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Zinc(II)-Dependent Synthesis of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate by *Escherichia coli* and Yeast Phenylalanyl Transfer Ribonucleic Acid Synthetases†

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ABSTRACT: A new activity of *Escherichia coli* and yeast phenylalanyl-tRNA synthetases, the conversion of adenosine 5′-triphosphate into diadenosine 5′,5‴-P^1,P^4-tetraphosphate, is reported. This activity is followed by ^{31}P NMR and chromatography on poly(ethylenimine)–cellulose. It is revealed by the addition of $ZnCl_2$ to a reaction mixture containing the enzyme, ATP–Mg^{2+}, L-phenylalanine, and pyrophosphatase. It reflects the reaction of enzyme-bound phenylalanyl adenylate with ATP instead of PP_i and strongly depends on the hydrolysis of pyrophosphate in the assay medium. The zinc dependence of this reaction parallels that of the inhibition of $tRNA^{Phe}$ aminoacylation which is described in the accompanying paper [Mayaux, J. F., & Blanquet, S. (1981) *Biochemistry* (preceding paper in this issue)]. In the presence of an unlimiting pyrophosphatase activity, diadenosine tetraphosphate synthesis by *E. coli* and yeast phenylalanyl-tRNA synthetases occurs at maximal rates of 0.5 and 2 s^{-1}, respectively (37 °C, pH 7.8, 150 mM KCl, 5 mM ATP, 10 mM $MgCl_2$, 2 mM L-phenylalanine, and 80 μM $ZnCl_2$). Under identical experimental conditions, *E. coli* isoleucyl-, methionyl-, and tyrosyl-tRNA synthetases produce small amounts of diadenosine tetraphosphate at rates 2 or 3 orders of magnitude lower than that achieved by phenylalanyl-tRNA synthetase. In the case of *E. coli* phenylalanyl-tRNA synthetase, it is shown that the diadenosine tetraphosphate synthetase activity is accompanied by a diadenosinetetraphosphatase activity. This activity, actually supported by phenylalanyl-tRNA synthetase, is responsible for the appearance of ADP in the assay medium. It requires also the presence of both $ZnCl_2$ and L-phenylalanine. The formation of ADP from diadenosine tetraphosphate and its reaction with enzyme-bound aminoacyl adenylate account for the appearance in the reaction mixture of diadenosine 5′,5‴-P^1,P^3-triphosphate, after that of diadenosine tetraphosphate. The significance of these findings in the context of the role of diadenosine tetraphosphate in controlling cellular growth is discussed.

Among the unusual nucleotides, diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA) deserves particular attention in so far as the intracellular concentration of this compound in mammalian cells is found to vary drastically in response to the proliferation rate (Rapaport & Zamecnik, 1976). Several mechanisms of action of AppppA in controlling growth have been recently proposed. Grummt et al. (1979) have reported that DNA[1] polymerase α was a target of AppppA and have suggested that AppppA could be an intracellular signal molecule for the initiation of DNA replication. On the other hand, Ono et al. (1980) describe that AppppA has no effect on the nucleotide-polymerizing activity of DNA polymerase α but that it specifically blocks the activity of the terminal deoxynucleotidyl transferase from calf thymus.

Diadenosine tetraphosphate was discovered by Zamecnik et al. (1966). Evidence was obtained that this nucleotide could be formed in a reaction mixture containing *Escherichia coli* lysyl-tRNA synthetase, lysine, ATP, and Mg^{2+}. Maximum formation was achieved when *E. coli* unfractionated tRNA was added. A smaller amount of diadenosine triphosphate (ApppA) has also been found under these conditions. AppppA was presumed to be made by a back reaction of the lysine adenylation reaction:

enzyme·lysyl-pA + pppA ⇌ AppppA + enzyme + lysine

The question as to whether the synthesis of AppppA is a general property within the class of prokaryotic or eukaryotic aminoacyl-tRNA synthetases is unresolved. Besides, the molecular signals that regulate AppppA synthesis have not yet been found. The present study investigates these two questions.

In the preceding paper (Mayaux & Blanquet, 1981) it has been shown that *E. coli* and yeast phenylalanyl-tRNA synthetases were able to bind zinc with concomitant reversible loss of their tRNA aminoacylation activity. This behavior markedly distinguished phenylalanyl-tRNA synthetase from

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[1] Abbreviations used: DNA, deoxyribonucleic acid; tRNA, transfer ribonucleic acid; FT, Fourier transform; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; PEI, poly(ethylenimine); AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; PP_i, inorganic pyrophosphate; poly(A), poly(adenylic acid).

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the other studied aminoacyl-tRNA synthetases. Following this observation and a recent report from Igloi et al. (1980), the possible zinc-dependent consumption of ATP during the overall aminoacylation process catalyzed by phenylalanyl-tRNA synthetase was investigated by ^{31}P NMR and thin-layer chromatography. Under the conditions of the tRNA aminoacylation assay with catalytic amounts of enzyme, no alteration of the ATP pool could be detected. On the other hand, with a higher enzyme concentration and provided that pyrophosphatase was present, a considerable synthesis of AppppA could be revealed. This novel enzymatic activity of phenylalanyl-tRNA synthetase strictly depends on the presence of the amino acid and of the zinc ion but not on that of tRNA.

Materials and Methods

Homogeneous isoleucyl-, methionyl-, tyrosyl-, and phenylalanyl-tRNA synthetases from *E. coli* strain EM 20031 carrying the F 32 episome were purified by using published procedures (Cassio & Waller, 1971; Fayat et al., 1974, 1978; M. Fromant, G. Fayat, P. Laufer, and S. Blanquet, unpublished experiments). Homogeneous phenylalanyl-tRNA synthetase from the overproducing strain IBPC 1671 pB_1 (Plumbridge et al., 1980) was obtained according to a similar simplified procedure (unpublished). Homogeneous phenylalanyl-tRNA synthetase from Baker's yeast was a gift from Dr. F. Fasiolo (IBMC, Strasbourg). The enzymes were stored in 50% glycerol at −15 °C. Yeast inorganic pyrophosphatase was purchased from Boehringer (Mannheim) (sp act., 200 units/mg at 25 °C). $tRNA^{Phe}$ from *E. coli* MRE 600 (1100 pmol of L-phenylalanine acceptance/A_{260} unit of tRNA) was from Boehringer and was freed of zinc as described in the preceding paper. Enzymes and tRNA concentrations were determined according to their specific absorption coefficients at 280 and 260 nm, respectively. Diadenosine 5′,5‴-P^1,P^4-tetraphosphate and diadenosine 5′,5‴-P^1,P^3-triphosphate were obtained from P-L Biochemicals and Boehringer (Mannheim). [γ-^{32}P]ATP (1600 Ci/mol) was purchased from the Radiochemical Centre (Amersham, United Kingdom). L-Phenylalaninol was from Fluka.

^{31}P NMR Measurements. ^{31}P spectra were measured at 111.7 MHz on a home-built FT spectrometer (Caron et al., 1980). The origin of chemical shifts was 85% H_3PO_4 whose frequency is computed from that of the lock signal provided by D_2O (concentration 25%). The spectra were obtained with broad-band proton decoupling; the repetition time was 2 s and the flip angle 60°. The integrated intensity of each peak is proportional to the concentration of the corresponding molecular species, multiplied by a correction factor which takes into account the deviation of the magnetization from its equilibrium value due to saturation and/or nuclear Overhauser effect. The corrections were determined in a separate experiment and were in every case smaller than 20%.

