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Some Properties and the Possible Role of Intrinsic ATPase of Rat Liver 80S Ribosomes in Peptide Bond Elongation

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The properties and role in peptide elongation of ATPase intrinsic to rat liver ribosomes were investigated. (i) Rat liver 80S ribosomes showed high ATPase and GTPase activities, whereas the GTPase activity of EF-1α and EF-2 was very low. mRNA, aminoacyl-tRNA, and elongation factors alone enhanced ribosomal ATPase activity and in combination stimulated it additively or synergistically. The results suggest that these translational components induce positive conformational changes of 80S ribosomes by binding to different regions of ribosomes. Translation inhibitors, tetracyclin and fusidic acid, inhibited ribosomal ATPase with or without elongational components. (ii) Two ATPase inhibitors, AMP-P(NH)P and vanadate, did not inhibit GTPase activities of EF-1α and EF-2 assayed as uncoupled GTPase, but they did inhibit poly(U)-dependent polyphe synthesis of 80S ribosomes. (iii) Effects of AMP-P(NH)P and ATP on poly(U)-dependent polyphe synthesis at various concentrations of GTP were examined. ATP enhanced the activity of polyphe synthesis even at high concentrations of GTP, suggesting a specific role of ATP. At low concentrations of GTP, the extent of inhibition by AMP-P(NH)P was very low, probably owing to the prevention of the reduction of the GTP concentration. (iv) Vanadate inhibited the translocation reaction by high KCl-washed polysomes. These findings together indicate that ribosomal ATPase participates in peptide translation by inducing positive conformational changes of mammalian ribosomes, in addition to its role of chasing tRNA from the E site.

Key words: ATPase, 80S ribosomes, GTPase, peptide-elongation, polysomes.

Considerable evidence now indicates that two elongation factors, EF-1α and EF-2, drive the elongation cycle in mammalian cells. The former mediates the binding of aminoacyl-tRNA to the ribosomal A site, and the latter catalyzes the translocation reaction. These two factors contain GTPase, which is essential for peptide bond elongation (*1*). On the other hand, mammalian ribosomes and ribosomal subunits contain an intrinsic ATPase, which shows a wide substrate specificity including GTP (*2–6*). However, the interaction of the ATPase with elongation components (mRNA, tRNA, elongation factors) has not been studied systematically, and its role in peptide bond elongation has not been clarified.

We previously reported (*6*) that the ATPase (GTPase) activity of rat liver 40S subunits or 30S-5SRNP particles, which were prepared by EDTA treatment of rat liver 80S ribosomes and consisted of unfolded 40S subunits with attached 5SRNP (*7*), was enhanced by natural and artificial mRNAs, phe-tRNA^{phe}, and elongation factors (EF-1α, EF-2, EF-1αβγ) alone or in combination, and that additive or synergic stimulations were observed. These results imply the possible involvement of ATPase of mammalian small subunits in peptide bond elongation.

To investigate the properties and role in peptide elongation of ribosomal ATPase, we extended the previous studies to ATPase of 80S ribosomes. We first investigated whether the ATPase activity was stimulated by the components of the elongation reaction. We also examined the effects of two elongation inhibitors (tetracyclin and fusidic acid). Next, we tried to clarify the role of the ATPase in peptide elongation using two ATPase inhibitors, AMP-P(NH)P, a competitive inhibitor, and vanadate, a widely used inhibitor of various ATPases. After showing the negligible effects of these ATPase inhibitors on the GTPase activity of the two elongation factors, we investigated their effects on poly(U)-dependent polyphe synthesis by 80S(PM) ribosomes. We also examined the effects of AMP-P(NH)P and ATP on poly(U)-dependent polyphe synthesis in the presence of various concentrations of GTP. Lastly, we investigated the inhibitory effects of vanadate on the translocation reaction on liver polysomes.

This report describes the results of these experiments, which may represent the first systematic study indicating

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Abbreviations: AMP-P(NH)P, 5′-adenylyl imidodiphosphate; DTT, dithiothreitol; GMP-P(NH)P, 5′-guanylyl imidodiphosphate; TCA, trichloroacetic acid; phe-tRNA^{phe}, phenylalanyl tRNA^{phe}; polyphe, polyphenylalanine; TMV, tobacco mosaic virus; 5SRNP, 5SrRNA-L5 protein particles.

the participation of ATPase intrinsic to 80S ribosomes in peptide bond elongation.

MATERIALS AND METHODS

Materials—Pure ATP and GTP preparations were obtained from Boehringer. [γ-^{32}P]ATP (10 Ci/mmol) and [γ-^{32}P]GTP (10 Ci/mmol) were purchased from the Institute of Isotopes, the Hungarian Academy of Science, and purified as described previously (*6*). L-[4-^{3}H]phenylalanine (25 Ci/mmol) and [^{3}H]puromycin dihydrochloride (9.1 Ci/mmol) were obtained from American Radiolabeled Chemicals and Havavek Biochemicals, respectively. Other chemicals were described in our previous reports (*6*).

Buffers—Medium A: 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6), and 1 mM DTT. Buffer E: 50 mM Tris-HCl (pH 7.8), 1.1 mM $MgCl_2$, 10 mM NH_4Cl, and 1 mM DTT. Buffer F: 25 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 M KCl, and 1 mM DTT. Buffer G: 50 mM Tris-HCl (pH 7.6), 5 mM Mg-acetate, 50 mM KCl, 50 mM NH_4Cl, and 0.2 mM DTT. Buffer P: 167 mM Tris-HCl (pH 7.6), 33 mM $MgCl_2$, and 1 M KCl.

Preparation of Rat Liver 80S(PU) Ribosomes, 60S and 40S Subunits, and High KCl-Washed Polysomes—Postmitochondrial ribosomes were prepared by the methods described previously (*6, 8*). In brief, the postmitochondrial supernatant of rat liver in Medium A was adjusted to 1.3% desoxycholate, 0.6 M KCl and 10 mM $MgCl_2$, 35 mM Tris-HCl, pH 7.6, and 1 mM DTT. The mixture was layered on 0.8 M sucrose containing 0.6 M KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6), and 1 mM DTT, and centrifuged at 145,000 $\times g$ for 2 h at 2°C. The pellet was suspended in Medium A by hand by using a loosely fitted Teflon-glass homogenizer, followed by centrifugation at 25,400 $\times g$ for 10 min. The supernatant was designated as post(mt) ribosomes. To prepare the 80S(PU) ribosomes used throughout these experiments, post(mt) ribosomes were treated with 0.2 mM puromycin at 37°C for 15 min, then incubated with a mixture containing 1 M KCl, 10 mM $MgCl_2$, and 2 mM DTT at 37°C for 15 min. The mixture was layered on 0.8 M sucrose–containing Medium A and centrifuged at 277,600 $\times g$ for 1 h. The pellet was suspended in Medium A and designated as 80S(PU) ribosomes (*9*). For the preparation of 60S and 40S subunits, post(mt) ribosomes were treated with puromycin and high KCl as described above, then layered on a 15 to 30% linear sucrose gradient which contained 0.85 M KCl, pH 7.6, and 2 mM DTT. Ribosomal subunits were separated by centrifugation at 95,000 $\times g$ for 5 h. The absorbance at 254 nm was continuously monitored with an ISCO automatic density-gradient fractionator. The 60S fraction was concentrated as described below. To remove contaminating 40S subunit dimers, the 60S fraction was dialyzed against Medium A containing 50 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6, then centrifuged on a 15 to 30% sucrose density-gradient in the same medium. The 60S fraction was then dialyzed against sucrose-free Medium A for 1 h, then concentrated with a Diaflow membrane (*8*).

