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Biochemistry

Stringent factor from *Escherichia coli* directs ribosomal binding and release of uncharged tRNA

[70S ribosome·poly(U)·tRNA·stringent factor complex/acceptor site/peptidyl site/ppGpp/pppGpp/ATP-PP_i exchange]

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ABSTRACT **Uncharged tRNA is preferentially bound to the peptidyl site of the ribosome in the absence of stringent factor, but in its presence is directed to the acceptor site. The synthesis of pppGpp and ppGpp is initiated by tRNA bound in the acceptor but not in the peptidyl site. In this reaction, tRNA is not permanently attached to the acceptor site. Uncharged [^{32}P]tRNA but not ^{3}H-labeled stringent factor is released from the ribosome after each round of stringent factor-dependent hydrolysis of ATP. ATP-$^{32}PP_i$-exchange experiments reveal that exchange is independent of the presence of GTP but strongly enhanced by the addition of stringent factor and tRNA. The tRNA release is suppressed when ATP is replaced by β,γ-imido adenosine 5′-triphosphate, 5′-AMP, or GTP.**

A ribosomal wash factor (stringent factor) of stringent *Escherichia coli* cells promotes the *in vitro* synthesis of guanosine polyphosphates, guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp), by transferring pyrophosphate from ATP to the 3′-OH group of GTP or GDP (1, 2). This transfer reaction occurs on ribosomes and is initiated when codon-specific, uncharged tRNA (3) or the tetranucleotide TpΨpCpGp (4) is bound to the ribosomal acceptor site. Synthesis of pppGpp + ppGpp is immediately stopped when the elongation factor Tu-aminoacyl-tRNA-GTP complex is bound to the ribosomal acceptor site (A-site), and the ribosomes are actively involved in the peptide chain elongation cycle (3).

The mechanism of the binding of uncharged tRNA to the ribosome has not yet been resolved. In particular it is not known whether the stringent factor is engaged in this reaction. It is reported here that the stringent factor is actively involved not only in the binding of tRNA, but also in its release from the ribosomal acceptor site.

MATERIALS

The 50S and 30S ribosomal subunits from *E. coli* strain CGSC 2834/a were isolated as described (5); the stringent factor from the same strain was purified as outlined in detail (see ref. 5). [α-^{32}P]GTP was purchased from New England Nuclear Corp., Boston. $tRNA^{Phe}_{yeast}$, poly(U), and β,γ-imido adenosine 5′-triphosphate (AMPPNP) came from Boehringer, Mannheim. *N*-Ac[^{14}C]Phe-tRNA and [^{14}C]Phe-tRNA were prepared from $tRNA^{Phe}_{yeast}$ and purified by benzoylated DEAE-cellulose (Schwarz BioResearch) chromatography (6). [^{32}P]tRNA was prepared by growing yeast cells with 5–10 mCi of NaH_2PO_4 (7). The isolated yeast [^{32}P]tRNA was charged with phenylalanine (8) and purified on a benzoylated DEAE-cellulose column (6). Phe-[^{32}P]tRNA was deacylated at pH 10 with NH_4OH, incubated at 37° for 30 min, and re-isolated by ethanol precipitation. The specific activity of this preparation was 20 Ci/mol (1 A_{260} unit of tRNA ≙ 1.5 nmol). ^{3}H-Labeled stringent factor was prepared by the reductive methylation method (ref. 9; specific activity 0.5–5.0 Ci/mol).

METHODS

Formation of Ribosome·Poly(U) Complex (Mixture 1). The standard reaction mixture (250 μl) contained 300 pmol of 50S subunits (1 A_{260} unit ≙39 pmol), 300 pmol of 30S subunits (1 A_{260} unit ≙67 pmol), 200 μg of poly(U), 40 mM Tris·HCl, pH 7.8, 10 mM Mg-acetate, and 4 mM dithiothreitol. The mixture was incubated at 30° for 10 min and cooled to 4° (5).