Samples (1.2–2 mL) in 20 mM Tris-HCl (pH 7.8), 150 mM KCl, and 5 mM free $MgCl_2$ contained ATP–Mg^{2+}, amino acid, $ZnCl_2$, or EDTA, and enzyme concentrations as given under Results. Kinetics were initiated by the addition of enzyme and followed at 37 ± 1 °C. Spectra were acquired in the block mode; typically, 600 spectra were averaged per block (one block corresponded to 20 min of the kinetics).

Thin-Layer Chromatography. Identification of nucleotides was performed by thin-layer chromatography on PEI–cellulose plates. Aliquots of 5 μL were spotted (6 mm diameter spots) on a plastic-backed sheet of PEI–cellulose [UV_{254} sheet (20 × 20 cm) Macherey and Nagel, Düren, West Germany]. The nucleotides were separated by chromatography with 1 M LiCl (Randerath et al., 1966). A paper wick was attached to the end of the chromatogram, and the solvent was allowed to advance to a distance of 24 cm from the origin. The sheet was dried and the spots were located by UV absorption. The chromatogram was photographed in short-wave ultraviolet light.

Enzymatic Cleavage of the γ-Phosphate of ATP. Initial rates of hydrolysis of [γ-^{32}P]ATP were followed at 37 °C. The assay mixture contained 20 mM Tris-HCl, pH 7.8, 150 mM KCl, and phenylalanyl-tRNA synthetase (0.4–0.6 μM) with various concentrations of $ZnCl_2$, $MgCl_2$, and labeled ATP. The reaction was initiated by the addition of L-phenylalanine. Control experiments were performed in parallel without the amino acid. From time zero, 20-μL aliquots of the reaction assay were periodically withdrawn and mixed with 2.5 mL of 0.35% perchloric acid in 50 mM sodium acetate (pH 4.5) containing 0.4% (w/w) activated charcoal and 100 mM PP_i. The Norit was filtered on Whatman No. 1 filter paper disks and washed, and the radioactivity was counted on an IDL crystal scintillation counter.

Results

Evidence of Synthesis of AppppA by E. coli Phenylalanyl-tRNA Synthetase. The effect of zinc on a reaction mixture containing phenylalanyl-tRNA synthetase, 7.5 mM ATP, and 2 mM L-phenylalanine in 20 mM Tris-HCl (pH 7.8), 12.5 mM $MgCl_2$, and 150 mM KCl was followed at 37 °C through ^{31}P NMR measurements. As shown in Figure 1, in the presence of 80 μM $ZnCl_2$ and ~1 μM enzyme, several unusual resonances appeared on the NMR spectrum at 10.9, 21.5, and 22.1 ppm. The area of these resonances progressively increased with time, at the expense of the resonances of the three phosphates of ATP (10.3, 18.6, and 5.1 ppm for the α-, β-, and γ-phosphates, respectively). In parallel, resonances developed at −2.3, 9.7, and 5.6 ppm which correspond to the phosphates of P_i and to the α- and β-phosphates of ADP, respectively. A minor peak at −3.7 ppm, the postion of the 5′-phosphate of AMP can be distinguished only in the latest spectrum. It is remarkable that pyrophosphate (expected resonance at 4.95 ppm) was not detectable on the spectra.

On the basis of chemical shift, fine structure, and coupling constants, the resonances at 10.9 and 21.5 ppm were respectively identified as those of the α- and β-phosphates of a sample of pure AppppA (Table I). Also the resonance at 22.1 ppm was identified as that of the β-phosphate of ApppA. The resonance of the α-phosphates of ApppA (11.0 ppm) was superimposed on that of the α-phosphates of AppppA in our experimental conditions.

Additional evidence supporting the synthesis of AppppA and of ApppA from ATP was provided by analytical thin-layer chromatography of the reaction mixture on PEI–cellulose according to Randerath et al. (1966). In agreement with the NMR spectra, the chromatogram showed the disappearance of ATP and the appearance of AppppA and ADP (Figure 2). The synthesis of ApppA started after that of ADP. No other nucleotides could be detected on the chromatogram.

The kinetics of disappearance of ATP and of formation of the various AppppA, ApppA, ADP, and P_i have been measured with the help of the spectra in Figure 1. In the cases of AppppA and ApppA, only the β-phosphate resonances have been followed since the α-phosphates of these two nucleotides cannot be resolved on the spectra. The areas corrected as described under Materials and Methods were converted into concentrations on the basis of the initial concentration of ATP in the reaction mixture and plotted as a function of time (Figure 3). It was verified that the total integral corresponding to total phosphate concentration was the same for

