ALTERED SPECIFICITY OF SYNTHESIS OF GUANOSINE TETRAPHOSPHATE (ppGpp) AND PENTAPHOSPHATE (pppGpp) BY SALT-WASHED RIBOSOMES

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Received March 18,1974

SUMMARY

Salt-washed ribosomes from Escherichia coli, plus stringent protein, form more ppGpp than pppGpp from GTP at all times, but unwashed ribosomes are shown to synthesize primarily pppGpp as the initial product.

Cells of stringent (rel$^+$) strains of Escherichia coli, when starved for a required amino acid, accumulate two guanosine nucleotides, ppGpp (MS I)† and pppGpp (MS II) (1), which appear to play a role in regulating ribosomal RNA synthesis. It is not clear whether both of these products play a regulatory role or whether one is instead a byproduct or an intermediate. Recently it was shown that preparations of ribosomes from stringent strains, in the presence of mRNA and uncharged tRNA, catalyze a pyrophosphoryl transfer from ATP to either GTP or GDP (2); the reaction requires an enzyme (3) called stringent protein (2), which appears to be present on the 50S subunit throughout the ribosome cycle (4). The reaction of salt-washed ribosomes (plus stringent protein) with GDP yielded ppGpp, while GTP yielded not only pppGpp but also considerable ppGpp; the latter might have been formed by dephosphorylation either of pppGpp or of its precursor GTP (2). To understand further the formation of the two products from GTP the activities of ribosomes were compared before and after treatment with high salt. The results show that this treatment markedly affects the specificity

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†Abbreviations: ppGpp, Guanosine 5'-diphosphate-3'-diphosphate; pppGpp, Guanosine 5'-triphosphate-3'-diphosphate; (p)ppGpp = pppGpp + ppGpp.

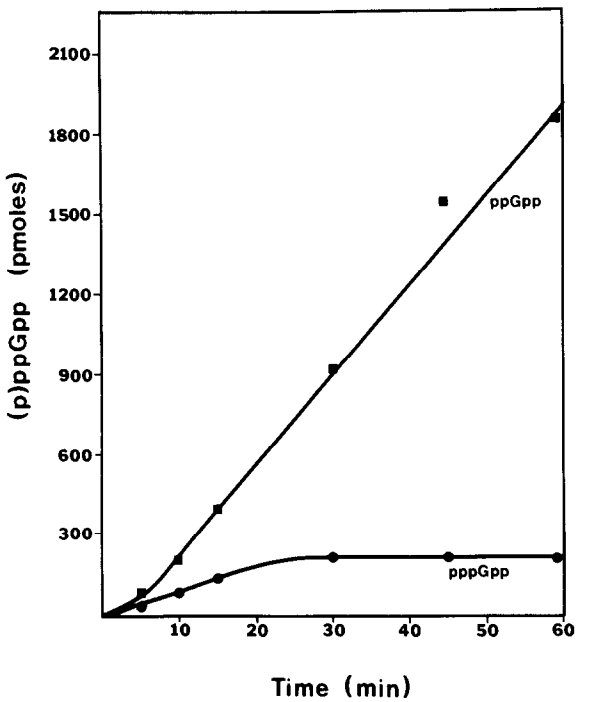

Figure 1. Synthesis of pppGpp and ppGpp with a fractionated system containing salt-washed ribosomes and crude stringent protein.

The ribosomes washed with 0.5 M NH_4Cl, and the wash containing the stringent protein, were prepared from E. coli, strain CP 78. The in vitro system synthesizing pppGpp and ppGpp included 2.98 A_{260} units of ribosomes and 9.6 µg of stringent protein in a 50 µl reaction mixture containing 50 mM Tris-acetate (pH 7.8), 2 mM dithiothreitol, 12 mM $Mg(OAc)_2$, 28 mM NH_4OAc, 10 mM KOAc, 0.55 mM GTP (0.8 µCi of [α-^{32}P]GTP, New England Nuclear Corp.) and 2.2 mM ATP. After incubation at 37° for the indicated times, the reaction mixtures were chilled and processed as described (4). Briefly, this was done by chromatographing the samples on polyethyleneimine cellulose thin layer plates, identification of the spots of pppGpp and ppGpp by autoradiography and counting the spots.

