transfer factor G_{80}, ribosome dependent GTP-ase may be uncoupled from peptide chain elongation. Indeed peptide chain elongation may be totally inhibited without impairing such GTP-ase activity or this activity may be drastically reduced without affecting the reactions required for peptide chain elongation.

2. Materials and methods

Conditions for the growth of cells and the preparation of subcellular fractions from *E. coli*, *P. zopfii* and *Saccharomyces cerevisiae* have been already reported [12, 14, 15, 17]. The procedure for preparing *E. coli* transfer factor G, *P. zopfii* transfer factors G_{70} and G_{80} to a considerable degree of purification (fig. 1) will be published elsewhere. The puri-

* For the sake of simplicity, we shall designate all transfer factors endowed with translocase activity as transfer factors G. In the case of the preparations from eukaryotic organisms, known to contain ribosome-specific translocases, we shall employ the designation transfer factor G_{70} or transfer factor G_{80} to identify the factors active on ribosomes of either the 70 S type or the 80 S type [17].

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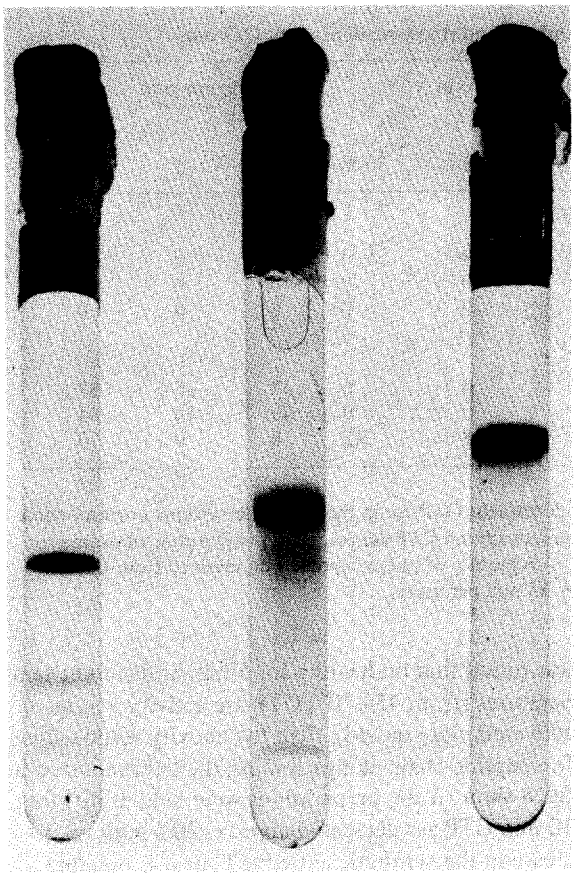

Fig. 1. Polyacrylamide gel electrophoresis of purified transfer factors. From left to right: *E. coli* transfer factor G, *P. zopfii* transfer factor G_{70}, *P. zopfii* transfer factor G_{80}. Electrophoresis was run at pH 8.9 and stained with amido schwartz.

fied transfer factors G were devoid of any aspecific, non-ribosome dependent GTP-ase activity.

Ribosome-dependent GTP-ase was assayed as reported by Conway and Lipmann [13] in a final volume of 100 μl containing 1 nmole of γ-^{32}P-GTP (approx. 1.3×10^5 cpm) and 140 μg of either *E. coli* or yeast ribosomes. *E. coli* transfer factor G corresponded to 12 μg of protein/assay; *P. zopfii* transfer factor G_{70} to 18 μg of protein/assay; *P. zopfii* transfer factor G_{80} to 20 μg of protein/assay.

Polyphenylalanine synthesis from L-U-^{14}C-phenylalanyl-tRNA was assayed as previously reported [15] using ribosomes and transfer factors G as in the GTP-ase assay but adding either *E. coli* transfer factor T corresponding to 18 μg of protein/assay or *P. zopfii* transfer factor T_{80} corresponding to 12 μg of protein/assay.

The activity of transfer factors specific for 70 S ribosomes was assayed on *E. coli* ribosomes while that of the factor specific for 80 S ribosomes was assayed on ribosomes from *S. cerevisiae* [14, 15].

In the experiments employing diphtheria toxin, the transfer factors G were preincubated with the toxin and NAD (0.3 μmole/assay) for 15 min at 30°. Appropriate controls showed that preincubation without the toxin did not inactivate the transfer factors and that the toxin-induced inhibition of the activity of transfer factor G_{80} was completely dependent on the presence of NAD.