Preparation of the 70S·Poly(U)·[^{32}P]tRNA·^{3}H-Labeled Stringent Factor Complex. Mixture 1 (250 μl), 140–350 pmol of ^{3}H-labeled stringent factor, and 350–600 pmol of [^{32}P]tRNA were mixed in a volume of 500 μl, the final concentration being 20 mM Tris·HCl, pH 7.8, 20 mM Mg-acetate, 2 mM dithiothreitol. The mixture was kept at 4° for 30 min, and then layered on top of a 5% sucrose solution (1.6 ml) that contained 20 mM Tris·HCl, pH 7.8, 20 mM Mg-acetate, 2 mM dithiothreitol, and finally centrifuged at 160,000 × *g* for 3.5 hr in a Beckman Ti rotor 40. The ribosomal pellet was rinsed three times with 2 ml of buffer 1 (20 mM Tris·HCl, pH 7.8, 10 mM Mg-acetate, 2 mM dithiothreitol) and then resuspended in 50 μl of the same buffer. The absorbance of the ribosomal suspension was determined at 260 and 280 nm; assuming that the suspension contained mostly 70S ribosomes, the yield was 125 pmol (1 A_{260} unit of 70S ribosomes ≙25 pmol). The complexed ribosomes were analyzed for ^{32}P or ^{3}H radioactivity and for ppGpp + pppGpp synthesizing activity. The latter reaction was carried out in a 50-μl volume that contained 20 mM Tris·HCl, pH 7.8, 2 mM dithiothreitol, 40 mM NH_4Cl, 5 pmol of complexed ribosomes, 2 mM ATP, 0.177 mM [α-^{32}P]GTP, and varying amounts of Mg^{++} as indicated in the legends to the figures and tables. The reaction mixture was incubated at 37° for 1 hr, and pppGpp + ppGpp were analyzed by polyethyleneimine thin-layer chromatography and autoradiography (10). Radioactive spots were cut out, and radioactivity was determined in Bray's solution (5). The conversion of radioactive GTP into ppGpp + pppGpp is given as percentage of added GTP.

Abbreviations: pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; AMPPNP, β,γ-imido adenosine 5′-triphosphate; A-site, acceptor site; P-site, peptidyl site; 1 A_{260} unit, that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent when the light path is 1 cm.

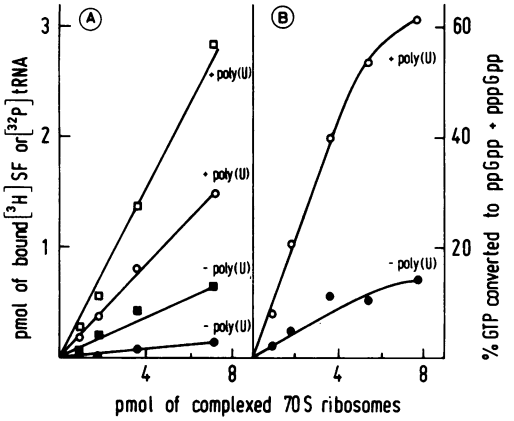

FIG. 1. Binding of [^{32}P]tRNA and ^{3}H-labeled stringent factor ([^{3}H]SF) to 70S ribosomes. The 70S·poly(U) complex was pre-incubated with [^{32}P]tRNA and ^{3}H-labeled stringent factor as shown in *Methods* . Where indicated, poly(U) was omitted from the pre-incubation step. The complexed ribosomes were isolated by centrifugation. (A) Aliquots were analyzed for ^{32}P and ^{3}H radioactivity; (○, ●) ^{3}H-labeled stringent factor; (□, ■) [^{32}P]tRNA. The curves were obtained from four experiments using different amounts of ^{3}H-labeled stringent factor and [^{32}P]tRNA in the pre-incubation step; optimal binding was observed with 350 pmol of ^{3}H-labeled stringent factor and 450 pmol of [^{32}P]tRNA. (B) Various concentrations of complexed ribosomes were complemented with ATP and [α-^{32}P]GTP and assayed for ppGpp + pppGpp synthesis. The Mg^{++} concentration in the assay mixture was 20 mM.

