Zinc-Dependent Synthesis of Various Dinucleoside 5′,5‴-P^1,P^3-Tri- or 5′,5‴-P^1,P^4-Tetraphosphates by *Escherichia coli* Lysyl-tRNA Synthetase†

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ABSTRACT: Upon addition of $ZnCl_2$, the $tRNA^{Lys}$ aminoacylation activity of *Escherichia coli* lysyl-tRNA synthetase is progressively inhibited (50% inhibition at 60 μM $ZnCl_2$). This inhibition is paralleled by the stimulation of a diadenosine 5′,5‴-P^1,P^4-tetraphosphate synthetase activity. This activity could be followed by ^{31}P or ^1H nuclear magnetic resonance spectroscopy. The rate of diadenosine tetraphosphate synthesis is enhanced from 0.02 to 4.2 s^{-1} (37 °C) upon the addition of 150 μM $ZnCl_2$ to a reaction mixture containing 20 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.8), 150 mM KCl, 5 mM ATP, 10 mM $MgCl_2$, 2 mM L-lysine, catalytic amounts of lysyl-tRNA synthetase, and unlimiting pyrophosphatase activity. Of all of the other divalent metals assayed at 150 μM, only cadmium and, to a lesser extent, manganese are able to sustain a noticeable production of diadenosine tetraphosphate (0.7 and 0.2 s^{-1}, respectively, in the same experimental conditions as above). The effect of 150 μM zinc on the rates of synthesis of mixed dinucleoside 5′,5‴-P^1,P^3-tri- or 5′,5‴-P^1,P^4-tetraphosphates (ApppX or AppppX) resulting from the reaction of lysyl adenylate with a variety of ribo- (or deoxyribo-) nucleotides 5′-tri- (or 5′-di-) phosphates was also examined. In each case, the presence of zinc markedly stimulated the synthesis of dinucleoside tri- or tetraphosphates. The stimulation was larger in the case of the reaction of an XTP to form AppppX (50–180-fold) than in the case of the reaction of an XDP to form ApppX (3–13-fold). The presence of a deoxyribose instead of a ribose had no effect on the reaction rate. Zinc did not stimulate the production of A(5′)ppp(5′)G(3′)pp from pp(5′)G(3′)pp. Nevertheless, in the absence of zinc, ApppGpp appears faster (0.045 s^{-1}) than AppppA (0.019 s^{-1}). The possible in vivo occurrence of mixed dinucleoside 5′,5‴-P^1,P^3-tri- or 5′,5‴-P^1,P^4-tetraphosphates is discussed in the light of the rate values determined in this study.

Unusual dinucleoside 5′,5‴-P^1,P^4-tetraphosphates such as diguanosine tetraphosphate (GppppG) or diadenosine tetraphosphate (AppppA) have been shown to occur in a variety of tissues or cells [reviewed by Silverman & Atherly (1979)]. In the case of AppppA, a relation has been established between the cellular concentration of this nucleotide and the rate of cell proliferation (Rapaport & Zamecnik, 1976; Ogilvie, 1981; C. Weinmann, A. Hedl, I. Grummt, F. J. Ferdinand, R. R. Friis, K. Geider, G. Pierron, A. Schimpl, and F. Grummt, unpublished results). In the latter study, it was observed that, upon mitogenic stimulation of G1-arrested mammalian cells, the cellular AppppA increased by 1000-fold during progression through the G1 phase. On the basis of such evidence and on the observation by Grummt (1978) that AppppA triggered initiation of in vitro DNA replication in baby hamster kidney cells, it was argued that AppppA could be a second messenger of cell cycle and proliferation control.

The AppppA molecule was first observed by Zamecnik et al. (1966), who showed that it could be synthesized in vitro from the back reaction of an aminoacyl-tRNA synthetase: lysyl-tRNA synthetase. Evidence was obtained that this nucleotide resulted from the reaction of lysyl adenylate with ATP instead of PP_i[1] (Zamecnik et al., 1966; Randerath et al., 1966). On the other hand, a molecular signal that regulates AppppA synthesis by an aminoacyl-tRNA synthetase has been very recently described (Mayaux & Blanquet, 1981; Plateau et al., 1981a,b). It was shown that the capability of *Escherichia coli* or yeast phenylalanyl-tRNA synthetase to convert ATP into AppppA depended in fact on the addition of zinc to the reaction mixture. The stimulation by the metal of the new AppppA-synthetase activity was paralleled by full inhibition of the normal tRNA aminoacylation activity.