3. Results and discussion

The data of table 1 demonstrate that as expected fusidic acid inhibits polyphenylalanine synthesis and GTP hydrolysis catalyzed by the ribosome-specific transfer factors G on either 70 S or 80 S ribosomes. As compared to *E. coli* transfer factor G, transfer factor G_{70} from *P. zopfii* appears to be more resistant to the inhibition by fusidic acid. A concentration more than 10-fold and possibly 100-fold higher of fusidic acid is indeed required to achieve an inhibition comparable to that obtained in the case of the factor from *E. coli*. Assuming, as reported for other eukaryotic organisms [16–19], that also in *P. zopfii* the transfer factor G specific for 70 S ribosomes is that responsible for protein synthesis in mitochondria (organelles known to contain ribosomes of the 70 S type), the reported data confirm a previous finding indicating that the transfer factors G from mitochondria are more resistant to the inhibition by fusidic acid than the corresponding factor isolated from *E. coli* [20].

Sporangiomycin, an antibiotic very similar if not identical to siomycin [21], appears to inhibit both polyphenylalanine synthesis and GTP-ase activities only in the systems acting on 70 S ribosomes, as reported for siomycin [6]. However it appears that polyphenylalanine synthesis and hence peptide chain elongation is much more sensitive to the action of this antibiotic than GTP-ase activity, as a concentra-

Table 1
Effect of specific inhibitors on polyphenylalanine synthesis and ribosome-dependent GTP-ase.

Transfer factor G from	Fusidic acid (M)			Diphteria toxin (μg/ml)			Sporangiomycin (μg/ml)		
	5×10^{-5}	5×10^{-4}	5×10^{-3}	3	15	3000	0.5	1	50
	Ribosome-dependent GTP-ase, inhibition (%)								
E. coli	0	49	80	0	0	0	0	0	66
P. zopfii G_{70}	0	0	48	0	0	0	0	0	47
P. zopfii G_{80}	0	37	81	0	0	0	0	0	0
	Polyphenylalanine synthesis, inhibition (%)								
E. coli	50	77	91	0	0	0	37	65	87
P. zopfii G_{70}	0	0	73	0	0	0	39	74	84
P. zopfii G_{80}	9	33	73	0	68	95	0	0	0

Assay conditions as reported under Materials and methods. Ribosome-dependent GTP-ase in the complete systems corresponded to approx. 800 pmoles of GTP hydrolyzed per 10 min per assay with practically no GTP-ase associated with either ribosomes or transfer factors G alone. Polyphenylalanine synthesis corresponded to 6–8 pmoles of phenylalanine polymerized from L-phenylalanyl-U-^{14}C-tRNA per 15 min per assay.

tion 100-fold higher of sporangiomycin is required to give an inhibition of GTP-ase comparable to that of polyphenylalanine synthesis. The uncoupling of translocase activity from GTP-ase activity is more clearly demonstrated in the case of the diphtheria toxin, a compound known to inhibit peptide chain elongation on 80 S ribosomes only by a specific NAD-dependent inactivation of the transfer factor G acting on such ribosomes [9–11]. While polyphenylalanine synthesis is inhibited at very low toxin concentration (approx. 15 μg of protein per ml) even a concentration 200-fold higher does not affect GTP-ase activity (fig. 2). Such results are at variance with those of some authors who have reported inhibition by the toxin of both GTP-ase and peptide chain elongation in the case of preparations of mammalian origin [11, 22]. If, as we have found, GTP-ase activity is not impaired under conditions leading to a complete inhibition of polyphenylalanine synthesis, the reverse (e.g. inhibition of GTP-ase but not of polyphenylalanine synthesis) should be possible. Albrecht et al. [23] have reported that heat treatment of transfer factor G_{80} from yeast decreases ribosome-dependent GTP-ase but not the synthetic activity. With such results in mind, we have subjected the preparations of the transfer factor G_{80} from *P. zopfii* to heat treatment and then assayed both activities. It was found that on heating for 5 min at different temperatures (from 45–70°) GTP-ase activity was inactivated more quickly than the activity responsible for peptide chain elongation. As the data reported in fig. 3 show, if the preparations were held at 60° for 10 min GTP-ase decreased approx. 70% with no decrease in the synthetic activity. Thus it is possible to obtain preparations practically devoid of ribosome-dependent GTP-ase but still capable of promoting polyphenylalanine synthesis. It may be added that polyphenylalanine synthesis in the presence of heat-treated preparations is still inhibited by fusidic acid and diphtheria toxin plus NAD. Analogous attempts to selectively inactivate GTP-ase activity in the preparations of transfer factor G_{70} from *P. zopfii* and transfer factor G from *E. coli* have been unsuccessful, as the loss of GTP-ase activity is parallelled by a loss of the capacity to catalyze polyphenylalanine synthesis. Such findings seem to indicate that in the transfer factors acting on ribosomes of the 70 S type, the two activities appear to be more tightly coupled.