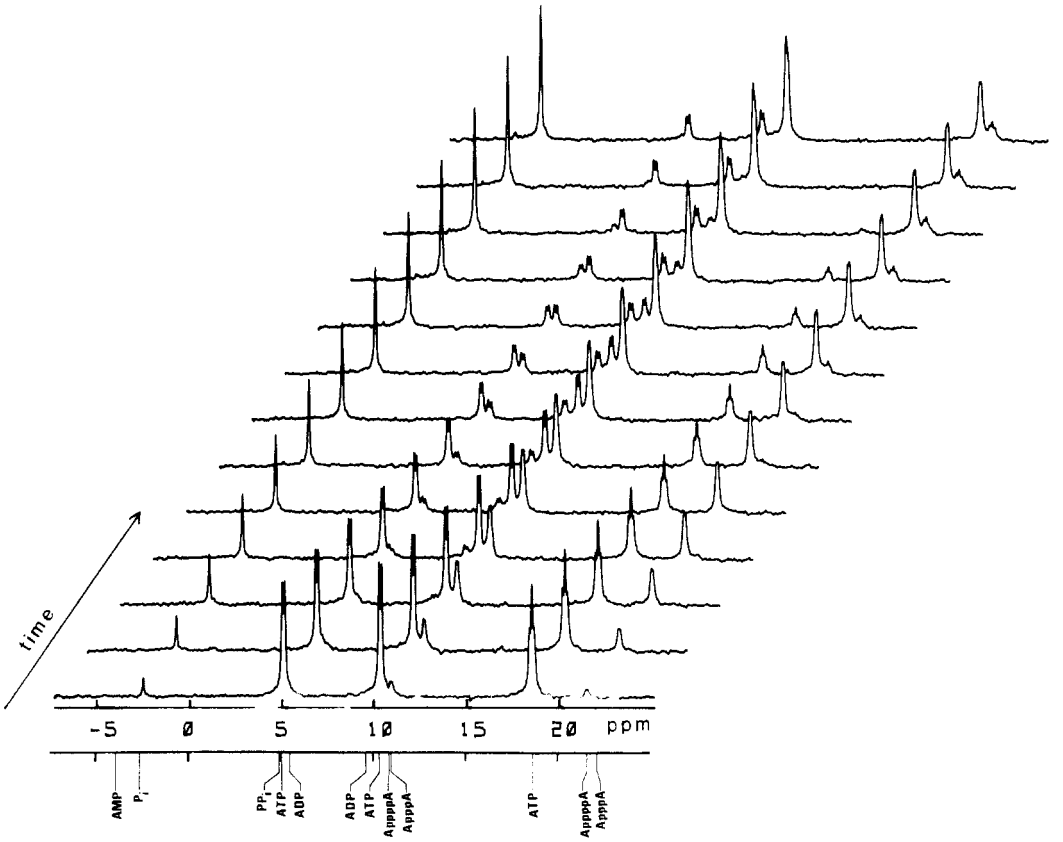

FIGURE 1: Evidence by ^{31}P NMR spectroscopy for synthesis of AppppA and ApppA by *E. coli* phenylalanyl-tRNA synthetase. The sample was buffered with 20 mM Tris-HCl, pH 7.8, 150 mM KCl, and 12.5 mM $MgCl_2$ including 25% D_2O and contained 7.5 mM ATP, 2 mM L-phenylalanine, and 80 μM $ZnCl_2$. The reaction (37 °C) was initiated by the addition of *E. coli* phenylalanyl-tRNA synthetase from strain EM 20031 at a final concentration of 0.6 μM. Each spectrum corresponds to the average of 600 scans: repetition time, 2 s, line broadening, 7.5 Hz. Spectra were acquired during consecutive 20-min periods.

Table I: ^{31}P Chemical Shifts (in ppm from 85% H_3PO_4) and Coupling Constants (in Hz) for the Various Phosphorus-Containing Compounds Appearing on NMR Spectra[a]

		chemical shift (ppm)	coupling constant (Hz)
AMP		−3.68	
P_i		−2.32	
PP_i		4.95	
ATP	α	10.31	$J_{\alpha\beta} = J_{\beta\gamma} = 15.4$
	β	18.57	
	γ	5.07	
ADP	α	9.66	$J_{\alpha\beta} = 18.0$
	β	5.63	
AppppA	α	10.90	$J_{\alpha\beta} = 16.3$
	β	21.49	$J_{\beta\beta'} = 13.7$
ApppA	α	10.98	$J_{\alpha\beta} = 16.3$
	β	22.09	

[a] The solution contained 20 mM Tris-HCl (pH 7.8), 150 mM KCl, and 5 mM free $MgCl_2$, T = 37 °C. Values in the table were obtained with pure samples (line broadening 0.5 Hz). In the case of AppppA, the ^{31}P NMR spectrum was that of an AA'XX' spin system (Corio, 1966).

each spectrum to better than 10%.

Figure 3 clearly shows the parallelism of ATP consumption and of orthophosphate formation. The initial rate of AppppA formation agreed well with that of P_i when it was assumed that two phosphates accompanied the synthesis of one AppppA. At longer times AppppA synthesis decreased and its concentration reached a plateau value while P_i still accumulated and ApppA formation was triggered. ADP seemed to appear from the beginning of the reaction in a sigmoidal manner; however, its rate of formation remained at least 4-fold lower than that of AppppA. After the total consumption of ATP, the concentration of AppppA slowly decreased with the consequent formation of ADP and ApppA (not shown). The origin of the various compounds appearing in the reaction will be discussed further below.

Enzymatic Synthesis of AppppA Is Stimulated in the Presence of Pyrophosphatase. If AppppA were formed from the reaction of ATP with the aminoacyl adenylate, pyrophosphate would also be produced. However, as shown on the spectra in Figure 1, orthophosphate, instead of pyrophosphate, accompanied the change of ATP into AppppA. In view of the high activity of *E. coli* pyrophosphatase (2×10^4 s^{-1}) and considering the large molecular weight (120K) of this enzyme (Josse & Wong, 1971), it was likely that contaminating pyrophosphatase had been copurified with phenylalanyl-tRNA synthetase (M_r 250K) and could account for the appearance of P_i instead of PP_i during the reaction.

This possibility was verified as follows. (i) The contaminating pyrophosphatase activity accompanying the phenylalanyl-tRNA synthetase was estimated by measuring the rate of hydrolysis of [^{32}P]PP_i. A contamination of 0.01% (w/w) with respect to phenylalanyl-tRNA synthetase was found. This corresponded to an apparent turnover rate of pyrophosphorolysis of 5 s^{-1} if calculated with respect to the synthetase concentration. This rate was larger than that calculated for AppppA formation in the presence of this contaminating activity (0.2 s^{-1} from Table II). (ii) The contaminating pyrophosphatase activity of phenylalanyl-tRNA synthetase was reduced by the addition of 10 mM KF which otherwise did not affect the activity of phenylalanyl-tRNA synthetase. Under this condition, the contaminating pyrophosphorolysis rate was reduced to 0.1 s^{-1}. The rate of AppppA formation in the presence of KF also decreased to

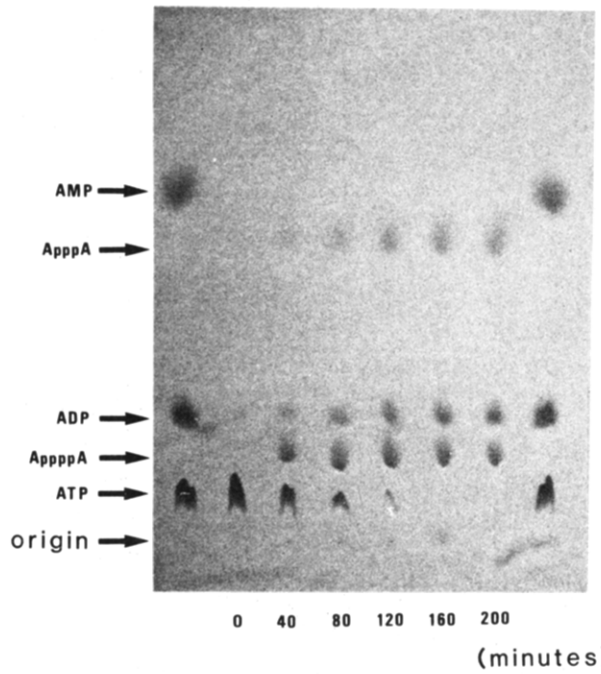

FIGURE 2: Evidence by thin-layer chromatography for synthesis of AppppA and ApppA by *E. coli* phenylalanyl-tRNA synthetase. The assay mixture (0.2 mL) contained 50 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, 4 mM ATP, 2 mM L-phenylalanine, 80 μM $ZnCl_2$, and 0.01 mg/mL yeast pyrophosphatase. The incubation was initiated by the addition of *E. coli* phenylalanyl-tRNA synthetase at a final concentration of 1 μM. The reaction mixture was maintained at 37 °C. Every 40 min, 5-μL aliquots were withdrawn and spotted on PEI–cellulose. The nucleotides were separated by chromatography with 1 M LiCl. ATP, ADP, and AMP (4 mM each in 50 mM Tris-HCl, pH 7.8, plus 11 mM $MgCl_2$) were used as standards on both sides of the chromatogram.