RESULTS

Binding of uncharged tRNA to the 70S·poly(U)·stringent factor complex

The assay for binding the stringent factor and uncharged tRNA to the ribosome is based on the finding that 70S ribosomes, poly(U), stringent factor, and tRNA form a stable complex at 13–20 mM Mg-acetate (5). The complexed ribosomes can be isolated by centrifugation, a step that also removes unbound tRNA and stringent factor. The binding of stringent factor and uncharged tRNA to the ribosome can be demonstrated either directly by using ^{3}H-labeled stringent factor and [^{32}P]tRNA, or indirectly by determining the ppGpp + pppGpp synthesizing activity of the complexed ribosomes. Results of a typical experiment are shown in Fig. 1, where the 70S·poly(U)·[^{32}P]tRNA·^{3}H-labeled stringent factor complex was analyzed either for [^{32}P]tRNA and ^{3}H-labeled stringent factor (Fig. 1A) or for ppGpp + pppGpp synthesizing activity (Fig. 1B). Optimal binding of the ^{3}H-labeled stringent factor and [^{32}P]tRNA to ribosomes occurred with the 70S·poly(U) complex; in the absence of poly(U), ribosomal binding of ^{3}H-labeled stringent factor and [^{32}P]tRNA as well as ppGpp + pppGpp synthesizing activity of the complexed ribosomes was significantly reduced (ref. 5; Fig. 1A and B, lower curves). As reported recently, 0.21 mol of ^{3}H-labeled stringent factor per mol of 70S ribosome was bound (Fig. 1A; ref. 5). In contrast, the binding of [^{32}P]tRNA per 70S ribosome was higher, 0.38 mol/mol of 70S ribosome, which is most likely due to binding of tRNA in a position other than the ribosomal A-site. In Fig. 1B it can be seen that synthesis of ppGpp + pppGpp was dependent on the concentration of the complexed ribosomes, the activity of the latter indirectly reflecting the amount of tRNA plus stringent factor bound to the ribosome. Synthesis of ppGpp + pppGpp could not be increased when additional tRNA and stringent factor were added to the complexed ribosomes, indicating that the latter was saturated with the tRNA and stringent factor (data not shown).

Table 1. Stringent factor-directed binding of [^{32}P]tRNA to ribosomes

70S·poly(U)·[^{32}P]tRNA complex formed	pmol of [^{32}P]tRNA bound/ pmol of 70S ribosomes
With stringent factor	0.35
Without stringent factor	0.16

The 70S·poly(U)·[^{32}P]tRNA complex was formed as described in *Methods;* where indicated, 350 pmol of stringent factor was present in the pre-incubation step. The ribosomal complex was isolated by centrifugation and analyzed for [^{32}P]tRNA.

That the stringent factor is specifically involved in the binding of tRNA to the ribosomal complex is shown in Table 1 and in the lower curve of Fig. 2, where the 70S·poly(U)·[^{32}P]tRNA complex was formed in the absence of the stringent factor. [^{32}P]tRNA binding to the isolated complex was significantly reduced. Although the mole ratio of [^{32}P]tRNA to ribosomes was still sufficiently high to trigger ppGpp + pppGpp production, there was only little activity in the stringency reaction with the complexed ribosomes (Fig. 2), suggesting that the tRNA was bound in a position other than the ribosomal A-site. The data in Table 2 indicate that the alternative position for the binding of the tRNA is the ribosomal peptidyl site (P-site). When incubated with the 70S·poly(U)·tRNA complex, Phe-tRNA but not *N*-acetyl-Phe-tRNA was bound. As known for the poly(U)-dependent system, *N*-acetyl-Phe-tRNA functions as an initiator tRNA in binding to the P-site, unlike aminoacyl-tRNA which is directed to the A-site (12). The results show that the ribosomal P-site but not the A-site was blocked by the uncharged tRNA. In contrast, when ribosomes carried stringent factor-directed tRNA in the A-site, Phe-tRNA but not *N*-acetyl-Phe-tRNA binding was prevented. The decreased binding of Phe-tRNA to the 70S·poly(U)·tRNA complex compared with the 70S·poly(U) complex is most likely due to some binding