The preparations of transfer factor G_{80} devoid of ribosome-dependent GTP-ase still split GTP to GDP in the course of polyphenylalanine synthesis (table 2). Nishizuka and Lipmann [2] have calculated that in *E. coli* one molecule of GTP is hydrolyzed per each peptide bond formed while two molecules of GTP

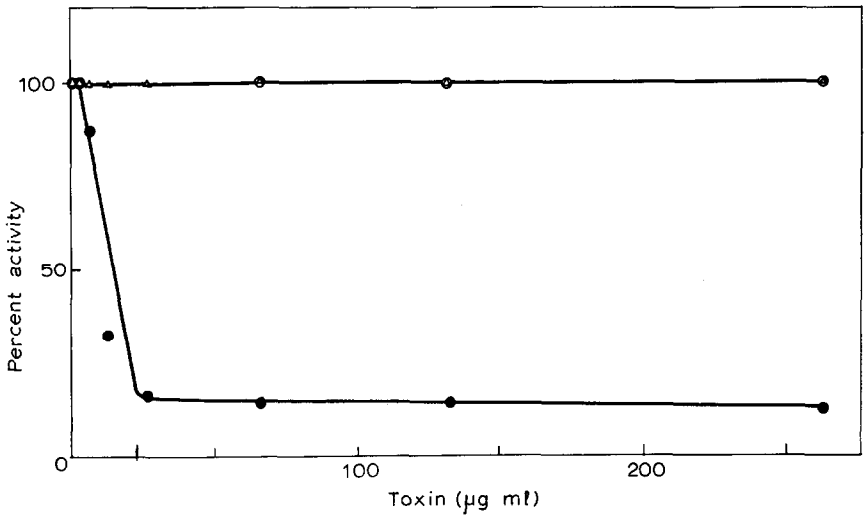

Fig. 2. Effect of diphtheria toxin on ribosome-dependent GTP-ase and polyphenylalanine synthesis catalyzed by *P. zopfii* transfer factor G_{80}. Assay conditions as reported in Materials and methods except that toxin concentration was varied as shown. •—•, polyphenylalanine synthesis; △—△, GTP-ase activity; ○—○, GTP-ase or polyphenylalanine synthesis in the absence of NAD.

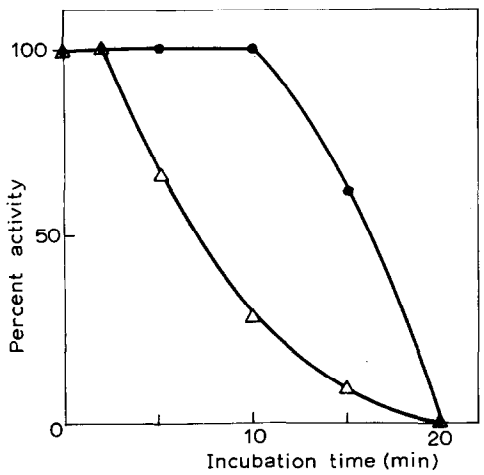

Fig.3. Effect of heat treatment on ribosome-dependent GTP-ase and polyphenylalanine synthesis catalyzed by *P. zopfii* transfer factor G_{80}. Aliquots of *P. zopfii* transfer factor G_{80} were heated at 60° for the shown time intervals. After cooling GTP-ase (△—△) and polyphenylalanine synthesis (•—•) were assayed as reported in Materials and methods.

are hydrolyzed in the case of *Bacillus stearothermophilus* [24]. Our estimate in the case of *P. zopfii* transfer factor G_{80} and yeast ribosomes is approx. 2 molecules of GTP hydrolyzed per each peptide bond formed. In the case of the heat treated preparations, practically devoid of ribosome-dependent GTP-ase, fusidic acid inhibits polyphenylalanine synthesis and the GTP-ase presumably linked to peptide bond formation. On the other hand this hydrolysis of GTP is not inhibited by diphtheria toxin at concentrations which inhibit completely polyphenylalanine synthesis.

The reported data show that it is possible to uncouple ribosome-dependent GTP-ase from polyphenylalanine synthesis. Support to this conclusion is offered also by the reported isolation from the same organism of multiple forms of a transfer factor G_{80}, some of which are devoid of GTP-ase activity [23]. In addition, we have reported that some preparations of transfer factors G from *P. zopfii* and yeast do not show appreciable ribosome-dependent GTP-ase even if they catalyze polyphenylalanine synthesis [12]. We wish to conclude that at least *in vitro* the ribosome-dependent hydrolysis of GTP associated with the transfer factors catalyzing translocation plays no apparent role in the process of protein synthesis. It may be that this activity has a more subtle signifi-

Table 2
Effects of inhibitors on GTP hydrolysis and polyphenylalanine synthesis by heat-treated preparations of *P. zopfii* transfer factor G_{80}.