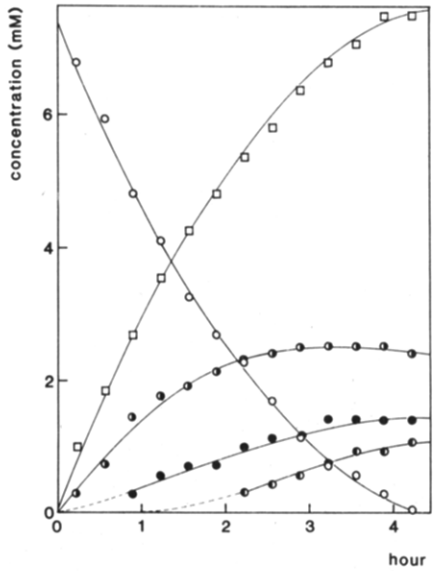

FIGURE 3: Kinetics of ATP consumption and of AppppA, ApppA, ADP, and P_i formation by *E. coli* phenylalanyl-tRNA synthetase as followed by ^{31}P NMR spectroscopy. Concentrations of the various ATP (○), AppppA (◑), ADP (●), ApppA (◐), and P_i (□) were measured on the NMR spectra of Figure 1 from the area of resonances corrected for T_1 and/or Overhauser effect differences. In the cases of AppppA and ApppA, concentrations were followed through the resonances of their β-phosphate. The calculation takes into account the fact that two β-phosphates per AppppA contribute to the ^{31}P resonance.

a value close to 0.1 s^{-1}, as estimated from the NMR measurements (Table II). (iii) A sample of phenylalanyl-tRNA synthetase was further purified by an additional chromatography on hydroxylapatite (pH 6.75), and the contaminating pyrophosphatase activity was reduced to 0.1 s^{-1}. This enzyme supported also a reduced rate of synthesis of AppppA (Table II). Again, the NMR spectra did not show any accumulation of PP_i. This suggested that AppppA synthesis could be rate limited by the hydrolysis of pyrophosphate. This possibility was examined by measuring the activity of AppppA synthesis supported by the repurified synthetase in the presence of added yeast pyrophosphatase. As shown in Table II, a rate of 0.25 s^{-1} for the conversion of ATP into AppppA was recovered. This rate value corresponded well, within experimental error, to the turnover rate initially measured with the enzyme from strain EM 20031 contaminated by *E. coli* pyrophosphatase.

Table II: Effect of Pyrophosphatase on Rates of AppppA and ADP Formation by *E. coli* Phenylalanyl-tRNA Synthetase[a]

	1		2	
	80 μM $ZnCl_2$	80 μM $ZnCl_2$ and 10 mM KF	80 μM $ZnCl_2$	80 μM $ZnCl_2$ and 0.01 mg/mL pyrophosphatase
AppppA	0.20 ± 0.03	0.095 ± 0.010	0.080 ± 0.01	0.25 ± 0.07
ADP	0.10 ± 0.02	0.040 ± 0.010	0.040 ± 0.020	0.15 ± 0.05

[a] Rates (s^{-1}) were measured at 37 °C from ^{31}P NMR spectra as shown in Figures 1 and 3. The reaction mixture in 20 mM Tris-HCl (pH 7.8) contained 7 mM $MgCl_2$, 150 mM KCl, 2 mM ATP, and 2 mM L-phenylalanine plus $ZnCl_2$, KF, and yeast pyrophosphatase concentrations as indicated in the table. The enzyme (0.6 μM) was (1) phenylalanyl-tRNA synthetase from *E. coli* strain EM 20031 containing endogeneous pyrophosphatase contamination and (2) phenylalanyl-tRNA synthetase from overproducing strain IBPC 1671 pB_1 with the lowest contaminating pyrophosphatase activity. Single-exponential kinetics were assumed for computing of the initial rates of AppppA and ADP formation. In the case of ADP, due to the sigmoidal nature of the kinetics of appearance of this nucleotide, this treatment overestimated the initial rate value. The rate values for ADP given in the table correspond better therefore to the rate of ADP formation in the presence of at least 0.5 mM AppppA in the reaction mixture.

This set of experiments clearly indicated that pyrophosphorolysis was the rate-limiting step in the reaction of AppppA formation. Since this nucleotide was presumed to arise from the reversion of the adenylate by ATP instead of PP_i (Zamecnik et al., 1966), it was reasonable to assume that PP_i could be a strong inhibitor of the reaction. Evidence in favor of this hypothesis was given by an experiment where 10 mM KF was added to the system with 2 mM ATP and 0.8 μM repurified phenylalanyl-tRNA synthetase. In this condition the pyrophosphatase activity of the enzyme sample was reduced well below 0.01 s^{-1}. The NMR spectra revealed that AppppA could be initially formed at a rate of 0.08 ± 0.02 s^{-1} with concomitant appearance of PP_i instead of P_i. However, within 60 min the rate of formation of AppppA decreased rapidly, and the reaction stopped when PP_i concentration reached a value on the order of 250 μM. The pyrophosphate resonance appeared as a shoulder on the resonance of the ATP γ-phosphate. Its peak area on the spectrum was compared to that of the phosphates of ATP after addition of 100 mM EDTA to the reaction mixture. This treatment increased by 1 ppm the distance between the PP_i and γ-phosphate resonances. Finally, it was verified that AppppA as well as ADP could not be formed if 1.5 mM PP_i was initially added to a reaction mixture containing 3 mM ATP, 4.6 mM $MgCl_2$, 150 mM KCl, 150 μM $ZnCl_2$, and 2 mM L-phenylalanine but devoid of pyrophosphatase activity.

Table III: Effects of Zinc, L-Phenylalanine, and $tRNA^{Phe}$ on Rates of AppppA and ADP Formation by *E. coli* Phenylalanyl-tRNA Synthetase[a]

	80 μM $ZnCl_2$ and 2 mM phenylalanine	100 μM EDTA and 2 mM phenylalanine	80 μM $ZnCl_2$	80 μM $ZnCl_2$ and 10 mM phenylalaninol	80 μM $ZnCl_2$, 2 mM phenylalanine, and 3 μM $tRNA^{Phe}$
AppppA	0.25 ± 0.07	<0.006	nd[b]	<0.007	0.055 ± 0.010
ADP	0.15 ± 0.05	0.01 ± 0.005	<0.010	<0.010	0.040 ± 0.020

[a] Rates (s^{-1}) were measured at 37 °C from ^{31}P NMR spectra as shown in Figures 1 and 3. The reaction mixture in 20 mM Tris-HCl (pH 7.8) contained 7 mM $MgCl_2$, 150 mM KCl, 2 mM ATP, and yeast pyrophosphatase (0.01 mg/mL) plus $ZnCl_2$, EDTA, amino acid or amino alcohol, and $tRNA^{Phe}$ concentrations as indicated in the table. The enzyme (0.6 μM) was from overproducing strain IBPC 1671 pB_1 and had the lowest contaminating pyrophosphatase activity. Single-exponential kinetics were assumed for computing of initial rates of AppppA and ADP formation (see Table II, footnote *a*). [b] nd, not detected.

Enzymatic Synthesis of AppppA Requires the Presence of Zinc and of L-*Phenylalanine.* A control experiment with 0.1 mM EDTA instead of zinc was performed. The NMR spectra showed that under this condition the rate of AppppA synthesis was reduced by at least 50-fold, compared to that measured under the conditions of Figures 1 and 3. It was verified that this effect did not occur from an indirect effect of EDTA on the activity of the pyrophosphatase present in the reaction mixture. Also, it was shown that the reaction of AppppA synthesis initiated in the presence of $ZnCl_2$ could be stopped later on by the addition of excess EDTA.