Crude polysomes were prepared from the rat liver postmitochondrial supernatant by a modification of the method of Falvey and Staehelin (*10*). In brief, after addition of desoxycholate at the final concentration 1%, the post-mitochondrial supernatant was layered on the top of a discontinuous gradient consisting of 3 ml of 1.38 M sucrose in Medium A and 3 ml of 2 M sucrose in Medium A. After centrifugation at 152,000 $\times g$ for 18 h at 2°C, the pellet of crude polysomes was suspended in Medium A. High KCl-washed polysomes were further prepared by the methods described in reference (*9*), as follows. To crude polysomes in Medium A, 4 M KCl was added to give 0.5 M, and the mixture was layered on top of a discontinuous sucrose density-gradient consisting of 3 ml of 0.5 M sucrose containing 20 mM Tris-HCl, pH 7.6, 3 mM Mg-acetate, 0.3 M KCl, and 1 mM DTT, and 4 ml of 1 M sucrose containing the same solution. The mixture was centrifuged at 145,000 $\times g$ for 2 h at 2°C. The pellet was washed with Medium A and suspended in Medium A.

The 80S(PU) ribosomes, high KCl-washed polysomes, and 60S and 40S subunits were stored in small portions at –85°C.

Elongation Factors—EF-1α (*11, 12*), EF-1$\beta\gamma$ (*13*), and EF-2 (*14*) were highly purified from pig liver by Iwasaki *et al.*, and were almost free from contamination by ATPase (*6*).

Assay of ATPase and GTPase Activities—(i) The ATPase and GTPase activities of 80S(PU) ribosomes were measured as described previously (*6*). In brief, the standard incubation mixture comprised 1 pmol of 80S(PU) ribosomes (in 2 µl of Medium A), 50 mM Tris-HCl, pH 7.6, 5 mM KCl, 5 mM $MgCl_2$, 2.5 mM DTT, and 0.125 mM [γ-^{32}P]ATP or GTP (1 µCi) in a total volume of 20 µl. Incubation was carried out at 37°C for 30 min. (ii) The uncoupled GTPase activity of EF-2 was measured by a slightly modified method of Iwasaki *et al.* (*15*). A reaction mixture (20 µl) containing EF-2, 60S subunits, and 0.01 mM [γ-^{32}P]GTP (1 µCi) in Buffer E was incubated at 37°C for 30 min. The amounts of EF-2 and 60S subunits are given in the legend to Fig. 4. (iii) The GTPase activity of EF-1α was measured by a slightly modified method of Crechet and Parmeggiani (*16*). EF-1α was incubated under the same conditions as in (ii) except that Buffer F was used. (iv) The GTPase and ATPase activities of the polyphe-synthesizing system were assayed by using 0.1 mM [γ-^{32}P]GTP or [γ-^{32}P]ATP (1 µCi) and 1 pmol of 80S(PU) ribosomes under the same reaction conditions as polyphe-synthesizing system described later.

The hydrolysis of [γ-^{32}P]GTP or [γ-^{32}P]ATP was followed by measuring the amounts of inorganic $^{32}PO_4$ hydrolyzed during the incubation by the isobutanol-benzene method as described previously (*6*). The results were expressed as pmol of inorganic $^{32}PO_4$ hydrolyzed during the incubation. Each curve in a figure or table shows the results of at least two or three runs which varied within 10%.

Poly(U)-Dependent Polyphe Synthesis—The slightly modified method of Iwasaki *et al.* (*14, 15*) was used: 32 pmol of [^{3}H]phe-tRNA^{phe} was incubated with 3.3 pmol of 80S ribosomes, 5 µg of poly(U), 5 pmol EF-1α, 10 pmol of EF-2, and 0.1 mM GTP in Buffer G (total volume of 20 µl). In some experiments 10 pmol of EF-1$\beta\gamma$ was further added. Incubation was carried out at 37°C for 20 min. The amount of polyphe synthesized was measured as hot TCA-insoluble [^{3}H]radioactivity (*14, 15*). The TCA-precipitated fraction was washed seven times, including four washings of the Eppendorf tube and glass membrane filter and three further washings of the glass filter. The results were expressed as pmol [^{3}H]phe-tRNA^{phe} incorporated into the hot TCA-insoluble fraction.

EF-2 Dependent Translocation—The slightly modified

method of Iwasaki *et al.* was used (*15*). The complete reaction mixture contained, in a total volume of 20 µl, 1.5 pmol of high KCl-washed polysomes, 12.5 pmol of EF-2, 40 mM Tris-HCl, pH 7.6, 5 mM Mg-acetate, 50 mM KCl, 50 mM NH_4Cl, 0.1 mM GTP, and 0.2 mM DTT. As a control, the mixture without EF-2 was used. After incubation at 2°C for 20 min, 0.5 µCi of [^{3}H]puromycin and 4 M KCl were added to 0.4 mM at the final concentration in a total volume of 30 µl. The mixture was further incubated at 2°C for 20 min. After incubation, the cold TCA-insoluble radioactivity was measured in the same way as for polyphe synthesis. The translocation activity was determined as EF-2–dependent cold TCA–insoluble radioactivity, which was calculated as [^{3}H]radioactivity observed in the complete reaction mixture minus that observed in the mixture without EF-2.

RESULTS

For the purpose of the present experiments, it is necessary to use rat liver 80S ribosomes that have a high ATPase activity and are free from contamination by elongation factors. It was also preferable to remove the nascent peptide on ribosomes for poly(U)-dependent polyphe synthesis (*14, 15*). Therefore, we used 80S(PU) ribosomes throughout the present experiments.

(I) Basic Properties of 80S(PU) Ribosomes—The patterns of sucrose density-gradient centrifugation of three kinds of ribosomal particles used are shown in Fig. 1. A typical pattern of polysomes was observed in the case of high KCl-washed polysomes [Fig. 1(1)]. The pattern of 80S(PU) ribosomes indicated that they consisted mainly of 80S monomers [Fig. 1(3)]. A pattern of partially degraded polysomes containing monomers and disomes was observed in the case of post(mt) ribosomes [Fig. 1(2)].

The ATPase activities of 80S(PU) ribosomes under our standard conditions in the presence of 0.125 mM ATP are summarized in Table I. The activity of post(mt) ribosomes was about twofold higher than that of 80S(PU) ribosomes. Since the former particles contained large amounts of poly-

TABLE I. **Basic properties of 80S(PU) ribosomes.**

(1) ATPase activities of rat liver post(mt) ribosomes and 80S(PU) ribosomes

ATPase activity (pmol inorganic P/pmol particles/min)	
Post(mt) ribosomes	80S(PU) ribosomes
13 ± 3.7 (4)*	6.1 ± 1.7 (16)*

(2) Polyphe synthesizing activity of 80S(PU) ribosomes

Polyphe synthesizing activity (pmol [^{3}H]phe/20 min)
12.7 ± 5 (17)*

*Number of independent experiments.