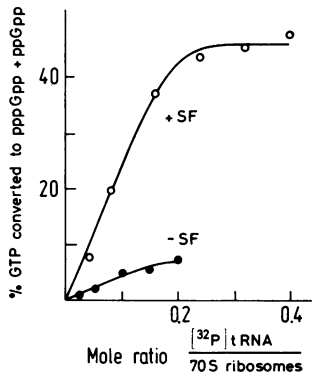

FIG. 2. Stringent factor (SF)-dependent binding of tRNA to 70S ribosomes. The 70S·poly(U) complex was pre-incubated with 450 pmol of [^{32}P]tRNA as shown in *Methods*. Where indicated, 350 pmol of ^{3}H-labeled stringent factor was present in the pre-incubation step. The complexed ribosomes were isolated by centrifugation. The various mole ratios of [^{32}P]tRNA to 70S ribosomes were obtained by the addition of uncomplexed 70S ribosomes. Various concentrations of the complexed ribosomes were complemented with ATP and [α-^{32}P]GTP and assayed for ppGpp + pppGpp synthesis. The Mg^{++} concentration in the assay mixture was 20 mM. In the experiment in which the stringent factor was omitted from the pre-incubation step, 1.2 μg of stringent factor was added to the assay.

Table 2. Inhibition of ribosomal Phe-tRNA or *N*-acetyl-Phe-tRNA binding to ribosomes carrying uncharged tRNA

Ribosome complex	Ribosomal binding (pmol) of [^{14}C]Phe-tRNA	*N*-acetyl-[^{14}C]Phe-tRNA
70S·poly(U)	4.5	3.9
70S·poly(U)·tRNA	3.8	0.7
70S·poly(U)·tRNA·stringent factor	0.9	3.1

The various ribosomal complexes were prepared and isolated by centrifugation (see *Methods*). Where indicated, tRNA and/or stringent factor were omitted from the precentrifugation step. Complexed ribosomes (15 pmol) were incubated with 40 pmol of [^{14}C]Phe-tRNA or *N*-acetyl-[^{14}C]Phe-tRNA. The reaction volume was 125 μl and contained 20 mM Tris·HCl, pH 7.8, 20 mM Mg-acetate, 40 mM NH_4Cl, 2 mM dithiothreitol. Ribosomal binding of the charged tRNAs was assayed by the Millipore filter technique (11).

of tRNA to the ribosomal A-site (Table 2). This is consistent with the observation that there is residual ppGpp + pppGpp synthesizing activity of the 70S·poly(U)·tRNA complex (Fig. 2). The above results suggest (*i*) that the stringent factor bound to the ribosome guides the tRNA to the A-site and (*ii*) that the tRNA bound to the A-site is responsible for the activation of the stringent factor.

Release of the tRNA from the ribosome complex

As reported recently, the *in vitro* transfer of pyrophosphate from ATP to GTP or GDP catalyzed by the stringent factor depends on relatively high Mg^{++} concentrations (13, 14), which are probably necessary to stabilize the 70S·mRNA·tRNA complex. For example, stringent factor but not tRNA is bound to the ribosome at 5–10 mM Mg-acetate whereas the tRNA binds only at higher Mg^{++} concentrations (13–20 mM; ref. 5). Thus, binding of the tRNA to the ribosomal A-site depends not only on the stringent factor but also on Mg^{++}. One might expect the Mg^{++} requirement of the ribosome·tRNA complex to be lower than that of ribosomes not precharged with tRNA. However, Table 3 shows that there is no difference in the Mg^{++}-dependence regardless of

Table 3. Mg^{++} requirement of the 70S·poly(U)·tRNA·stringent factor complex

Ribosome complex	Conversion of GTP to ppGpp + pppGpp (%) 9 mM*	12 mM*	15 mM*
70S·poly(U)	6.7	42.1	67.5
70S·poly(U)·tRNA	4.5	38.9	67.0
70S·poly(U)·tRNA·stringent factor	6.9	45.2	59.1