In the presence of Zn^{2+} and pyrophosphatase but in the absence of phenylalanine, AppppA formation could not be shown on the NMR spectra, even after 15 h of incubation in the presence of 1 μM enzyme. Formation of ADP, AMP, or P_i was negligible (Table III). AppppA synthesis was also undetectable when L-phenylalaninol instead of L-phenylalanine was added in the reaction mixture. This analogue which couples with ATP binding to the enzyme (Kosakowski & Holler, 1973) lacks the reacting COO^- group and therefore cannot form the adenylate. This result supports the assumption that phenylalanyl adenylate is a prerequisite for the synthesis of AppppA.

Enzymatic Synthesis of AppppA Is Reduced by the Presence of Phenylalanine-Specific tRNA. The rate of AppppA formation sustained by the addition to *E. coli* phenylalanyl-tRNA synthetase of 2 mM L-phenylalanine, 80 μM $ZnCl_2$, and pyrophosphatase was measured in the absence or presence of 3 μM *E. coli* $tRNA^{Phe}$.

Table III shows that the initial rate of AppppA formation decreased 5-fold upon the addition of tRNA. As shown in the preceding paper, the rate of tRNA aminoacylation was markedly decreased by the presence of 80 μM zinc. Nevertheless, the residual aminoacylating activity of the enzyme was enough to complete the acylation of 3 μM $tRNA^{Phe}$ within 10 min under the conditions of the NMR experiment. The effect described here corresponded therefore to that of the aminoacylated $tRNA^{Phe}$.

The Rate of AppppA Synthesis Depends on ATP, Phenylalanine, and $ZnCl_2$ Concentrations. The initial rate of AppppA formation was measured by ^{31}P NMR in the presence of various initial concentrations of ATP. $ZnCl_2$ and L-phenylalanine were held constant at 80 μM and 2 mM, respectively, in this set of experiments. Yeast pyrophosphatase was present in the reaction mixture. As shown in Figure 4, the rate of AppppA formation increased with ATP concentration. An approximate K_m of 2 mM for ATP in the reaction could be determined. At saturating ATP conditions the reaction rate reached 0.5 s^{-1}.

The dependence of the reaction rate on ATP concentration was also followed by [γ-^{32}P]ATP hydrolysis. The obtained rate values of hydrolysis fitted well with those derived from ^{31}P NMR for AppppA formation under identical experimental conditions (Figure 4). This correspondence indicated that the initial rate of ADP formation in the presence of zinc did not contribute significantly per se to the initial rate of the overall loss of ^{32}P from [γ-^{32}P]ATP.

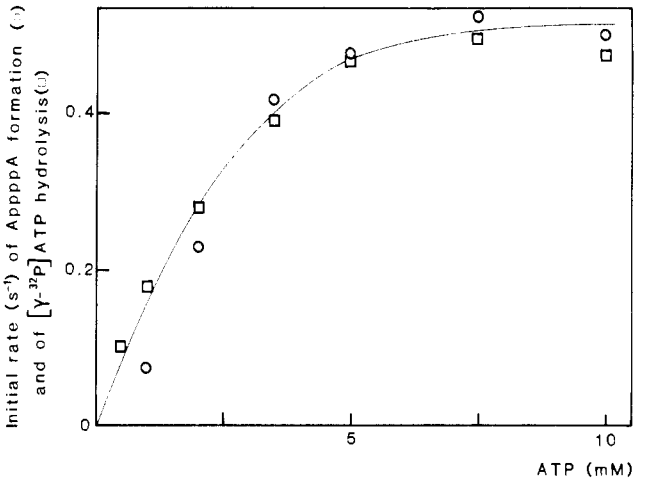

FIGURE 4: Dependence on ATP concentration of the initial rate of AppppA formation (○) and of the initial rate of [γ-^{32}P]ATP hydrolysis (□). Initial rates of AppppA synthesis were followed by ^{31}P NMR measurements at 37 °C in 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM free $MgCl_2$, 2 mM L-phenylalanine, 80 μM $ZnCl_2$, and 0.01 mg/mL yeast pyrophosphatase. ATP-Mg^{2+} was varied in the sample in the presence of 0.6 μM *E. coli* phenylalanyl-tRNA synthetase. The β-phosphate resonance of AppppA was used for calculating the concentrations. Initial rates of [γ-^{32}P]ATP hydrolysis were measured under the same buffer conditions without D_2O. [γ-^{32}P]ATP (1–10 Ci/mol) complexed with $MgCl_2$ was varied in the assay in the presence of 0.6 μM *E. coli* phenylalanyl-tRNA synthetase.

In view of the slight sigmoidal nature of the kinetics of ADP formation (Figure 3), it could be imagined that ADP originated from AppppA decomposition rather than direclty from ATP. This possibility was sustained by the observation by ^{31}P NMR that, provided both Zn^{2+} and phenylalanine were present, phenylalanyl-tRNA synthetase itself triggered the conversion of 2 mM pure AppppA into ADP at a rate of 0.1 s^{-1} (20 mM Tris-HCl, pH 7.8 (37 °C), 150 mM KCl, 7 mM $MgCl_2$, 5 μM EDTA, 200 μM $ZnCl_2$, 2 mM phenylalanine, 2 mM AppppA, 1.5 μM phenylalanyl-tRNA synthetase, and 0.01 mg/mL yeast pyrophosphatase). This rate value was close to that measured in Figure 3 for ADP synthesis when the AppppA concentration reached 2 mM. With 0.5 mM pure AppppA instead of 2 mM, the rate of ADP appearance decreased to 0.05 s^{-1}. This reduced rate probably reflected a smaller degree of saturation of the enzyme by AppppA in the reaction of hydrolysis. In the presence of $ZnCl_2$, L-phenylalanine, and yeast pyrophosphatase but in the absence of phenylalanyl-tRNA synthetase, the AppppA sample remained intact for at least 15 h.

Further evidence for the formation of ADP at the expense of AppppA was provided by thin-layer chromatography on

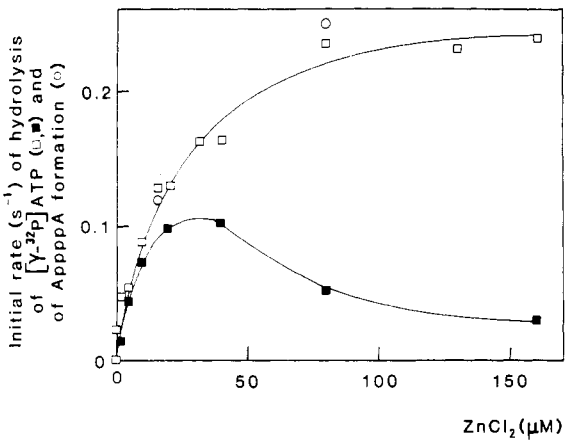

FIGURE 5: Dependence on $ZnCl_2$ concentration of the initial rate of [γ-^{32}P]ATP hydrolysis. Initial rates were followed at 37 °C in 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM free $MgCl_2$, 2 mM ATP-Mg^{2+} (5 Ci/mol), 0.01 mg/mL yeast pyrophosphatase, and 2 mM (□) or 30 μM (■) L-phenylalanine. $ZnCl_2$ was varied in the assay in the presence of 0.6 μM *E. coli* phenylalanyl-tRNA synthetase. For comparison, initial rates of AppppA formation (○) under the same experimental conditions with 25% D_2O are also shown on the figure.

