ON THE FUNCTION OF GUANOSINE TRIPHOSPHATE IN THE FORMATION OF N-ACETYL-PHENYLALANYL PUROMYCIN

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Summary: In the presence of washed E. coli ribosomes, the formation of N-Ac-phenylalanyl puromycin from N-Ac-phe-RNA and puromycin at 5 mM Mg^{++} does not proceed unless initiation factors (ribosomal wash) and GTP are also added. GMPPCP cannot replace GTP under these conditions. However, if the N-Ac-Phe-RNA is prebound to the ribosomes in the presence of initiation factors and GMPPCP, and the N-Ac-phe-RNA-ribosome-polyU complex is first diluted and adsorbed on a cellulose nitrate filter, then the bound N-Ac-phe-RNA reacts efficiently with puromycin. Thus, in this system the conversion of GTP to GDP does not appear to be a prerequisite for the binding of N-Ac-phe-RNA to the ribosome in a puromycin-reactive state.

In the presence of polyU, GTP, elongation factors and at low concentrations of Mg^{++}, E. coli ribosomes previously washed with buffered NH_4Cl solutions can carry out the formation of polyphenylalanine from Phe-RNA provided that initiation factors and initiator N-Ac-phe-RNA are added (1). When puromycin is used in place of Phe-RNA and the elongation factors, this model system can be used for the study of peptide chain initiation as reflected by the formation of N-Ac-phe-pu (1). This latter reaction can proceed efficiently only in the presence of GTP which is converted to GDP and inorganic phosphate, and GMPPCP cannot replace GTP presumably due to its inability to be cleaved analogously. Similar observations have been made (2,3) in a system in which N-Formyl-methionyl-RNA replaces N-Ac-phe-RNA to formN-Formyl-methionyl-puromycin. These results have led to the view (4,5) that the conversion of GTP to GDP is necessary for binding the N-protected amino acyl-RNA to the ribosomal "peptide" or "donor" site.

Abbreviations used: PolyU, polyuridylic acid; FWR, "factors washable from ribosomes"; N-Ac-phe-RNA, N-Acetyl-phenylalanyl-t-RNA; N-Ac-phe-pu, N-Acetyl-phenylalanyl-puromycin; N-Ac-phe, N-Acetyl-phenylalanine; GMP, GDP, GTP, 5'-guanosine mono-, di-, tri-phosphate, respectively; GMPPCP, 5'-guanylyl-methylene diphosphonate; AMPPCP, 5'-adenylyl-methylene diphosphonate; UMPPCP, 5'-uridylyl-methylene diphosphonate; t-complex, N-Ac-phe-RNA-ribosome-polyU ternary complex.

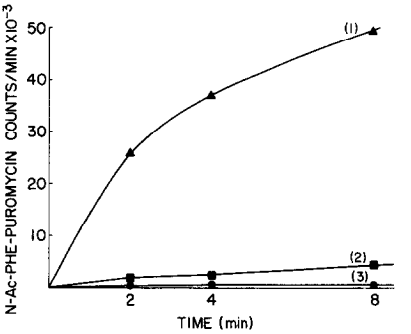
We have reevaluated this postulate by examining the formation of N-Ac-phe-pu at 5 mM Mg^{++} in a reaction mixture containing initiation factors (ribosomal wash), and have also observed that under these conditions GMPPCP cannot replace GTP in this reaction. However, as we show in this communication, when N-Ac-phe-RNA is prebound to the ribosomes in the presence of GMPPCP and "ribosomal wash", and the reaction mixture is then diluted with buffer and passed through a cellulose nitrate filter, then the N-Ac-phe-RNA bound to the adsorbed ribosomal t-complex is reactive towards puromycin. This observation suggests that the conversion of GTP to GDP may not be a prerequisite for binding the N-protected amino acyl-RNA to the ribosomal "peptide" site.