The various ribosomal complexes were isolated by centrifugation and suspended in 50 μl of buffer 1. In the first experiment [70S·poly(U)], 5 pmol of the complexed ribosomes were combined with 1 μg of tRNA and 0.4 μg of stringent factor. In the second experiment [70S·poly(U)·tRNA], 5 pmol of ribosomes were combined with 0.4 μg of stringent factor. In the third experiment [70S·poly(U)·tRNA·stringent factor], 5 pmol only of the complex were used. In all three experiments ATP, [α-^{32}P]GTP, and salts were present as listed in *Methods;* the reaction volume was 50 μl.
* The assay was carried out at the Mg^{++} concentrations indicated.

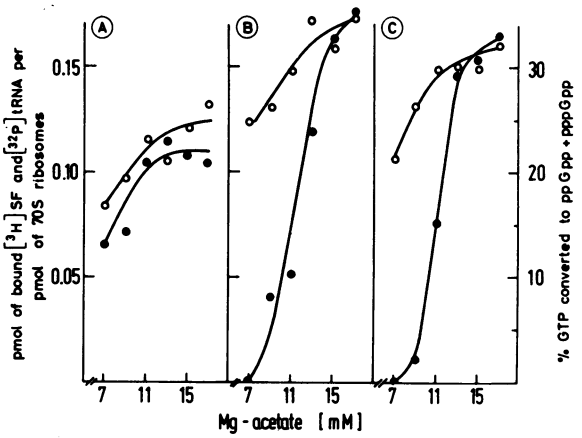

FIG. 3. Stability of the 70S·poly(U)·tRNA·stringent factor complex at various Mg^{++} concentrations. The 70S·poly(U)·[^{32}P]tRNA·^{3}H-labeled stringent factor complex was isolated and dissolved in 50 μl of buffer 1 (see *Methods*). Aliquots (75 pmol) were incubated with (●) or without (○) 2 mM ATP + 0.177 mM GTP in a volume of 500 μl; the final concentration was 20 mM Tris·HCl, pH 7.8, 2 mM dithiothreitol, 40 mM NH_4Cl. Mg^{++} concentrations were as indicated. Incubation was carried out at 37° for 1 hr. The ribosomal complex was recovered from the reaction mixture by centrifugation through a 5% sucrose solution (see *Methods*). The ribosomal pellets were resuspended in 30 μl of buffer 1. Aliquots were assayed for [^{32}P]tRNA (B) and ^{3}H-labeled stringent factor ([^{3}H]SF) (A), and for ppGpp + pppGpp synthesis (C). The stringency reaction was carried out in a 50-μl volume that contained 5 pmol of complexed ribosomes, 20 mM Tris·HCl, pH 7.8, 2 mM dithiothreitol, 20 mM Mg-acetate, 2 mM ATP, and 0.177 mM [α-^{32}P]GTP (5). Due to the recentrifugation step there was some loss in ribosomal-bound ^{3}H-labeled stringent factor and [^{32}P]tRNA.

whether the tRNA was prebound to the ribosome or not. This suggests that Mg^{++} was probably necessary to sustain the preformed 70S·poly(U)·tRNA complex. To test this possibility, the 70S·poly(U)·[^{32}P]tRNA·^{3}H-labeled stringent factor complex was preincubated at 37° for 1 hr with various Mg^{++} concentrations; it was then re-isolated by centrifugation and assayed for binding of [^{32}P]tRNA and ^{3}H-labeled stringent factor as well as for ppGpp + pppGpp production. Fig. 3 (A and B) shows that the complex remained stable even when treated at 9 mM Mg-acetate; neither [^{32}P]tRNA nor ^{3}H-labeled stringent factor were released during the low Mg^{++} incubation step. However, when the pre-incubation step was carried out in the presence of ATP and GTP and at 9 mM Mg-acetate, the re-isolated ribosomes lost significant amounts of [^{32}P]tRNA (Fig. 3B) and most of their guanosine polyphosphate synthesizing activity (Fig. 3C). At the elevated Mg^{++} concentration (15 mM) the complex remained active. In contrast, little ^{3}H-labeled stringent factor was released from the complexed ribosomes (Fig. 3A). Obviously incubation with ATP and GTP at suboptimal Mg^{++} concentration altered the stability of the ribosomal complex. Most likely the released tRNA could not be re-attached to the ribosomal A-site because of the low Mg^{++} concentration (9 mM), while this could occur at optimal Mg^{++} concentration.