PEI–cellulose. The reaction as described under Materials and Methods was monitored with [γ-^{32}P]ATP (0.12 Ci/mol). Autoradiography of the sheet clearly showed ^{32}P radioactivity incorporated into ADP. This observation excludes the possibility that ADP has been formed solely through hydrolysis of the γ-phosphate labeled ATP.

The conversion of AppppA into ADP required phenylalanyl-tRNA synthetase (AppppA remained intact in the presence of pyrophosphatase alone). As shown in Table II, the ratio of the rates of AppppA and ADP synthesis did not depend markedly on the degree of purification of the used enzyme. It is therefore unlikely that a contaminant enzyme rather than phenylalanyl-tRNA synthetase was responsible for the appearance of ADP.

When reactions were started from pure AppppA under the conditions given above, formations of ApppA, P_i, and AMP were also revealed by the ^{31}P NMR spectra. The appearance of ApppA from AppppA clearly supported the possibility of the formation of phenylalanyl adenylate from AppppA and phenylalanine, the adenylate being further allowed to react with ADP and to form ApppA. AMP began to accumulate in the reaction mixture after the formation of ApppA. It is possible that this nucleotide appeared at the expense of ApppA in a manner similar to that of the formation of ADP from AppppA or that it resulted from the slow hydrolysis of enzyme-bound aminoacyl adenylate.

The dependence of the synthesis of AppppA on the $ZnCl_2$ concentration was followed by [γ-^{32}P]ATP hydrolysis (Figure 5) in the presence of 2 mM ATP. At constant 2 mM amino acid, the rate of hydrolysis increased from 0 to nearly 0.24 s^{-1} upon raising the Zn^{2+} concentration in the reaction mixture. Another experiment was performed in the presence of 30 μM L-phenylalanine, in order to compare the reaction of AppppA formation and the inhibition by Zn^{2+} of tRNA aminoacylation as described in the accompanying paper (Mayaux & Blanquet, 1981). A maximal rate of 0.11 s^{-1} was reached at a $ZnCl_2$ concentration on the order of 20–40 μM. The half-maximal rate corresponded to 6 μM $ZnCl_2$. This concentration of $ZnCl_2$ was in close agreement with that ensuring 50% inhibition of the $tRNA^{Phe}$ aminoacylation reaction followed at 37 °C under identical buffer conditions. It could be noted that $ZnCl_2$ concentrations larger than 40 μM inhibited the rate of hydrolysis. This behavior was not observed in the experiment with 2 mM L-phenylalanine. A possible explanation for this effect was that excess of $ZnCl_2$ increased the K_m of phenylalanine in the reaction of [γ-^{32}P]ATP hydrolysis and therefore decreased the saturation rate of the enzyme at the limiting amino acid concentration. Such an hypothesis was supported by the observation that $ZnCl_2$ increased the K_m of the amino acid in the L-phenylalanine-dependent isotopic ATP–PP_i exchange reaction catalyzed by phenylalanyl-tRNA synthetase (results not shown).

Yeast Phenylalanyl-tRNA Synthetase Also Catalyzes the Synthesis of AppppA in the Presence of Zinc. Three other homogeneous *E. coli* aminoacyl-tRNA synthetases, tyrosyl-, methionyl-, and isoleucyl-tRNA synthetases, were assayed for AppppA production in the presence of $ZnCl_2$. Enzymes (1–3 μM) were mixed with 1–2 mM amino acid, 2–5 mM ATP, 80 μM $ZnCl_2$, and 0.01 mg/mL yeast pyrophosphatase in 20 mM Tris-HCl (pH 7.8), 7–10 mM $MgCl_2$, and 150 mM KCl. Small amounts of AppppA could be distinguished on the NMR spectrum after 12 h of incubation, corresponding to rates of synthesis of 7×10^{-4}, 1.5×10^{-3}, and 4×10^{-3} s^{-1} for tyrosyl-, methionyl-, and isoleucyl-tRNA synthetases, respectively. These rates were 2–3 orders of magnitude lower than that measured with *E. coli* phenylalanyl-tRNA synthetase under identical conditions. It was verified that the activities of tyrosyl-, isoleucyl-, and methionyl-tRNA synhtetases had remained at least 50% intact at the end of the incubation.

The different behavior of these three enzymes with respect to that of phenylalanyl-tRNA synthetase could be related to the insensitivity to zinc of their tRNA aminoacylation reaction, as described in the preceding paper. For this reason, yeast phenylalanyl-tRNA synthetase, another enzyme the activity of which has been found sensitive to the addition of zinc, was investigated for the possible synthesis of AppppA.

Due likely to the low turnover rate of yeast pyrophosphatase [$<10^3$ s^{-1} from Moe & Butler (1972)] compared to that of *E. coli* enzyme, our sample of yeast phenylalanyl-tRNA synthetase was devoid of detectable interfering pyrophosphatase activity. In the absence of added pyrophosphatase, zinc triggered the production of AMP and PP_i by yeast phenylalanyl-tRNA synthetase at the expense of ATP. The hydrolysis was phenylalanine dependent (Table IV). These data confirmed a recent observation made by Igloi et al. (1980) that yeast phenylalanyl-tRNA synthetase catalyzed a Zn^{2+}-induced, tRNA-independent hydrolysis of ATP.

However, when yeast pyrophosphatase was added to the reaction mixture, a considerable production of AppppA was revealed on the ^{31}P NMR spectrum (Figure 6). The synthesis occurred at a rate of 2.1 ± 0.1 s^{-1} and was accompanied by that of ADP and AMP at similar rates. It was verified that, in the presence of pyrophosphatase, the formation of these nucleotides strictly required the presence of both $ZnCl_2$ and L-phenylalanine (Table IV).

Discussion

This study clearly indicates the importance of zinc in promoting the synthesis of diadenosine tetraphosphate by phenylalanyl-tRNA synthetase. The various data enable us to confirm that this synthesis does proceed from a back reaction of the phenylalanyl adenylate formation. Provided $ZnCl_2$ is present and pyrophosphate is prevented from reacting with the adenylate by the action of pyrophosphatase, the γ- and β-phosphates of ATP mimic the pyrophosphate and reverse the adenylate to form diadenosine tetraphosphate and phenylalanine. As indicated by the experiment with pure AppppA, this reaction is reversible and AppppA may react with phenylalanine to give the normal adenylate plus ATP. However,

Table IV: Rates of AppppA, ADP, and AMP Formation by Yeast Phenylalanyl-tRNA Synthetase[a]

	80 μM $ZnCl_2$ and 2 mM phenylalanine	80 μM $ZnCl_2$, 2 mM phenylalanine, and 0.01 mg/mL pyrophosphatase	100 μM EDTA, 2.mM phenylalanine, and 0.01 mg/mL pyrophosphatase	80 μM $ZnCl_2$ and 0.01 mg/mL pyrophosphatase
AppppA	nd[b]	2.1 ± 0.1	0.06	nd
ADP	nd	1.4 ± 0.1	0.10	nd
AMP	1.5 ± 0.2	3.2 ± 0.2	nd	nd

[a] Rates (s^{-1}) were measured at 37 °C from ^{31}P NMR spectra as shown in Figure 6. The reaction mixture in 20 mM Tris-HCl (pH 7.8) contained 7 mM $MgCl_2$, 150 mM KCl, and 5 mM ATP plus $ZnCl_2$, EDTA, amino acid, and yeast pyrophosphatase as indicated in the table. The enzyme (0.1–0.7 μM) was freed from detectable contaminating pyrophosphatase activity. Single-exponential kinetics were assumed for computing of initial rates of AppppA, ADP, and AMP formation (see Table II, footnote *a*). [b] nd, not detected.