System	Ribosome-dependent GTP-ase (pmoles/assay)	Peptide bond-dependent GTP-ase (pmoles/assay)	Phenylalanine polymerized (pmoles/assay)
Complete	0.72	4.93	2.4
Complete + diphtheria toxin + NAD		6.02	0.5
Complete + fusidic acid		0.21	0.5

Assay conditions as reported under Materials and methods except that transfer factor G_{80} was heated at 60° for 10 min to inactivate ribosome-dependent GTP-ase. Ribosome-dependent GTP-ase was assayed in the absence of transfer factor T_{80}. Under such conditions polyphenylalanine synthesis was negligible. Control experiments employing untreated transfer factor G_{80} gave a ribosome-dependent GTP-ase corresponding to 16.6 pmoles/assay. Fusidic acid concentration was 5×10^{-3} M while diphtheria toxin used was at a concentration of 250 μg/ml.

cance that has eluded our attention or that this activity is necessary only for the synthesis of protein *in vivo*. Alternatively, ribosome-dependent GTP-ase activity reported also for *E. coli* transfer factor T [25] may be an artifact of the isolated proteins. As already pointed out by Nishizuka and Lipmann [4] the energetic requirement for peptide bond formation appears to be adequately met by the hydrolysis of the bond between the amino acid (or the peptide) and the tRNA and the hydrolysis of one molecule of GTP for every peptide bond formed.

The data of table 2 further show that it is possible to inhibit polyphenylalanine synthesis without affecting the hydrolysis of GTP presumably linked to the formation of the peptide bond. Thus even this hydrolytic activity may be uncoupled from peptide bond synthesis.

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References

[1] F. Lipmann, Science 164 (1969) 1024.
[2] Y.Nishizuka and F. Lipmann, Proc. Natl. Acad. Sci. U.S. 55 (1966) 212.
[3] Y. Kuriki, N. Inoue and Y.Kaziro, Biochim. Biophys. Acta 224 (1970) 487.
[4] Y. Nishizuka and F. Lipmann, Arch. Biochem. Biophys. 116 (1966) 344.
[5] L. Felicetti and F. Lipmann, Arch. Biochem. Biophys. 125 (1968) 548.
[6] J. Modolell, D. Vazquez and R. E. Monro, Nature 230 (1971) 109.
[7] T. Tanaka, T. Kinoshita and H. Masukawa, Biochem. Biophys. Res. Commun. 30 (1968) 278.
[8] M. Malkin and F. Lipmann, Science 164 (1969) 71.
[9] R. J. Collier and A. M. Pappenheimer, J. Exp. Med. 120 (1964) 1010.
[10] R. J. Collier and J. A. Traugh, Cold Spring Harbor Symp. Quant. Biol. 34 (1969) 589.
[11] T. Honjio, Y. Nishizuka and O. Hayashi, Cold Spring Harbor Symp. Quant. Biol. 34 (1969) 503.
[12] O. Tiboni, B. Parisi, A. Perani and O. Ciferri, J. Mol. Biol. 47 (1970) 467.
[13] T. W. Conway and F. Lipmann, Proc. Natl. Acad. Sci. U.S. 52 (1964) 1462.
[14] B. Parisi, G. Milanesi, J. Van Etten, A. Perani and O. Ciferri, J. Mol. Biol. 28 (1967) 295.
[15] O. Ciferri, B. Parisi, A. Perani and M. Grandi, J. Mol. Biol. 37 (1968) 528
[16] R. D. Richter and F. Lipmann, Biochemistry 9 (1970) 5065.
[17] A. Perani, O.Tiboni and O. Ciferri, J. Mol. Biol. 55 (1971) 107.
[18] M. Grandi and H. Küntzel, FEBS Letters 10 (1070) 25.
[19] L. Felicetti and G. E. Di Matteo, J. Mol. Biol. 54 (1970) 611.

[20] M. Grandi, A. Helms and H. Küntzel, Biochem. Biophys. Res. Commun. 44 (1971) 864.
[21] J. E. Thiemann, C. Coronelli, H. Pagani, G. Beretta, G. Tamoni and V. Arioli, J. Antibiotics 21 (1968) 525.
[22] S. Raeburn, R. S. Goor, J. A. Schneider and E.S. Maxwell, Proc. Natl. Acad. Sci. U.S. 61 (1968) 1428.
[23] U. Albrecht, K. Prenzel and D. Richter, Biochemistry 9 (1970) 361.
[24] Y. Ono, A. Skoultchi, J. Waterson and P. Lengyel, Nature 223 (1969) 697.
[25] J. Gordon, J. Biol. Chem. 244 (1969) 5680.