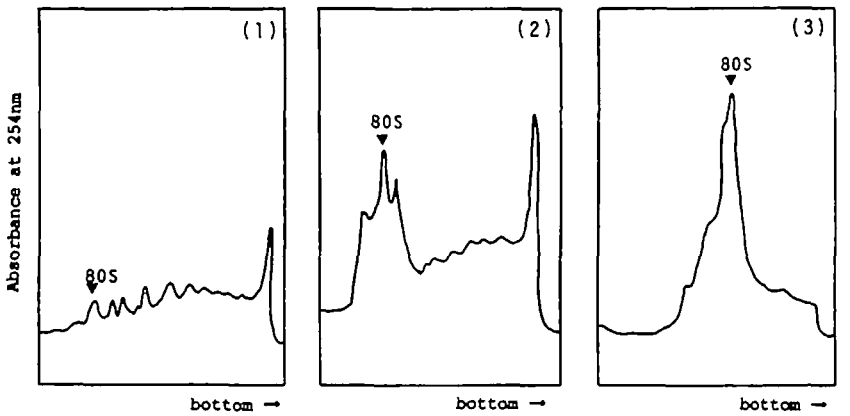

Fig. 1. **Sedimentation patterns of (1) high KCl-washed polysomes, (2) post(mt) ribosomes, and (3) 80S(PU) ribosomes.** (1) 20 A_{260} *units of* 0.5 M KCl–*washed polysomes* were subjected to linear density-gradient centrifugation from 0.4 M to 1.0 M sucrose containing 20 mM Tris-HCl (pH 7.6), 5 mM Mg-acetate and 0.15 M NH_4Cl. After centrifugation at 156,500 ×*g* for 45 min, the absorbance at 254 nm was continuously monitored with an ISCO automatic density-gradient fractionator. (2) 20 A_{260} units of post(mt) ribosomes were analyzed as described in (1). (3) 40 A_{260} units of 80S(PU) ribosomes were analyzed as described in (1) except for centrifugation at 156,500 ×*g* for 90 min.

TABLE II. **Effects of polyamine on the ATPase and poly(U)-dependent polyphe synthesizing activities of 80S ribosomes obtained by reassociation of 40S and 60S subunits, and 80S(PU) ribosomes.**

(1) Effects of spermidine on ATPase activity of reassociated 80S ribosomes and 80S(PU) ribosomes

	ATPase activity				
	Complete system	+ spermidine (mM)			
		0.4	0.8	1.6	2.4
Reassociated ribosomes	100 (16 pmol)	97	110	131	138
80S(PU) ribosomes	100 (156 pmol)	97	100	92	93

(2) Effects of spermidine on polyphe synthesizing activity of reassociated 80S ribosomes and 80S(PU) ribosomes

	Polyphe synthesizing activity				
	Complete system	+ spermidine (mM)			
		0.4	0.8	1.6	2.4
Reassociated ribosomes	100 (7.5 pmol)	210	290	320	270
80S(PU) ribosomes	100 (22 pmol)		103		

somes in which bound peptidyl-tRNA and mRNA were present [Fig. 1(2)], they had a more positive conformation for translation than 80S(PU) ribosomes and thus a higher ATPase activity, as described later. The ratio of the GTPase activity to the ATPase activity of 80S(PU) ribosomes was about 0.75 for all three kinds of preparations (data not shown). The polyphe synthesizing activity of 80S(PU) ribosomes, which is shown in Table I(2), was rather variable among preparations, as shown by the standard deviations of the activities.

The effect of $MgCl_2$ concentration on the ribosomal ATPase activities was examined. The highest activity (100%) was observed at 4 mM, 5 mM, and 6 mM $MgCl_2$, with slightly lower activity at 1 mM (89%), 8 mM (95%), and 10 mM (86%). The activity was highest (100%) at 10 mM KCl and slightly lower at 2 mM (93%), 25 mM (93%), 50 mM (91%), and 100 mM (90%). The heat stabilities of ATPase and polyphe synthesis were as follows. On heat treatment at 50°C for 4 min, 93% and 99% respectively of the control activities were retained; while on treatment at 60°C for 4 min, these decreased markedly to 28% and 25% of the control, respectively. The results indicate that 80S(PU) ribosomes have a stable structure for ATPase.

(II) Effects of Polyamines—Much evidence has been accumulated that polyamine stimulates protein synthesis by inducing positive conformational changes of ribosomes. In our previous experiments (*6*), we compared the ATPase activity of 30S-5SRNP, which was prepared by EDTA treatment of post(mt) 80S ribosomes (*7*) and consisted of unfolded small subunits with 5SRNP, with that of 40S subunits, which were prepared by puromycin and high KCl treatment of 80S ribosomes and lacked 5SRNP. We found that the ATPase activity of 40S subunits was significantly lower than that of 30S-5SRNP. Spermidine enhanced concentration-dependently the ATPase activity of 40S subunits up to the activity level of 30S-5SRNP, which was not affected by spermidine [Fig. 6 in Ref. *6*]. Therefore, we assumed that the lack of 5SRNP induced the negative conformation of 40S subunits for ATPase and spermidine restored it, whereas 30S-5SRNP had the positive conformation for ATPase and so its ATPase activity was not affected by spermidine. 5SRNP was attached to 60S subunits by the usual puromycin and high KCl treatment of 80S ribosomes, but attached to unfolded small subunits by EDTA treatment of 80S ribosomes (30S-5SRNP particles) (*7*). Recently, Ellskaya *et al.*, using 80S ribosomes obtained by reassociation of rabbit liver 40S and 60S subunits, showed that spermine stimulated the activity of poly(U)-dependent polyphe synthesis of reassociated ribosomes (*18*). Prompted by these findings, we examined the effects of spermidine on poly(U)-dependent polyphe synthesizing activities and ATPase activities of various ribosome preparations. As shown in

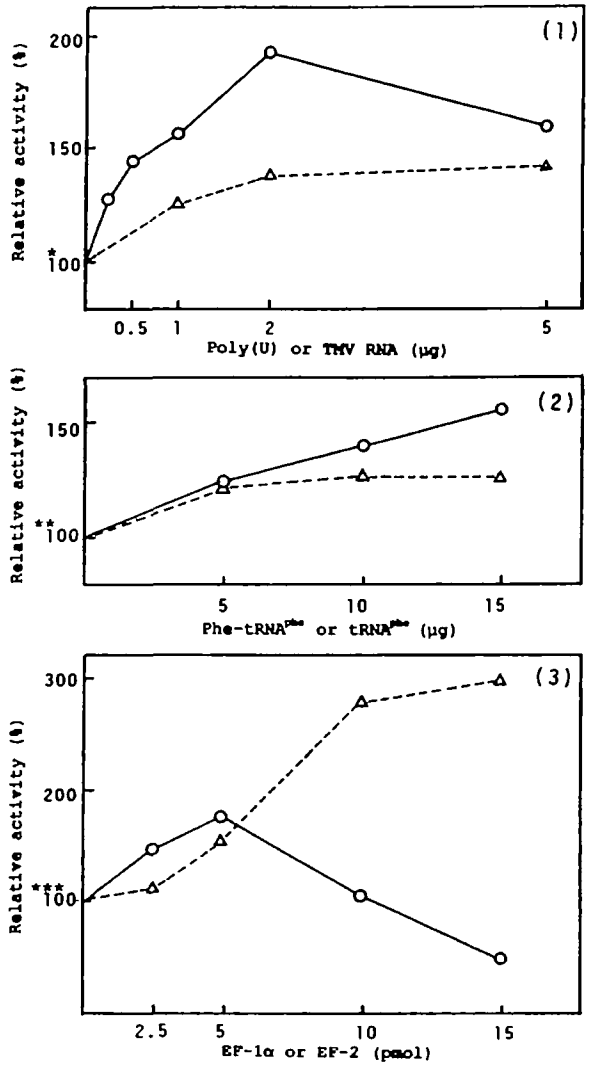

Fig. 2. **Effects of components of peptide elongation on the ATPase activity of 80S(PU) ribosomes.** (1) Effects of poly(U) and TMV RNA. (2) Effects of phe-tRNA^{phe}. (3) Effects of EF-1α and EF-2. The ATPase activity of 80S(PU) ribosomes alone is set as 100%. (1) ○, ATPase in the presence of poly(U). △, ATPase in the presence of TMV RNA. (2) ○, ATPase in the presence of phe-tRNA^{phe}. △, ATPase in the presence of tRNA^{phe}. (3) ○, ATPase in the presence of EF-1α. △, ATPase in the presence of EF-2. *141 and 127 pmol of ATP hydrolyzed in the case of poly(U) and TMV RNA, respectively. **125 and 170 pmol of ATP hydrolyzed in the case of phe-tRNA^{phe} and tRNA^{phe}, respectively. ***150 and 200 pmol of ATP hydrolyzed in the case of EF-1α and EF-2, respectively.