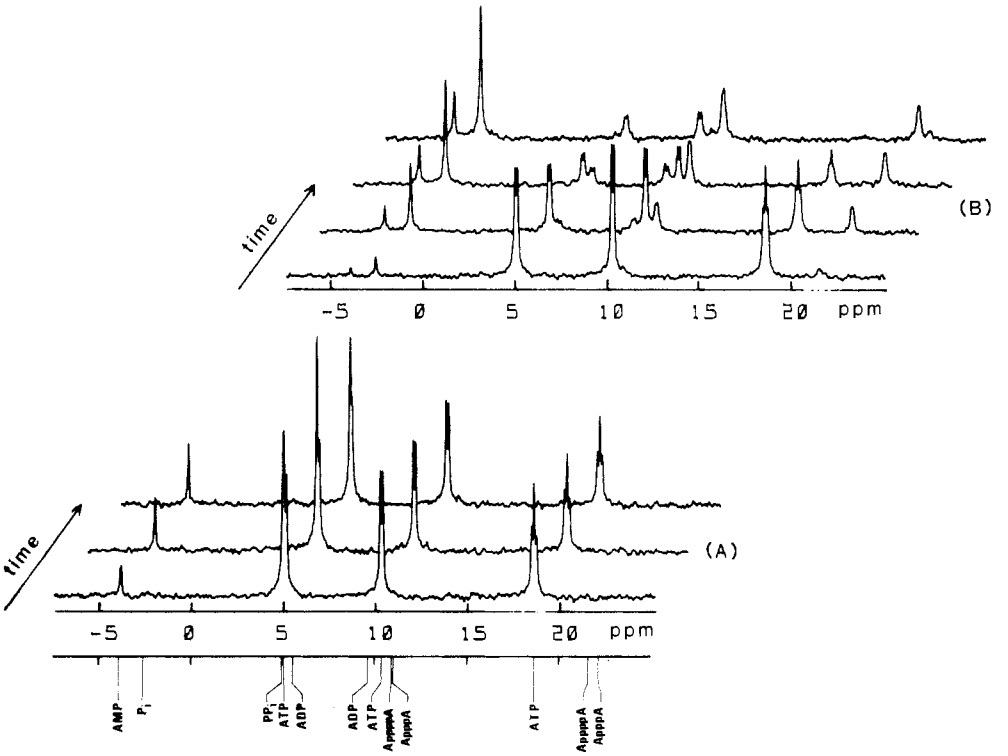

FIGURE 6: Evidence by ^{31}P NMR spectroscopy for synthesis of AppppA by yeast phenylalanyl-tRNA synthetase. The sample buffered with 20 mM Tris-HCl, pH 7.8, 150 mM KCl, and 10 mM $MgCl_2$ including 25% D_2O contained 5 mM ATP, 2 mM L-phenylalanine, and 80 μM $ZnCl_2$ in the absence (A) or presence (B) of 0.01 mg/mL yeast pyrophosphatase. The reaction (37 °C) was initiated by the addition of yeast phenylalanyl-tRNA synthetase at a final concentration of 0.73 (A) or 0.092 (B) μM. Each spectrum is the average of 600 scans: repetition time, 2 s; line broadening, 7.5 Hz. In panel A, the spectra correspond to times 3–23, 23–43, and 43–63 min of the kinetics. In panel B, the spectra correspond to times 3–23, 43–63, 83–103, and 123–143 min of the kinetics.

this pathway is paralleled by a diadenosine tetraphosphatase activity assignable to phenylalanyl-tRNA synthetase. This activity requires the presence of both $ZnCl_2$ and phenylalanine and transforms AppppA into ADP.

The formation of ADP from diadenosine tetraphosphate accounts for a sigmoidal nature of its kinetics of synthesis when starting from ATP. The rate of ADP formation increases with the increasing AppppA concentration in the assay medium, i.e., with the degree of saturation of the enzyme by AppppA. ADP synthesis also accounts for the appearance of ApppA during the incubation of phenylalanyl-tRNA synthetase with amino acid, ATP, $ZnCl_2$, and pyrophosphatase. The 5′-phosphoryl end of ADP is also able to mimic pyrophosphate and to form ApppA from adenylate. The efficiency of this synthesis compared with that of AppppA increases when the concentration of free ATP is reduced. This scheme is supported by the observation that diadenosine triphosphate can also be formed from homogeneous AppppA incubated with phenylalanyl-tRNA synthetase, amino acid, and $ZnCl_2$. In this case, it is clear that an adenylate is required for ApppA formation and that this adenylate can only be formed at the expense of diadenosine tetraphosphate.

It can be noted that the reversible reaction of AppppA formation may possibly account for a "direct interchange reaction" resembling that recently reported by Rossomando et al. (1979) and Smith & Cohn (1981), in which the β- and γ-phosphates of ATP are interchanged independently of the ATP–PP_i exchange reaction.

E. coli and yeast phenylalanyl-tRNA synthetases are changed into a diadenosine tetraphosphate synthetase in the presence of $ZnCl_2$, amino acid, and pyrophosphatase. This new activity has to be related with the unique effect of $ZnCl_2$ on the tRNA aminoacylation reaction catalyzed by these two enzymes. As shown in the preceding paper, among the various studied aminoacyl-tRNA synthetases, only *E. coli* and yeast phenylalanyl-tRNA synthetase activities were found to be sensitive to the addition of micromolar concentrations of $ZnCl_2$. We demonstrated that the inhibitory effect of zinc on the $tRNA^{Phe}$ aminoacylation activity of *E. coli* phenylalanyl-tRNA synthetase was assignable to a reversible binding of zinc to the enzyme.

The main difference in this study between the two phenylalanyl-tRNA synthetases resides in their effect on ATP in the presence of $ZnCl_2$ but in the absence of pyrophosphatase.

While ATP remains intact in the presence of the *E. coli* enzyme, it is rapidly hydrolyzed into AMP and PP_i by the yeast enzyme. This zinc-dependent hydrolytic activity of yeast phenylalanyl-tRNA synthetase has already been noted by Igloi et al. (1980). These authors showed that it required the presence of both phenylalanine and $ZnCl_2$. Under our experimental conditions, hydrolysis of ATP occurs at a rate of 1.5 s^{-1}. On the other hand, in the presence of both $ZnCl_2$ and pyrophosphatase the yeast enzyme becomes a diadenosine tetraphosphate synthetase, as happens with the *E. coli* enzyme.