Materials and Methods: "Washed ribosomes" were prepared from frozen E. coli B cells. The non-preincubated S-30 fraction (6) was spun at 100,000 x g for 4 hours. The supernatant was decanted and the pellets were suspended in 0.5 M NH_4Cl - 0.01 M $MgCl_2$ - 0.01 M Tris-HCl, pH 7.4 ("NH_4Cl-wash buffer") and left overnight in ice. After centrifuging at 100,000 x g for 6 hrs (the supernatant represents the "first NH_4Cl ribosomal wash") the pellets were resuspended in the "NH_4Cl-wash buffer", kept overnight in ice, and centrifuged at 100,000 x g for 6 hrs. Washing of the ribosomes was repeated once. The washed ribosomes were finally suspended in "standard buffer" (10 mM Tris-HCl pH 7.8, 10 mM $MgAc_2$, 60 mM KCl, 6 mM β-mercaptoethanol) dialyzed for 4 hours at 4°C against the same buffer and stored at -70°C in small aliquots. Fraction FWR (Factors washable from ribosomes) was used as a source of initiation factors and was prepared from the "first NH_4Cl ribosomal wash" which was brought to 80% saturation with ammonium sulfate while maintaining the pH at 7.2 with 1 N NaOH. The resulting precipitate was dissolved in "standard buffer" and dialyzed overnight at 4°C against this buffer. Any precipitate which developed after the dialysis was removed, by centrifugation at 30,000 x g for 15 min, and the supernatant passed through a Sephadex G-25 column equilibrated with "standard buffer". This fraction (FWR) was divided into small aliquots, kept at -70°C and thawed immediately before use. Its protein content was determined according to Lowry et al. (7). H^3-L-phe-RNA was prepared as previously described (8) and then converted to N-Ac-H^3-phe-RNA according to Haenni and Chapeville (9). It was then purified on a Sephadex G-25 column equilibrated with 0.01 M sodium

acetate buffer of pH 5.5, at 4°C. GMP, GDP and GTP were purchased from PL Biochemicals. PolyU, GMPPCP, AMPPCP and UMPPCP were purchased from Miles Chem. Co. Millipore cellulose nitrate filters were purchased from Millipore Corporation.

Preincubation (Prebinding of N-Ac-H^3-phe-RNA to the ribosomes). The "complete system" contained in 200 μl: 25 μmoles Tris-HCl buffer (pH 7.2), 25 μmoles NH_4Cl adjusted with NH_4OH to pH 7.2, 1.0 μmole total Mg^{++} (acetate), 80 μg polyU, "nucleotide" (GTP, GDP, GMPPCP, etc.) at 5 x 10^{-4} M as indicated, 8.0 A_{260} units of washed ribosomes, 45 μg (protein) FWR fraction, and 6.4 A_{260} units N-Ac-H^3-phe-RNA charged with 100 pmoles of H^3-phe (300,000 cpm total). After incubation at 25°C for 8 min, the reaction mixture was cooled in ice and one of the three following assays was performed:

(1) Assay A (N-Ac-phe-pu formation without adsorption on millipore discs) carried out



Legend to Fig. 1

Time course of N-Ac-H^3-phe-pu formation when the FWR fraction, the "nucleotide" and puromycin were contained in the same reaction vessel. Curve 1 (triangles) with GTP; curve 2 (squares) with GDP; curve 3 (circles) with GMP or GMPPCP. Curve 3 represents also the course of the reaction when no "nucleotide" was added or, when the FWR fraction was omitted and GTP was present or absent.

The amount of N-Ac-H^3-phe-pu formed was determined by Assay A which was carried out as follows: To the preincubated reaction mixture (200 μl) given in Methods, were added 50 μl of an ice-cold mixture containing 0.25 μmole magnesium acetate and 0.025 μmole puromycin monohydrochloride. After mixing quickly, the mixture was incubated at 25° for the desired amount of time, and, after recooling in ice, the reaction was stopped by adding 25 μl of 4 N KOH. A 200 μl aliquot was transferred to 1.0 ml of potassium acetate pH 5.5 buffer, and N-Ac-H^3-phe-pu was extracted in 2.0 ml of ethyl acetate essentially as described by Leder and Bursztyn (13). One and one-half ml of the ethyl acetate phase was mixed with 15 ml of Bray's phosphor and counted as described under Assay B. After correcting for radioactivity quenching the values were multiplied by 1.33 in order to estimate the total amount of N-Ac-H^3-phe-pu formed in the 200 μl aliquot. Controls without puromycin were included in each determination and the values obtained were subtracted. These results are given on the ordinate. A further computation is needed for estimating the total N-Ac-H^3-phe-pu formed in the whole reaction mixture of 275 μl.

as described in the legend of Fig. 1, (2) Assay B (Binding Assay) and (3) Assay C (N-Ac-phe-pu formation with the disc-adsorbed t-complex) both described in the legend of Table I. All values given in counts/min throughout this paper have been corrected for radioactivity quenching.