TABLE III. **Effects of translational components on ATPase activity of 80S(PU) ribosomes.**

(1) Effects of poly(U), EF-1α, and phe-tRNA^{phe} or tRNA^{phe}

	Additions						
Ribosomes (1 pmol)	+	+	+	+	+	+	+
Poly(U) (0.5 μg)	–	+	–	–	–	+	+
EF-1α (5 pmol)	–	–	+	–	–	+	+
Phe-tRNA^{phe} (10 μg)	–	–	–	+	–	+	–
tRNA^{phe} (10 μg)	–	–	–	–	+	–	+
Relative activity	100 (192 pmol)	121	137	127	104	199 (185#)	157 (162#)

The values in parenthesis denoted by # were calculated from the relative activity in the presence of each component examined.

(2) Effects of poly(U), phe-tRNA^{phe}, EF-1α, and EF-2

	Additions					
Ribosomes (1 pmol)	+	+	+	+	+	+
Poly(U) (2 μg)	–	+	–	–	–	+
Phe-tRNA^{phe} (10 μg)	–	–	+	–	–	+
EF-1α (5 pmol)	–	–	–	+	–	+
EF-2 (10 pmol)	–	–	–	–	+	+
Relative activity	100** (72 pmol)	163	238	154	461	799 (716#)

The results of seven individual experiments. # the same as (1).

Table II(1), spermidine enhanced the ATPase activity of reassociated 80S ribosomes, depending on the concentration. Table II(2) shows that depending on its concentration, spermidine markedly stimulated the polyphe synthesizing activity of reassociated 80S ribosomes, in agreement with the results shown in Ref. *18*. On the other hand, spermidine or spermidine plus spermine did not affect ATPase activity or polyphe synthesizing activity of 80S(PU) ribosomes, as discussed later [Table II, (1) and (2)]. In this respect, the ATPase activity of the reassociated ribosomes was the sum of the activity of each subunit, as also reported in Ref. *4*, and markedly lower than that of 80S(PU) ribosomes, indicating the importance of the conformation of ribosomes for their ATPase and translation activities.

(III) Effects of Elongational Components on the ATPase Activity of 80S(PU) Ribosomes—The correlation between the ATPase activity of 80S(PU) ribosomes and the binding of components involved in peptide bond elongation (mRNA, aminoacyl-tRNA, and elongation factors) was examined.

(1) Effects of mRNA: We used poly(U) as artificial mRNA and TMV RNA as natural mRNA. As shown in Fig. 2(1), the ATPase activity was increased markedly and concentration-dependently by poly(U) up to 2 μg. Depending on its concentration up to 5 μg, TMV RNA also stimulated the ATPase activity. These results were in good agreement with our previous results with 30S-5SRNP particles or 40S subunits (*6*), probably owing to binding of mRNA to small subunits.

(2) Effects of phe-tRNA^{phe}: Figure 2(2) shows the dose-response curve for the effect of phe-tRNA^{phe} on the ATPase activity of 80S(PU) ribosomes. phe-tRNA^{phe} stimulated the activity concentration-dependently. tRNA^{phe} also stimulated it, although to a lower extent. In general, these results are in agreement to the results obtained with 30S-5SRNP particles or 40S subunits previously reported (*6*).

(3) Effects of EF-1α and EF-2: Figure 2(3) shows that EF-1α stimulated the ATPase activity up to 5 pmol, then depressed it. EF-2 stimulated the activity strongly, depending on the concentration, up to 15 pmol.

(4) Effects of the combination of peptide elongation components: Since it is well known that EF-1α, phe-tRNA^{phe}, and GTP form a ternary complex which binds to poly(U) on ribosomes, the effects of the combination of poly(U), phe-tRNA^{phe}, and EF-1α on ATPase activities of 30S-5SRNP or 40S subunits were examined in our previous experiments. The results showed that the extent of stimulation was similar to the sum of those by individual components (additive stimulation) (*6*). Similar experiments were performed with 80S(PU) ribosomes. As shown in Table III(1), the combination of these components showed additive stimulatory effects on the ATPase activity of 80S(PU) ribosomes, as in the case of 30S-5SRNP [Table III in Ref. *6*].

It is of interest that the combination of four elongation components necessary for poly(U)-dependent polyphe synthesis [poly(U), phe-tRNA^{phe}, EF-1α, and EF-2] showed synergic effects on the ATPase activity of 80S(PU) ribosomes [Table III(2)]. These results suggest that the binding of individual components to different regions of 80S ribosomes, as described later, induce positive conformational changes in each region. This may be the reason for additive or synergic stimulatory effects of combined elongation factors on the ribosomal ATPase activity.

(5) Effects of translation inhibitors on the ATPase activity of 80S(PU) ribosomes: We examined the effects of two translation inhibitors with different inhibitory mechanisms which are known to interact with ribosomes.

(5-1) Fusidic acid is known to form a stable fusidic acid–GDP–EF-2–ribosome complex and sequester EF-2, resulting in the inhibition of translocation [see the review article (*19*), pp. 64–67]. As shown in Table IV(1), fusidic acid inhibited concentration-dependently the ATPase activity of 80S-

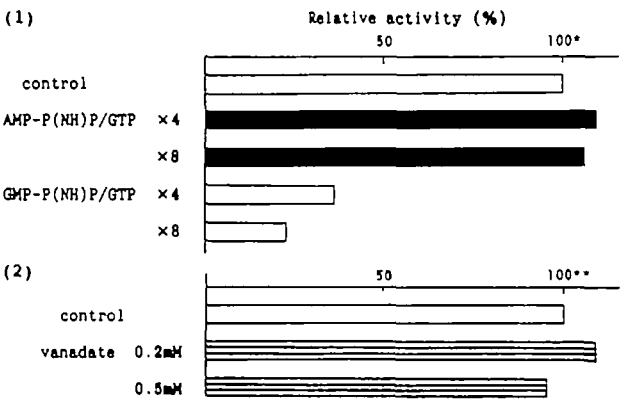

Fig. 3. **Effects of (1) AMP-P(NH)P and GMP-P(NH)P and (2) vanadate on the uncoupled GTPase of EF-2 in the presence of 60S subunits.** (1) Rat liver 60S subunits (1 pmol) ribosomes (1 pmol) were incubated with 0.01 mM [γ-^{32}P]GTP (1 μCi) in the presence of 10 pmol EF-2 in Buffer E (total volume of 20 μl) at 37°C for 30 min. *98 pmol of GTP hydrolyzed (0.09 pmol GTP hydrolyzed by 60S subunits alone). (2) 60S subunits (2 pmol) were incubated under the same conditions as (1). **117 pmol of GTP hydrolyzed.