The importance of this new activity of phenylalanyl-tRNA synthetase is underlined by the magnitude of the observed rates of diadenosine tetraphosphate synthesis in the presence of $ZnCl_2$ and pyrophosphatase. Maximal rates of 0.5 and 2.1 s^{-1} have been measured in the case of the *E. coli* and of the yeast enzyme, respectively. These rates can be compared with the rates of $tRNA^{Phe}$ aminoacylation measured at 25 °C in the absence of added $ZnCl_2$ [see Mayaux & Blanquet (1981)]. These rates are equal to 3 and 5 s^{-1} for *E. coli* and yeast phenylalanyl-tRNA synthetases, respectively.

Diadenosine tetraphosphate has been found in a variety of mammalian cell types as well as in *E. coli* [Rapaport & Zamecnik (1976) and references cited therein]. The question of knowing whether the reactions described above can be responsible for the in vivo synthesis of AppppA arises.

Undoubtedly, there is enough pyrophosphatase activity within the cytoplasm of *E. coli* to hydrolyze pyrophosphate (Josse & Wong, 1971). The absence of free pyrophosphate in *E. coli* living cells has in fact been demonstrated by ^{31}P NMR studies (Navon et al., 1977). Moreover it has been shown that Zn^{2+} could be concentrated in the *E. coli* cell from very dilute solutions. This uptake strongly depends on the energy supply through fermentation or, better, respiration (Bucheder & Broda, 1974). A similar energy-dependent uptake of Co^{2+} by *E. coli* cells has been reported by Nelson & Kennedy (1972).

In the case of yeast, a resonance at the position of pyrophosphate could be distinguished on the ^{31}P NMR spectrum of living cells; however, some ambiguity remains as to the origin of this resonance due to the fact that the peak of the terminal phosphate groups of the polyphosphates is undistinguishable from those of the pyrophosphate (Salhany et al., 1975; Navon et al., 1979). On the other hand, the total concentration of Zn^{2+} in the cytosol of eukaryotic cells has been estimated to be ~0.1 mM (Thiers & Vallee, 1957). Considering all these data, it is likely that suitable Zn^{2+} and pyrophosphatase conditions may exist in prokaryotic and eukaryotic cytoplasms, enabling phenylalanyl-tRNA synthetase to synthesize AppppA and ApppA.

It is striking that a correlation should exist between the growth properties of cells and the level of AppppA found in vivo (Rapaport & Zamecnik, 1976). Human hepatoma cells grown in nude mice contained 0.8–1.2 μM AppppA, whereas normal mouse livers contained much lower levels of AppppA, 0.03–0.06 μM. In the case of cells in culture, it was shown that conditions which inhibit growth reduced the concentration of diadenosine tetraphosphate. It is tempting to establish a parallel between AppppA which reflects the degree of cellular proliferation and Zn^{2+} which is actively concentrated within *E. coli* cells in the presence of substrate for energy metabolism. Also, consistent with a functional role for zinc in the growth of cells, there is the observation by Rose et al. (1978) of a preferential uptake of Zn^{2+} into hepatoma nuclei relative to host liver cells. This uptake has been related to the activation by Zn^{2+} of nuclear poly(A) polymerase, an enzyme involved in the processing of newly transcribed mRNA (Rose et al., 1977a,b). In fact, many studies have indicated that zinc may play a role in tumor growth (Addink & Frank, 1959; De Wys et al., 1970; McQuitty et al., 1970; Andronikashvili et al., 1973).

Beyond a possible role of diadenosine tetraphosphate in controlling growth, another interesting possibility resides in the fact that the synthetase can form ApppA as well as AppppA and that the synthesis of one or another of these two compounds depends on the ATP to ADP ratio in the assay. Therefore, the AppppA to ApppA ratio itself reflects the energy balance of the cell. Also these nucleotides could serve as a source of purine and phosphate bond energy during development. This raises the question of an ATP/ADP level in cells being regulated via aminoacyl-tRNA synthetase activity, for instance, through the binding of AppppA to adenylate kinase (Purich & Fromm, 1972; Gupta & Yushok, 1980).

At this stage it is surprising that, among the aminoacyl-tRNA synthetases examined here, phenylalanyl-tRNA synthetase is the only one to massively synthesize AppppA and ApppA and that this synthesis requires Zn^{2+} as a cofactor. This statement must remain tentative until other aminoacyl-tRNA synthetases have been examined for the sensitivity to zinc of their tRNA aminoacylation activity and for their ability to acquire the diadenosine tetraphosphate synthetase activity. The case of *E. coli* lysyl-tRNA synthetase is an example of this. Zamecnik et al. (1966) first reported a biosynthesis of AppppA which was dependent on the presence of a purified (but not homogeneous) preparation of *E. coli* lysyl-tRNA synthetase. This synthesis required ATP, L-lysine, and Mg^{2+}. The compound AppppA was formed maximally when pyrophosphatase was added. Also, its synthesis was stimulated by the addition of unfractionated *E. coli* tRNA in a final concentration of 2 mg/mL. In our study the presence of aminoacylated $tRNA^{Phe}$ inhibits the synthesis of AppppA by *E. coli* phenylalanyl-tRNA synthetase. This is likely to reflect the esterified amino acid competing with free phenylalanine for the formation of the adenylate. Also, an indirect but specific effect of the binding of tRNA on the ability for the enzyme to synthesize adenylate and AppppA cannot be excluded (Santi et al., 1971; Jacques & Blanquet, 1977).

In the accompanying paper, samples of unfractionated *E. coli* tRNA have been shown to contain nearly 10^{-8} g-atom of contaminating zinc/mg of tRNA. This suggested the possibility that the role of Zn^{2+} in promoting the synthesis of AppppA by lysyl-tRNA synthetase might have been overlooked.

Preliminary experiments in our laboratory on samples of partially purified *E. coli* lysyl-tRNA synthetase in 20 mM Tris-HCl, pH 7.8 (37 °C), containing 150 mM KCl and 5 mM free $MgCl_2$, confirm the massive production of AppppA in the presence of 100 μM $ZnCl_2$ and pyrophosphatase. This synthesis is actually dependent on the addition of L-lysine. A maximum stimulation is reached with amino acid concentrations lower than 100 μM. However, the diadenosine tetraphosphate synthetase activity of lysyl-tRNA synthetase can be blocked by the omission of $ZnCl_2$ and the addition of 100 μM EDTA.

These experiments argue strongly in favor of a behavior of lysyl-tRNA synthetase similar to that of phenylalanine-tRNA synthetase. Nevertheless, it remains difficult to explain why these synthetases, but not the isoleucyl-, tyrosyl-, or methionyl-tRNA synthetases, can adopt this unusual activity in the presence of the effector Zn^{2+}. It is worthwhile to mention that the unusual reactivity of enzyme-bound aminoacyl adenylate

can theoretically lead to a variety of complex nucleotides. The only requirement for reaction is a free 5′-diphosphoryl end, including the 5′ end of an RNA. Such a general mechanism has already been stated in Rapaport et al. (1975). These authors have reported that A(5′)ppp(5′)Gpp could be formed from ppGpp in the back reaction of the lysine activation process. Also ApppG and AppppG were prepared from GDP and GTP. Apppp can be formed from tripolyphosphate (Zamecnik & Stephenson, 1968). It is likely that diadenosine pentaphosphate, a very strong inhibitor of adenylate kinase (Feldhaus et al., 1975), could be formed from adenosine 5′-tetraphosphate. The in vivo occurrence and possible role of these highly phosphorylated nucleotides are reviewed by Silverman & Atherly (1979).

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