To determine the precise conditions for the tRNA releasing mechanism, the 70S·poly(U)·[^{32}P]tRNA·stringent factor complex was combined with various nucleotides at suboptimal Mg^{++} concentration (9 mM) and incubated as indicated below. The ribosomal complex was re-isolated by centrifugation and assayed for binding of [^{32}P]tRNA and for ppGpp + pppGpp synthesizing activity, which is a measure of ribo-

Table 4. Release of [^{32}P]tRNA under various conditions

70S·poly(U)·[^{32}P]tRNA·stringent factor complex incubated	pmol of [^{32}P]tRNA bound/pmol of 70 S	% GTP converted to ppGpp + pppGpp
Alone	0.24	41.2
+ ATP, GTP	0.13	11.3
+ ATP	0.12	17.2
+ AMPPNP, GTP	0.25	43.5
+ 5'-AMP	0.20	38.9
+ GTP	0.21	35.2

For preparation of the 70S·poly(U)·[^{32}P]tRNA·stringent factor complex, see *Methods*. In a volume of 500 μl, 75 pmol of the complexed ribosomes were incubated with the final concentration of 20 mM Tris·HCl, pH 7.8, 9 mM Mg-acetate, 40 mM NH_4Cl, 2 mM dithiothreitol and, where indicated, 2 mM ATP + 0.177 mM GTP; 2 mM ATP or 0.177 mM GTP; 2 mM AMPPNP + 0.177 mM GTP; or 2 mM 5'-AMP. Incubation was carried out at 37° for 1 hr. After incubation the complexed ribosomes were recovered from the reaction mixture by centrifugation through a 5% sucrose solution (see *Methods*). The pellets were resuspended in 30 μl of buffer 1 and analyzed for [^{32}P]tRNA binding and ppGpp + pppGpp production; aliquots of 5 pmol of complexed ribosomes were assayed for ppGpp + pppGpp synthesis.

somal bound tRNA. As shown in Table 4, ATP + GTP or ATP alone were most effective in releasing the [^{32}P]tRNA. Neither GTP or 5'-AMP alone, nor AMPPNP + GTP, were sufficient to cause the [^{32}P]tRNA release. Similar results were obtained when the re-isolated ribosomes were assayed for ppGpp + pppGpp production. No stringent reaction was observed when the ribosomes were pre-incubated with ATP + GTP or ATP alone (Table 4).

Since the data presented above seem to indicate that hydrolysis of ATP is coupled to the ribosomal release of the