TABLE IV. **Effects of fusidic acid (FA), tetracycline (TC), and pactamycin on the ATPase activity of 80S(PU) ribosomes.** The *figures in parenthesis represent percent inhibitions by inhibitors.*

(1) Effects of FA

	None	Additions				
		Poly(U) (2 μg)	Phe-tRNA^{phe} (10 μg)	EF-1α (5 pmol)	EF-2 (10 pmol)	4 components
Ribosomes	100 (175 pmol)	205 (100)	118 (100)	145 (100)	326 (100)	413 (100)
+ FA (2 mM)	82	87 (44)	75 (63)	128 (88)	163 (56)	327 (79)
Ribosomes	100 (120 pmol)	216 (100)	146 (100)	145 (100)	496 (100)	1182 (100)
+ FA (4 mM)	70	91 (42)	128 (77)	82 (55)	184 (37)	654 (55)

(2) Effects of TC

	None	Additions				
		Poly(U) (2 μg)	Phe-tRNA^{phe} (10 μg)	EF-1α (5 pmol)	EF-2 (10 pmol)	4 components
Ribosomes	100 (62 pmol)	221 (100)	118 (100)	155 (100)	552 (100)	663 (100)
+ TC (1.4 mM)	74	84 (38)	86 (77)	113 (73)	226 (41)	338 (51)

(PU) ribosomes, probably by binding to the specific site of ribosomes. It also inhibited more markedly the ATPase activity enhanced by elongation components, especially by EF-2. The inhibition of the activity enhanced by EF-1α may be explained by the overlapping interaction site of the two elongation factors on ribosomes, as described later.

(5-2) Tetracycline blocks the protein synthesis of both bacterial and mammalian systems. In the bacterial system, the EF-Tu–dependent binding of aminoacyl-tRNA to 70S ribosomes was specifically inhibited by low concentrations of tetracycline (pp. 54–57 in Ref. *19*). As shown in Table IV(2), tetracycline inhibited not only the ATPase activity of 80S(PU) ribosomes but also the ATPase activity enhanced by the four elongation components, especially EF-2.

These results may indicate that these two elongation inhibitors inhibit (i) the ATPase of 80S(PU) ribosomes by binding to the specific sites of ribosomes, and (ii) the ATPase enhanced by elongation components by interacting with these components. It must be added that pactamycin, which inhibits specifically the peptide bond initiation at low concentrations (pp. 36–38 in Ref. *19*) did not affect the ribosomal ATPase, which showed 98% of the control activity at 5 μM pactamycin and 101% at 10 μM.

(IV) Effects of ATPase Inhibitors on ATPase and Polyphe Synthesizing Activities of 80S(PU) Ribosomes—On the basis of the findings described above, we attempted to get more direct evidence for the participation of ATPase intrinsic to 80S(PU) ribosomes in peptide bond elongation. Our strategy was as follows. When GTPase of mammalian elongation factors would be stringent for GTP as bacterial elongation factors and the inhibition of the translation reaction by ATPase inhibitors would be proved, the participation of ribosomal ATPase in translation might be indicated. For this purpose we used two kinds of ATPase inhibitor, AMP-P(NH)P, a competitor of ATP, and vanadate, an inhibitor of various kinds of ATPase. The ATPase activity of 80S(PU) ribosomes was actually significantly inhibited by AMP-P(NH)P (44% of the control at 25 mM, 37% at 0.5 mM, 29% at 1 mM) and vanadate (72% of the control at 0.2 mM, 59% at 0.4 mM, 46% at 1 mM), depending on their concentra-

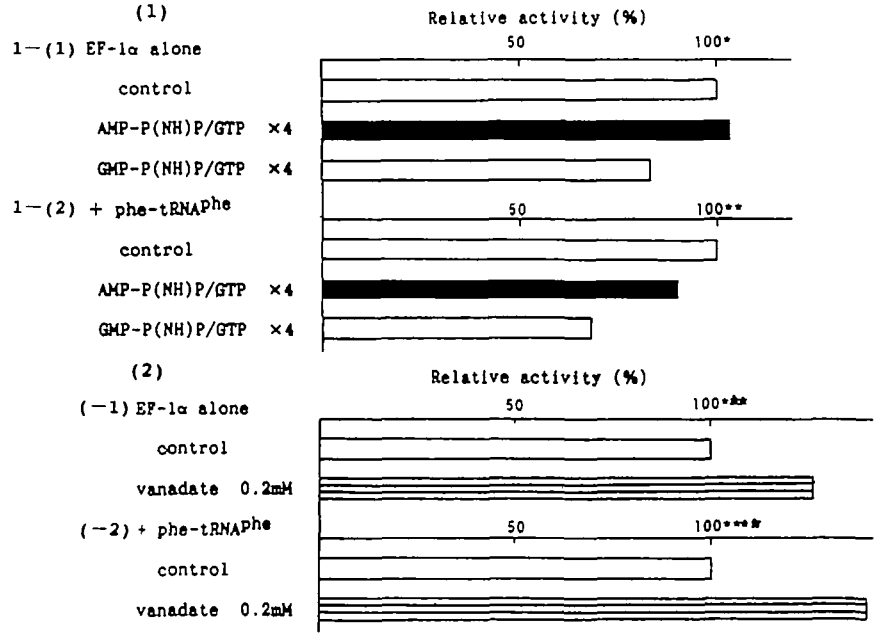

Fig. 4. **Effects of (1) AMP-P(NH)P and GMP-P(NH)P and (2) vanadate on the GTPase activity of EF-1α.** EF-1α (10 pmol) was incubated with 0.01 mM [γ-^{32}P]GTP in 0.1 M KCl–containing buffer E. (1) EF-1α alone, (2) plus 20 pmol of phe-tRNA^{phe}. *4.2 pmol, **6.3 pmol, ***11 pmol, ****16 pmol GTP hydrolyzed.

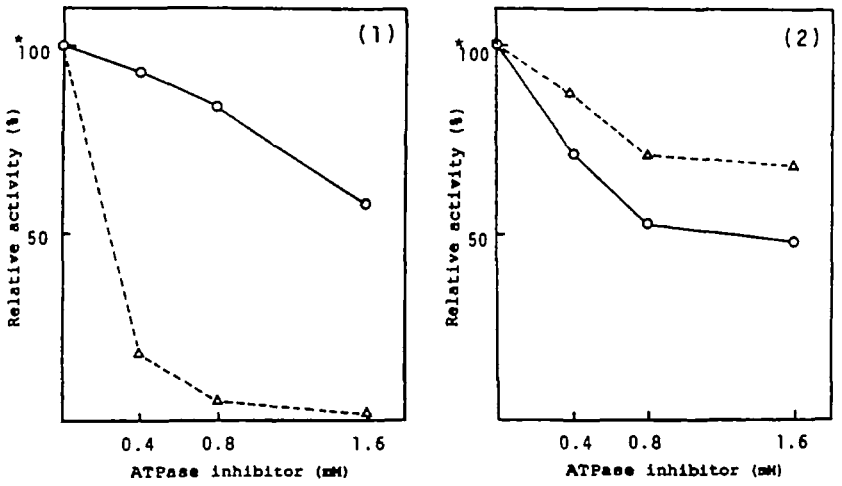

Fig. 5. **Effects of AMP-P(NH)P and GMP-P(NH)P on the activity of (1) poly(U)-dependent polyphe synthesis and (2) ATPase activity of the same system.** (1) 2.4 pmol of 80S(PU) ribosomes was incubated in the complete system (without EF-1βγ) containing 32 pmol of [^{3}H]phe-tRNA^{phe}. (2) ATPase activity was assayed under the same conditions except that 0.1 mM [γ-^{32}P]ATP was substituted for 0.1 mM GTP. *17 pmol of phe-tRNA^{phe} polymerized. **145 pmol of GTP hydrolyzed (GTPase activity of 80S(PU) ribosomes alone was 96 pmol). ○, AMP-P(NH)P; △, GMP-P(NH)P.

tions, as reported for 80S ribosomes reassociated from both subunits (*4*), and 30S-5SRNP particles are 40S subunits (*6*).