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of the synthesis.

The time course of pppGpp and ppGpp synthesis was studied in the in vitro system described earlier (2), in which the ribosomes were fractionated by washing with high salt to separate the stringent protein; this component, with or without further purification, was then recombined with the (salt-washed) ribosomes. It was anticipated that stopping the reaction earlier than the reported 30 min period (2) might reveal initial conversion of GTP only into pppGpp. However, as Figure 1 shows, not only

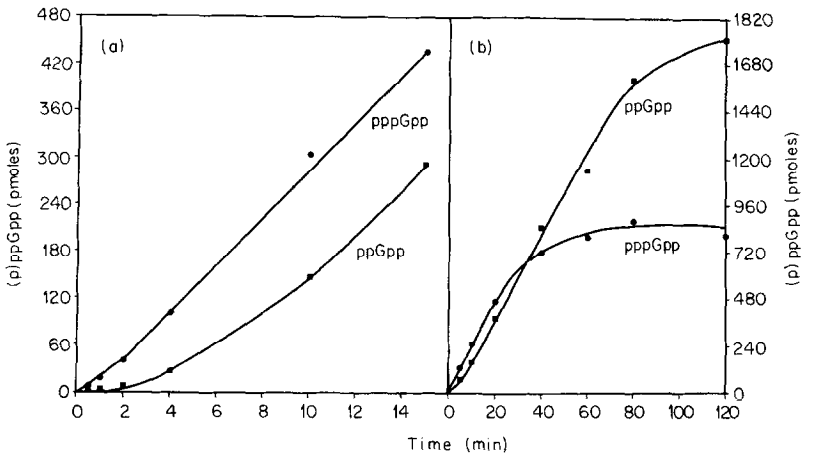

Figure 2. Kinetics of pppGpp and ppGpp synthesis by unfractionated ribosomes.

A solution of unwashed ribosomes was layered over an equal volume of a sucrose cushion (40% sucrose in 10 mM Tris-acetate pH 7.8, 14 mM $Mg(OAc)_2$, 60 mM KOAc, and 1 mM dithiothreitol) and centrifuged for 12 hr at 40,000 rpm (4). The pelleted ribosomes were resuspended in the same buffer and washed once. The resulting sucrose-washed ribosomes, with the associated stringent protein, were used as the source of all ribosomal components required for the reaction. The reaction mixtures contained 3.76 A_{260} units of ribosomes besides the buffer and salts indicated in Figure 1. Other details are described in Figure 1.

Early (a) and prolonged (b) kinetics of pppGpp and ppGpp synthesis.

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the rate but the extent of this synthesis was low at all times: the major product was always ppGpp. Similar results have also been obtained in a highly purified system (5), where the stringent protein and the ribosomes were further fractionated. Thus, both the crude and the highly purified systems that have been employed so far, with salt-washed ribosomes, preferentially accumulate ppGpp when GTP is provided as the substrate. It therefore appeared that the synthesis of pppGpp might not be dissociable from that of ppGpp.

However, during the course of studies on the location of the stringent protein (4) it was found that if ribosomes were not fractionated by washing with high salt, and if they were passed through a sucrose cushion (which apparently removed inhibitors that blocked the reactions), they then synthesized about equal amounts of pppGpp and ppGpp in 30 min. Moreover, when

the kinetics of this synthesis was examined only pppGpp could be detected at first: ppGpp began to appear after a 4 to 5 min lag, and the rate of its synthesis approached that of pppGpp after about 10 min (Fig. 2a). After about 30 min the concentration of pppGpp gradually leveled off but that of ppGpp continued to rise linearly for at least 70 min (Fig. 2b). The GTP level fell steadily, and was very low by 120 min, while the GDP level remained relatively constant after 5 min (estimates from chromatographic spots).

Whatever the paths to the two products, it is clear that salt fractionation of ribosomes, though helpful in analyzing the reaction, seriously distorts it in favor of the synthesis of ppGpp. This effect suggests the possibility of an additional regulatory component in the system.

Acknowledgements: This work was supported by grants from the U.S. Public Health Service and the American Cancer Society to Bernard D. Davis. I am grateful for the critical comments of Dr. Davis on the manuscript.

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