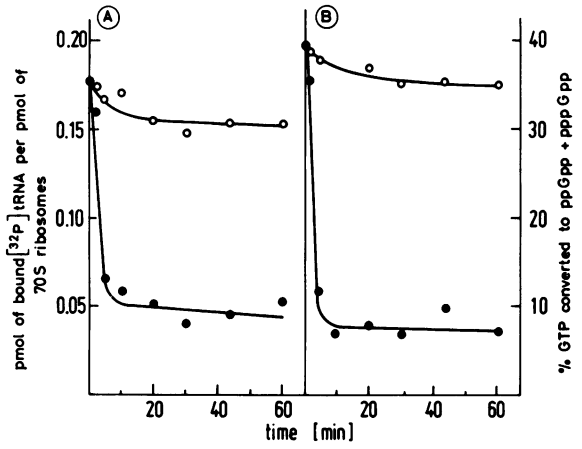

FIG. 4. Kinetics of the tRNA release. The 70S·poly(U)·[^{32}P]tRNA·stringent factor complex was isolated by centrifugation (see *Methods*). In a volume of 500 μl, 75 pmol of complexed ribosomes were incubated with 2 mM ATP and 0.177 mM GTP (●); the final buffer concentration was 20 mM Tris·HCl, pH 7.8, 9 mM Mg-acetate, 40 mM NH_4Cl, 2 mM dithiothreitol. In the control experiment incubation was performed with 2 mM AMPPNP and 0.177 mM GTP (○) under the same ionic conditions. Incubation was carried out at 37° for the time indicated. After incubation the mixture was rapidly cooled and immediately centrifuged through a 5% sucrose solution. The centrifuged ribosomes were analyzed for [^{32}P]tRNA binding (A) and ppGpp + pppGpp production (B) as described in *Methods*. The Mg^{++} concentration in the assay mixture was 20 mM.

Table 5. ATP-$^{32}PP_i$ exchange in the presence of the complexed ribosomes

Ribosome complex	ATP-$^{32}PP_i$ exchange −GTP (cpm)	ATP-$^{32}PP_i$ exchange +GTP (cpm)
70S·poly(U)	1,800	1,720
70S·poly(U)·tRNA	2,510	2,415
70S·poly(U)·stringent factor	5,870	4,350
70S·poly(U)·tRNA·stringent factor	15,320	16,280

The various ribosomal complexes were isolated as described in *Methods*, and, where indicated, stringent factor and/or tRNA was omitted from the pre-centrifugation step. The ATP-$^{32}PP_i$ exchange reaction was carried out with sodium pyrophosphate (400 Ci/mol) and ATP as reported (15). Where indicated, 0.1 mM GTP was present.

tRNA, it is suggested that this reaction occurs after each round of ATP hydrolysis. Thus, even short incubations of the 70S·poly(U)·[^{32}P]tRNA·stringent factor complex should cause a significant release of [^{32}P]tRNA and consequently result in a decrease in ppGpp + pppGpp production. Indeed, the tRNA release was observed after less than 5 min of incubation time (Fig. 4A); also, the guanosine polyphosphate synthesizing activity of the complexed ribosomes was drastically reduced (Fig. 4B). In the control experiment, where ATP was replaced by AMPPNP, the complex remained intact.

The data in Table 4 and Fig. 4 suggest that hydrolysis of ATP is a prerequisite of the tRNA releasing mechanism and that this hydrolysis presumably also occurs in the absence of the PP_i acceptor, GTP or GDP. This assumption is confirmed by the data shown in Table 5, where the incorporation of ^{32}P-labeled pyrophosphate into ATP was measured. The exchange reaction was significantly enhanced when the ribosomes carried both stringent factor and tRNA; the reaction is independent of the presence of GTP. Most likely the stringent factor interacts with ATP and binds PP_i, which in the absence of GTP can be released into the supernatant fraction.

CONCLUSION

The binding of the tRNA to the ribosome depends on Mg^{++} and stringent factor, the latter promoting the binding of the tRNA in the correct position. Incubation of the 70S·mRNA·tRNA·stringent factor complex at suboptimal Mg^{++} concentrations resulted in the release of tRNA but not of stringent factor from the ribosome. The possibility that this tRNA release was due to an indirect effect, such as a change in the effective Mg^{++} concentration by the nucleotides, is excluded by the fact that a similar change could not be observed when other nucleotides were tested (such as AMPPNP). Since AMPPNP cannot replace ATP, the tRNA releasing reaction seems to be coupled with the hydrolysis of ATP. This may suggest that binding and release of the tRNA is due to a pulsating ribosomal contraction mechanism similar to that proposed for the peptide chain elongation cycle (16). Thus, after each round of ATP hydrolysis the tRNA is rejected by a change in the ribosomal conformation. The cycle is started again by rebinding of the tRNA to the A-site of the ribosome.

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