(1) Effects of AMP-P(NH)P on GTPase activities of EF-2 and F-1α: For the purpose of the present experiments, it was important to show that AMP-P(NH)P did not inhibit the GTPase activity of elongation factors. Therefore, we examined the effects of this inhibitor and compared then with those of GMP-P(NH)P. The GTPase activity of EF-2 was very low, about 1/200 of that of 80S(PU) ribosomes, although it is known to be markedly stimulated by 60S subunits or 80S ribosomes, and has been designated as uncoupled GTPase (*9, 20*). Therefore, we examined the effects of AMP-P(NH)P and GMP-P(NH)P on the GTPase of EF-2 in the presence of 60S subunits. Sixty S subunits used were purified by repeating the sucrose density-gradient centrifugation to remove contaminating 40S subunit dimers. In these experiments we used 0.01 mM [γ-^{32}P]GTP as described in Ref. *15*.

As shown in Fig. 3(1), the stimulated GTPase activity of EF-2 in the presence of 60S subunits (about 1,000-fold that of 60S subunits) was not affected by AMP-P(NH)P, although it was markedly inhibited by GMP-P(NH)P. Therefore, we concluded that GTPase of EF-2 is stringent for GTP.

It was reported that EF-1α from rabbit reticulocytes (*21*) or calf brain (*16*) had GTPase activity which was enhanced by aminoacyl-tRNA in the presence of 0.1 M KCl (*16*) or poly(U) (*21*). The GTPase activity of EF-1α used was 0.011 ± 0.003 pmol inorganic P hydrolyzed/pmol EF-1α/min (average of nine independent experiments) in the presence of 0.01 mM GTP, which was comparable to the activity reported previously (*17, 18*). The GTPase activity of 80S(PU) ribosomes was markedly higher than that of EF-1α: 0.62 ± 0.05 pmol inorganic P/pmol ribosomes/min (average of six experiments).

As shown in Fig. 4, the GTPase activity of EF-1α alone or stimulated by phe-tRNA^{phe} was not affected by AMP-P(NH)P, although it was clearly inhibited by GMP-P(NH)P. The activity stimulated by poly(U) was also not affected by AMP-P(NH)P (data not shown).

(2) Effects of AMP-P(NH)P and GMP-P(NH)P on the poly(U)-dependent polyphe synthesis and GTPase activities of the same system: Since AMP-P(NH)P inhibited strongly the ATPase activity of 80S ribosomes but did not affect the GTPase activities of EF-1α and EF-2, we examined the effects of AMP-P(NH)P and GMP-P(NH)P on poly(U)-dependent polyphe synthesis, using the system of Iwasaki *et al.* (*15*). The polyphe synthesis was carried out by using [^{3}H]phe-tRNA^{phe} in the presence of 80S(PU) ribosomes, EF-1α, EF-2, poly(U), and GTP (*15*). After incubation, the hot TCA-insoluble radioactivities were measured.

The results are shown in Fig. 5(1). The polyphe synthesis was inhibited not only by GMP-P(NH)P but also by AMP-P(NH)P, depending on their concentrations, although the inhibition by GMP-P(NH)P was stronger. The ATPase activity of the polyphe synthesizing system was inhibited by AMP-P(NH)P, depending on the concentration [Fig. 5(2)]. The results may indicate directly that ribosomal ATPase participates in peptide elongation.

(3) Effects of vanadate on GTPase activities of EF-1α and EF-2: As described earlier, vanadate inhibited significantly and concentration-dependently the ATPase activity of 80S(PU) ribosomes. Then we examined whether vanadate inhibited the GTPase activity of EF-1α and EF-2.

As shown in Fig. 4(2), 0.2 mM vanadate enhanced the GTPase activity of EF-1α alone and stimulated by phe-tRNA^{phe}. The effects of vanadate on the uncoupled GTPase activity of EF-2 in the presence of 60S subunits are shown

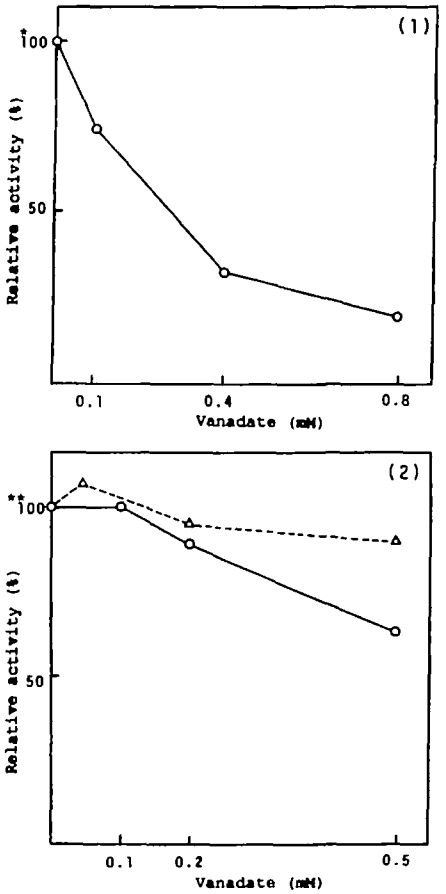

Fig. 6. **Effects of vanadate on (1) poly(U)-dependent polyphe synthesis and (2) the ATPase and GTPase activity of the same system.** (1) 3 pmol of 80S ribosomes was incubated in the complete system containing EF-1βγ. Average of three independent experiments. (2) 1 pmol of 80S(PU) ribosomes was incubated with 0.125 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP in the same system. *9 pmol of phe-tRNA^{phe} polymerized. **ATPase activity: 65 pmol ATP hydrolyzed; GTPase activity: 40 pmol GTP hydrolyzed. ○, ATPase; △, GTPase.

TABLE V. **GTPase and ATPase activities of poly(U)-dependent polyphe-synthesizing system (containing 0.1 mM [γ-^{32}P]GTP or [γ-^{32}P]ATP).**

	Additions						
Ribosomes (3 pmol)	+	+	+	+	+	+	+
Poly(U) (5 μg)	+	−	+	−	+	+	−
Phe-tRNA^{phe} (1 μg)	+	+	−	−	+	+	−
EF-1α (5 pmol)	+	+	+	+	+	−	−
EF-2 (10 pmol)	+	+	+	+	−	+	−
Relative activity of							
GTPase	100 (575 pmol)	100	96	97	22	102	17 (97 pmol)
ATPase	100 (279 pmol)	80	96	72	78	77	53 (147 pmol)

in Fig. 3(2). The stimulated GTPase activity of EF-2 was not affected by vanadate.

(4) Effects of vanadate on poly(U)-dependent polyphe synthesis and on the ATPase and GTPase activities of the polyphe synthesizing system: On the basis of the above results, we examined the effects of 0.1 mM and 0.2 mM vanadate on poly(U)-dependent polyphe synthesis by 80S(PU) ribosomes. As shown in Fig. 6(1), vanadate inhibited strongly polyphe synthesis, dependent on the concentration. The results were similar to those obtained with AMP-P(NH)P. We further examined the effects of vanadate on the GTPase activity of the polyphe synthesizing system. As shown in Fig. 6(2), vanadate little affected the GTPase activity, whereas it inhibited concentration-dependently the ATPase activity.