Results and Discussion: As shown in Fig. 1, when GTP at 5×10^{-4} M was used in the prebinding of N-Ac-phe-RNA to the ribosome, approx. 32% of the prebound N-Ac-phe-RNA (determined by Assay B) was converted in 2 min to N-Ac-phe-pu on the subsequent addition of puromycin (Assay A). In contrast, GMPPCP, GDP or GMP, at 5×10^{-4} M, did not support N-Ac-phe-pu formation. The same results were obtained when the concentration of GMPPCP, GDP or GMP was lowered to 5×10^{-5} M, or when the concentration of the FWR fraction was doubled. In the absence of the FWR fraction, virtually no reaction took place (Fig. 1, curve 3). Similarly, when the prebinding stage was omitted and puromycin was added before the N-Ac-phe-RNA and the mixture incubated once, only GTP but not GMPPCP, GDP or GMP could promote N-Ac-phe-pu formation (results not shown).

TABLE 1

ABILITY OF GMPPCP TO PROMOTE N-AC-PHE-PU FORMATION

Prebinding Conditions	N-Ac-H^3-phe-RNA Bound cpm	Percent N-Ac-H^3-phe-RNA converted to N-Ac-H^3-phe-pu in 1 min	2 min	4 min
C.S.* with GTP	53,714	16.5	29.2	47.2
" minus nucleotide	2,596	1.9	3.8	6.8
" minus FWR and nucleotide	5,747	0.9	2.1	3.1
" with GMPPCP	22,516	13.2	23.2	37.3
" with GDP	15,788	14.1	23.4	35.8
" with GMP	6,323	1.9	2.6	2.5
" with AMPPCP	2,865	1.8	1.8	2.2
" with UMPPCP	2,335	1.5	1.9	2.5

* Complete System (see Methods)

Legend to Table I

The amount of N-Ac-H^3-phe-RNA bound in the t-complex (second column) was determined with Assay B as follows: To the preincubated mixture given in Methods (200 µl), 50 µl of ice-cold water containing 0.25 µmole magnesium acetate were added. To 100 µl of the mixture, were added 4 ml of ice-cold buffer containing 100 mM Tris-HCl (pH 7.2), 50 mM KCl and 5.0 mM magnesium chloride (binding buffer). After mixing, the solution was immediately passed through a millipore cellulose nitrate filter (type HA 24 mm diameter; 0.45 micron pore size) and the filter was washed quickly with three 4 ml portions of the "binding buffer", without allowing any air to pass through the filter until the end of the third washing. These operations (filtering and washings) were carried out within 2 min from the time of dilution of the preincubated reaction mixture. The millipore disc was air-dried, dissolved in 15 ml of Bray's (14) phosphor, and counted in a Packard liquid scintillation spectrometer equipped with automatic external standardization which was used in correcting for quenching.

The percent of the N-Ac-H^3-phe-RNA, bound in the disc-adsorbed t-complex, which could react with puromycin was determined with Assay C which was carried out as follows: To the preincubated mixture (200 µl), given in Methods, were added 50 µl of ice cold water containing 0.25 µmole magnesium acetate. Four ml of ice cold "binding buffer" were added to a 200 µl aliquot of this mixture, and filtration through a millipore disc and washings, were carried out exactly as described in Assay B. After the third washing the millipore disc was not air-dried but was immersed quickly into ice-cold buffer consisting of 100 mM Tris-HCl (pH 7.2), 100 mM NH_4Cl adjusted with NH_4OH to pH 7.2, 5.0 mM $MgCl_2$ and 6.0 mM β-mercaptoethanol. Several such discs with pre-bound t-complex could be prepared by scaling up the original preincubation mixture. The discs could stay in this buffer up to two hrs without decomposition of the adsorbed t-complex. Each disc was cut in two halves, the exact size of which was not critical for the assay. Quarters or whole discs can be also used if so desired. The reaction between N-Ac-H^3-phe-RNA, bound to the adsorbed t-complex, and puromycin was carried out as follows: To each one of a series of small beakers (empty scintillation vials can be conveniently used for this purpose) was added 1.0 ml of a mixture consisting of 100 mM Tris-HCl (pH 7.2), 100 mM NH_4Cl adjusted to pH 7.2 with NH_4OH, 5.0 mM $MgCl_2$, 6.0 mM β-mercaptoethanol and 1×10^{-4} M puromycin monohydrochloride. The beakers were brought to 25°C and gently shaken by the use of a water bath provided with a shaking platform (e.g. Dubnoff shaker). At the desired intervals the puromycin reaction was started by adding to each beaker the half disc bearing the t-complex, and, after the desired reaction time, 1.0 ml of 1 N NaOH was added to stop the reaction. Shaking continued at 25° for 30 minutes to assure hydrolysis of the unreacted N-Ac-phe-RNA to N-Ac-phe and t-RNA. Of this alkaline mixture (2.0 ml) one aliquot (0.80 ml) was transferred to a scintillation vial and the sum of N-Ac-H^3-phe and N-Ac-H^3-phe-pu present in the aliquot was determined after adding 0.5 ml of 1.0 N HCl, 15 ml of Bray's phosphor and counting. An equal aliquot (0.80 ml) was transferred to a centrifuge tube and N-Ac-H^3-phe-pu was extracted in 2.0 ml of ethyl acetate, adapting the method of Leder and Bursztyn (13). One and one-half ml of the ethyl acetate phase was mixed with 15 ml of Bray's phosphor and counted. After correction, for radioactivity quenching the values were multiplied by 1.33 in order to estimate to total amount of N-Ac-H^3-phe-pu formed in the whole aliquot of 0.80 ml. The percent of the bound N-Ac-H^3-phe-RNA that was converted to N-Ac-H^3-phe-pu was calculated by dividing the value for the sum [N-Ac-H^3-phe-pu plus N-Ac-H^3-phe] into the value for N-Ac-H^3-phe-pu, and multiplying by 100. Controls without puromycin were included in each determination and the values obtained were subtracted. The results are given in the last three columns of this Table.