As evidenced in Zamecnik et al. (1967), Zamecnik & Stephenson (1968), and Rapaport et al. (1975), an enzyme-bound aminoacyl adenylate can theoretically react with any nucleotide, provided that this nucleotide possesses a free 5′-diphosphoryl end. In particular, these authors have observed that AppppG or ApppGpp could be obtained from the reaction of 5′-GTP or pp(5′)G(3′)pp with lysyl adenylate.

In this study, we explored the effect of zinc on the rates of synthesis of mixed dinucleoside tri- or tetraphosphates by *E. coli* lysyl-tRNA synthetase. The reaction of lysyl adenylate with a variety of ribo- (or deoxyribo-) nucleotides 5′-tri- (or 5′-di-) phosphates was followed by the NMR techniques already described in Plateau et al. (1981a,b). In each case, the presence of zinc markedly stimulated the synthesis of dinucleoside tri- or tetraphosphates by the aminoacyl-tRNA synthetase. On the other hand, zinc had no effect on the rate of conversion of pp(5′)G(3′)pp into A(5′)ppp(5′)G(3′)pp.

Materials and Methods

Homogeneous lysyl-tRNA synthetase from *E. coli* strain EM 20031 carrying the F32 episome was purified according to Fromant et al. (1981) and Plateau et al. (1981b). The enzyme was stored in 50% glycerol at −15 °C. Yeast inorganic pyrophosphatase (sp act. 200 units/mg at 25 °C) and unfractionated tRNA (40 pmol of L-lysine acceptance/A_{260} unit of tRNA) were obtained from Boehringer (Mannheim). The various 5′ nucleotides were also purchased from Boehringer,

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[1] Abbreviations: PP_i, inorganic pyrophosphate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate.

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Table I: Effects of Zinc, L-Lysine, and tRNA on the Rates of AppppA and ADP Formation by *E. coli* Lysyl-tRNA Synthetase[a]

	130 μM $ZnCl_2$ and 2 mM lysine	10 μM EDTA and 2 mM lysine	130 μM $ZnCl_2$	10 μM EDTA	130 μM $ZnCl_2$, 2 mM lysine, and 150 μM tRNA	10 μM EDTA, 2 mM lysine, and 150 μM tRNA
AppppA	4.1	0.020	<0.015	<0.003	4.3	0.021
ADP	0.060	0.011	<0.015	0.009	0.150	0.070

[a] Rates (s^{-1}) were measured at 37 °C from ^{31}P NMR spectra (Plateau et al., 1981a,b). The reaction mixture in 20 mM Tris-HCl, pH 7.8, contained 150 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 25% D_2O, and 0.01 mg/mL yeast pyrophosphatase, plus $ZnCl_2$, EDTA, amino acid, and unfractionated tRNA concentrations as indicated in the table. The aminoacyl-tRNA synthetase concentration ranged from 0.05 to 1 μM, depending on the rates to be measured. Note that when incubated in the presence of zinc (plus ATP) but in the absence of amino acid, lysyl-tRNA synthetase lost 50% of its initial $^{32}PP_i$-ATP isotopic exchange activity in 3 h. In the presence of both amino acid and zinc (plus ATP), the enzyme remained fully active over at least 15 h.

except dTDP and dGDP (from Sigma) and pp(5′)G(3′)pp (from P-L Biochemicals). The purity of ppGpp, as assessed by ^{31}P NMR analysis, was greater than 95%. D_2O was from the Commissariat à l'Energie Atomique (Saclay, France).

tRNA Aminoacylation. Initial rates of $tRNA^{Lys}$ aminoacylation were measured at 37 °C in 20 mM Tris-HCl buffer (pH 7.8) containing 5 mM ATP, 30 μM ^{14}C-labeled L-lysine (50 Ci/mol from the Commissariat à l'Energie Atomique, Saclay, France), and 150 μM unfractionated tRNA, plus 10 mM $MgCl_2$, 150 mM KCl, and the various metals. The final volume of the reactants was 0.1 mL. Initial velocities were measured with a reaction time of 10 min and an enzyme concentration of 0.7 nM. Prior to the reaction, the stock solution of lysyl-tRNA synthetase was diluted appropriately (greater than 1000-fold) in Tris-HCl buffer, pH 7.8, containing 200 μg/mL bovine serum albumin (Serva). The final concentration of albumin in the assay never exceeded 50 μg/mL. Aminoacylated $tRNA^{Lys}$ was precipitated with 5% trichloroacetic acid containing 2 mM DL-lysine and measured according to Lawrence et al. (1973).