(V) GTPase and ATPase Activities of the Poly(U)-Dependent Polyphe Synthesizing System—To obtain useful information on the participation of ATPase of 80S ribosomes in peptide elongation, we investigated the GTPase and ATPase activities of this polyphe synthesizing system using the same concentration of [γ-^{32}P]ATP or [γ-^{32}P]GTP (0.1 mM) as that of GTP used in this system. The effects of the elongation components were also studied. The results are shown in Table V. Concerning the GTPase activity, the high value observed in the complete system was mainly due to the uncoupled GTPase activity of EF-2, because the GTPase activity without EF-2 was very low (about 20% of that observed in the complete system), and omitting the other elongation components had little affect on the GTPase activity. On the other hand, the ATPase activity of the complete system was also high, about one half of the GTPase activity. As also described in Table V, the ATPase activity was enhanced by each elongation component, because omitting any one component resulted in the decrease of the ATPase activity. The results suggest that the ribosomal ATPase may undergo highly positive conformational changes in the presence of the full elongational components, which may contribute to the polyphe synthesizing activity.

(VI) Effects of ATP and AMP-P(NH)P on the Poly(U)-Dependent Polyphe Synthesis in the Present of Various Concentrations of GTP—Kovalchuke and Chakraburtty reported that ATP, ADP, or AMP-P(NH)P did not affect poly(U)-dependent polyphe synthesis in the presence of a low concentration of GTP (*5*). To elucidate the cause for the difference from our findings, we examined the effects of ATP and AMP-P(NH)P on the poly(U)-dependent polyphe synthesis in the presence of various concentrations of GTP.

The results are shown in Fig. 7. Surprisingly, ATP (300 μM) enhanced the polyphe synthesizing activity at all concentrations of GTP, even at enough concentrations of GTP (>100 μM). The results suggest that ATP contributes in some specific way to the polyphe synthesizing activity in addition to maintaining the GTP pool, although further studies should be done to elucidate the specific role of ATP.

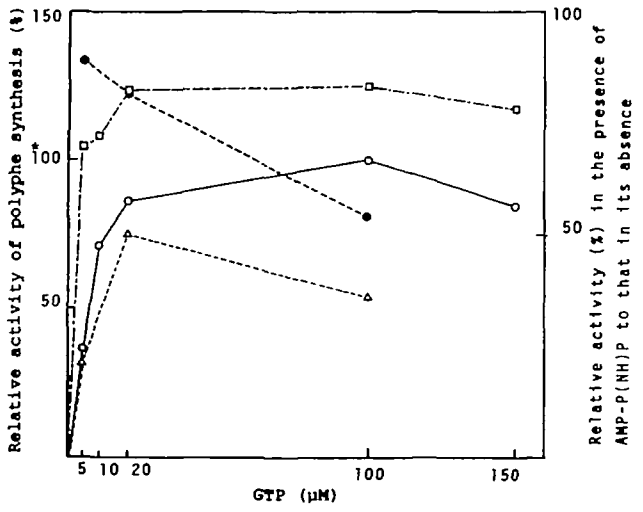

Fig. 7. **Effects of ATP and AMP-P(NH)P on the poly(U)-dependent polyphe synthesis by 80S(PU) ribosomes in the presence of various concentrations of GTP.** Results summarized from three independent experiments. 2.4 pmol of ribosomes was incubated in the complete system as described in Fig. 5. The hot TCA-insoluble activity in the absence of GTP (about 2% of the control at 0.1 mM GTP) was substituted from each value and the value in the presence of 0.1 mM GTP, which was 12.7 pmol of phe-tRNA^{phe} polymerized, was taken as 100%. ○, GTP alone; △, + 300 μM AMP-P(NH)P; □, + 300 μM ATP; ●, the extent of inhibition of AMP-P(NH)P at various concentrations of GTP.

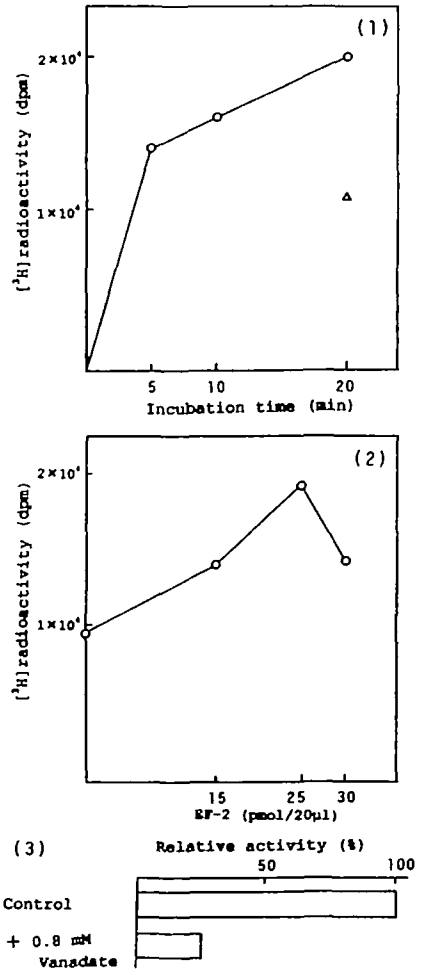

Fig. 8. **The translocation reaction by purified rat liver free polysomes using [^{3}H]puromycin.** (1) Time courses of the reaction. (2) Dose responses of EF-2. (3) Effects of vanadate on translocation. The complete reaction mixture in a total volume of 20 μl was used. ○, complete system; △, minus EF-2.

At low concentrations of GTP (5 μM or 10 μM), the extent of inhibition of AMP-P(NH)P (300 μM), expressed as percent of the control value without AMP-P(NH)P, was much lower than that observed at the high concentration of GTP (100 μM) that we usually used, probably because prevented the reduction of the GTP concentration due to GTPase intrinsic to ribosomes (*6*).

(VII) Effects of Vanadate on the Translocation Reaction—Since EF-2 markedly enhanced the ATPase activity of 80S ribosomes, we examined the effects of vanadate on the translocation by measuring the incorporation of [^{3}H]puromycin into the cold TCA-insoluble fraction in the presence of EF-2 and high KCl-washed polysomes (*15*), which showed a typical polysomal pattern even after washing with high KCl, as shown in Fig. 1(1), and a higher translocation activity than post(mt) ribosomes (data not shown). The reaction proceeded for at least 20 min at 2°C, and the optimal concentration of EF-2 was 25 pmol/20 μl [Fig. 8, (1) and (2)]. The effects of vanadate on the EF-2–dependent incorporation of [^{3}H]puromycin are shown in Fig. 8(3). The finding that vanadate inhibited the incorporation of [^{3}H]-puromycin into the cold TCA-insoluble fraction may indicate that ribosomal ATPase participates in the translocation reaction on polysomes.

DISCUSSION

Rat liver 80S(PU) ribosomes have intrinsic ATPase-(GTPase) with a markedly higher activity than GTPase of EF-2 [about 0.3% of ATPase activity of 80S(PU) ribosomes] and EF-1α (about 2%), although the activity of EF-2 is enhanced markedly by 60S subunits (up to 1,000 times of that of 60S subunits) or by 80S(PU) ribosomes [up to 12 times of that of 80S(PU) ribosomes, which is referred to uncoupled GTPase], and that of EF-1α is enhanced somewhat by poly(U) or phe-tRNA^{phe}.