The above results were obtained without separating the t-complex from the pre-incubated reaction mixture. When however the prebinding of N-Ac-phe-RNA was carried out in the presence of GMPPCP but, instead of adding puromycin, the mixture was first

diluted with buffer and passed through a millipore disc, then the N-Ac-phe-RNA bound to the disc-adsorbed t-complex was reactive towards puromycin (Table 1). The ability of the adsorbed t-complex to react with puromycin has been studied in detail (10) and forms the subject of a separate communication (11). As shown in Table 1, when either GMPPCP or the FWR fraction were omitted, the amount of N-Ac-phe-RNA bound to the t-complex was low and whatever was bound was only slightly reactive towards puromycin. GMP, AMPPCP or UMPPCP could not replace GMPPCP or GTP in supporting either efficient binding of N-Ac-phe-RNA or its reactivity towards puromycin. However, GDP could promote N-Ac-phe-pu formation to the same extent as GMPPCP, although its effect in stimulating the binding of N-Ac-phe-RNA was lower (Table 1). It should be recalled that, like GMPPCP, GDP was inactive in bringing about N-Ac-phe-pu formation under the conditions of Assay A (Fig. 1). It remains to be established whether, under the conditions of Assay C, GDP acts per se in promoting N-Ac-phe-pu formation, or it acts via a previous conversion to GTP.

The concentration of the FWR fraction is critical for the ability of GMPPCP to replace GTP in N-Ac-phe-pu formation under the conditions of Assay C. When the amount of FWR was doubled to 90 μg, the reactivity towards puromycin of the N-Ac-phe-RNA prebound in the presence of GMPPCP was decreased by 50%, whereas that of the N-Ac-phe-RNA prebound in the presence of GTP was virtually unchanged. Under the optimal conditions used in the experiments reported in Table 1, the reactivity towards puromycin of the N-Ac-phe-RNA bound in the presence of GMPPCP was 75-80% of that obtained when GTP was used. Other incubation conditions are at present being examined in an attempt to determine the parameters that govern the efficiency of GMPPCP. In this respect, it may be relevant to note that at 5 mM Mg^{++} the t-complex formed in the presence of GMPPCP is labile when in solution in ice-cold "binding buffer". After being kept in ice for 1 hr the t-complex in this solution was decomposed by 50%, whereas the complex formed in the presence of GTP was decomposed at this time by only 15%. In contrast, after its adsorption on the cellulose nitrate disc the t-complex formed in the presence of GMPPCP was stable for up to two hrs when kept in the ice-cold buffer used in Assay C.

The experiments reported in this communication demonstrate that GMPPCP can sub-

stitute for GTP in bringing N-Ac-phe-RNA to a puromycin-reactive state on the ribosome. It is suggested thereby, that the conversion of GTP to GDP is not necessary for this process.

After this work was completed we became aware of a very recent short communication (12) in which a similar requirement for GTP in a different system, namely the formation of N-Formyl-methionyl puromycin at 7 mM Mg^{++}, was satisfied by GMPPCP provided that the ternary complex was first isolated by column chromatography on Sephadex G-200. The methodology used in the present investigation is more rapid and convenient than column chromatography, and provides a stable t-complex formed at lower Mg^{++} concentrations where the ribosomal binding of the initiator amino acyl-RNA, which is independent of initiation factors and GTP, becomes minimal.

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