It is of interest that the ATPase activity of 80S(PM) ribosomes is stimulated by components of peptide elongation, poly(U), phe-tRNA^{phe}, EF-1α, and EF-2, alone and in combinations. With the combinations, additive or synergic stimulations are observed. Therefore, the ATPase activity of the polyphe synthesizing system is enhanced up to about one half of the GTPase activity of the same system, a greater part of which is owing to uncoupled GTPase, since without EF-2 the activity is reduced to 20% of that of the complete system with EF-2. Similar enhancement by translational components was observed for the ATPase activity of 30S-5SRNP, which consists of unfolded 40S subunits containing 5SRNP (*7*), and 40S subunits (*6*). Since these translational components interact with different regions of ribosomes, as described later, we assume that positive conformational changes of 80S(PU) ribosomes are induced additively or synergistically by these interactions, resulting in the additive or synergic stimulation of the ATPase centre of ribosomes. On the contrary, two elongation inhibitors (tetracycline and fusidic acid) inhibited ATPase activities of ribosomes alone and stimulated by elongational components by binding to ribosomes and by interaction with elongational components.

Much evidence has been accumulated that polyamines stimulate protein synthesis by inducing positive conformational changes of ribosomes [see review article (*17*)]. Previously, we showed that spermidine enhanced the ATPase activity of rat liver 40S subunits. Recently, the importance of spermidine for poly(U)-dependent polyphe synthesizing activity of 80S ribosomes obtained by reassociation of 40S and 60S subunits was reported by El' skaya *et al.* (*18*). We also showed that polyamine stimulated polyphe synthesizing and ATPase activities of reassociated 80S ribosomes [Table II(2)]. The lack of effect of polyamine on the ATPase activity of 80S(PU) ribosomes may be explained by assuming that they already have the positive conformation, as shown their higher ATPase activities than reassociated ribosomes. These results may indicate the importance of the conformation of ribosomes for ATPase and translational activities.

In this respect, it is important that dynamic properties of the conformation of *Escherichia coli* ribosomes have been indicated since the report of Conway and Lipmann (*22–27* and references therein). Dabrowski *et al.* recently showed that ribosomal contact sites of two tRNAs at the A and P sites hardly changed during the translocation reaction to the P and E sites, suggesting that a movable ribosomal domain exists that tightly binds to two tRNAs and carries them together with the translocation (*26*). Using three-dimensional cryo-electron microscopy, Gabashvili *et al.* observed the relative and independent movement of the head, platform, and main body of 30S subunits and suggested the existence of a dynamic property of the 30S subunit that might be required for facilitating its interaction with mRNA, tRNA, and other ligands during protein biosynthesis (*27*).

Considering the mode of action of elongation components in enhancing the ATPase activity of 80S(PU) ribosomes and 30S-5SRNP particles together with the importance of 5SRNP located at the interface of both subunits, for ATPase activities of both particles, we propose the ATPase center of 80S ribosomes consisting of 5SRNP connecting both subunits and their specific regions interacting with elongational components as described below. As for ribosomal proteins, crosslinking studies of rat liver ribosomes indicate the interactions of S3a, S6, L5, and L6 with mRNA; S23/24, S26, L12, L23, and L39 with EF-1α; and S3a, S6, S23/24, P2, L3, L4, L5, L9, L12, L23, and LA33 with EF-2 (*28–32* and references in Ref. *32*). As for rRNA, two specific domains of 28S rRNA were identified as the sites of interaction with elongation factors: α-sarcin and ricin sites (*33, 34*) (residues 4316–4332) and the GTPase centre (*35*) (residues 1841–1939). It is of interest that ricin and α-sarcin alter the conformation of 60S subunits at neighbouring but different sites (*36*). Therefore, it is possible that the interactions of mRNA, EF-1α, and EF-2 with the specific regions of the ribosomal ATPase centre result in the enhancement of the ribosomal ATPase activity through its positive conformational changes.

While the ATPase centre of 80S ribosomes shows a wide substrate specificity, GTPase activities of EF-1α and EF-2 of the liver were found to be stringent for GTP as Tu and G factors. Therefore, we considered that when ATPase inhibitors [AMP-P(NH)P and vanadate] would inhibit poly(U)-dependent polyphe synthesis, it may be indicated that ribosomal ATPase participates in peptide bond elongation. It was found that both ATPase inhibitors inhibit polyphe synthesis, suggesting the participation of ribosomal ATPase in peptide bond elongation.

In the experiments on the effects of AMP-P(NH)P and

ATP on the poly(U)-dependent polyphe synthesis in the presence of various concentrations of GTP, it was found that ATP enhanced the polyphe synthesizing activity not only at low concentrations but also high concentrations of GTP, suggesting that ATP has a specific role other than GTP in peptide bond elongation, probably inducing the positive conformation of ribosomes, although further study must be done to elucidate these points. The extent of inhibition of AMP-P(NH)P was very small at low concentrations of GTP (Fig. 8), because probably AMP-P(NH)P prevents the reduction of the GTP pool, as reported in reference (*5*). Furthermore, it is possible that AMP-P(NH)P may play a role in peptide elongation as an ATP analogue, like the role of GMP-P(NH)P as a GTP analogue (*1*), in addition to being a competitive inhibitor of ATP.

Since the ribosomal ATPase activity was markedly enhanced by EF-2, we examined the effects of vanadate on the translocation by high-KCl washed polysomes. The translocation was inhibited by vanadate, suggesting that ribosomal ATPase has multiple functions in translation, as described below.

Concerning the participation of ATPase in peptide bond elongation, a third elongation factor was found in yeast cell by Skogerson and Wakatama (*37*), which was designated as elongation factor 3 (EF-3). EF-3 is specific for fungal species and exhibits ribosome-dependent ATPase and GTPase activities. EF-3 is essential for peptide bond elongation in addition to EF-1α and EF-2 in fungal cells (*37*) [see also the review article (*38*)]. The question of whether EF-3 is specific to fungi has attracted the interest of many investigators. It was shown that there is a significant sequence homology between yeast EF-3 and *E. coli* S5 protein which is important for retaining translational accuracy (*38*). Furthermore, it was reported that EF-3 plus ATP stimulated the EF-1α–dependent binding of phe-tRNA^{phe} to the poly(U)-programmed ribosomes in the presence of ATP, accompanied with ATP-hydrolysis, and the EF-3–stimulated reaction was suggested to allow strict binding of cognate aminoacyl-tRNA to the A site (*39*, *40*).

Until recently, however, it was not known whether the ATPase intrinsic to ribosomes has the equivalent function to EF-3. In a study on the role of the E site in yeast, Triana-Alonso *et al.* found that the ATP-dependent activity of EF-3 is required for the release of deacylated tRNA from the E site in the yeast cell-free system (*41*). Furthermore, in experiments to show that rabbit liver ribosomes contain three tRNA binding sites, El'skaya *et al.* suggested that ATPase of 80S ribosomes fulfilled the same function as EF-3, from the finding that the chasing efficiency of tRNA at the E site by cognate tRNA was stimulated by ATP or GTP, whereas AMP-P(NH)P or GMP-P(NH)P reduced it (*18*).

These results, together with our finding of the participation of ribosomal ATPase in translocation, indicate that this ATPase may have multiple functions in peptide elongation in mammalian cells, as follows: (i) a direct function, through similar mechanisms to the case of EF-3 (*18*), and (ii) an indirect function, through conformational changes of ribosomes, as shown by the inhibition of translocation by vanadate. Further study is needed to elucidate the mechanisms of participation of ATP and ribosomal ATPase in translation of mammalian cells.

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