^{31}P and ^{1}H NMR Measurements. NMR spectra were measured and assigned according to Plateau et al. (1981a,b) on a home-built 276-MHz (proton) FT spectrometer (Caron et al., 1980), operating in the block mode. For phosphorus NMR, each block, unless specified otherwise, lasted 60 min, during which 720 free precessions were accumulated. Corresponding values for proton NMR are 5 min and 38 free precessions.

Dependences of the initial rate of AppppA formation on ATP or on $ZnCl_2$ concentration were followed by ^{1}H NMR at 37 °C in 20 mM Tris-HCl (pH 7.8), 150 mM KCl, 2 mM L-lysine, 20% D_2O, 0.05 mg/mL yeast pyrophosphatase, and 400 nM lysyl-tRNA synthetase. In addition, the sample contained 5 mM free $MgCl_2$, 150 μM $ZnCl_2$, and variable amounts of ATP–Mg^{2+} or 5 mM free $MgCl_2$, 5 mM ATP–Mg^{2+}, and variable amounts of $ZnCl_2$, respectively.

Enzymatic Cleavage of the γ-Phosphate of ATP. Initial rates of hydrolysis of [γ-^{32}P]ATP were measured at 37 °C. The assay mixture contained 20 mM Tris-HCl (pH 7.8), 150 mM KCl, 10 mM $MgCl_2$, 2 mM L-lysine, 5 mM [γ-^{32}P]ATP (5 Ci/mol, from the Radiochemical Centre, Amersham, U.K.), 400 nM *E. coli* lysyl-tRNA synthetase, 0.05 mg/mL yeast pyrophosphatase, and 150 μM amounts of the various studied metals. The reaction was initiated by the addition of L-lysine. Control experiments were performed in parallel without the amino acid or without the enzyme. From time zero, 20-μL aliquots of the reaction assay were periodically withdrawn and analyzed as in Plateau et al. (1981a).

Results

Zinc Reduces Rate of tRNA Aminoacylation by E. coli Lysyl-tRNA Synthetase. The rate of $tRNA^{Lys}$ aminoacylation decreased sharply upon the addition of zinc to the assay mixture (Figure 1). An inhibition of 50% was obtained in the presence of 60 μM $ZnCl_2$.

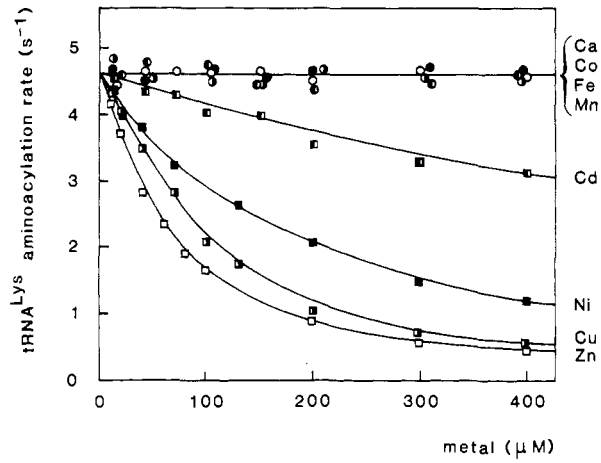

FIGURE 1: Effect of the addition of various metals on tRNA aminoacylation activity of *E. coli* lysyl-tRNA synthetase. Initial rates of aminoacylation by L-[^{14}C]lysine of 150 μM unfractionated tRNA were measured in the presence of various concentrations of the following metal salts: $ZnCl_2$ (□), $CdCl_2$ (left-filled box), $CuSO_4$ (right-filled box), $NiCl_2$ (■), $CoCl_2$ (○), $CaCl_2$ (◐), $MnCl_2$ (◑), and $FeCl_2$ (●). Initial velocities were measured using a reaction time of 10 minutes and an enzyme concentration of 0.7 nM.

The effect of other metal ions was also studied in the lysyl-tRNA synthetase dependent tRNA aminoacylation assay. Figure 1 shows that Zn(II) was the strongest inhibitor. Weaker inhibitions were obtained with Cu(II), Ni(II), and Cd(II), while Ca(II), Co(II), Fe(II), and Mn(II) had no measurable effect in the range of concentrations studied (<400 μM).

Zinc Stimulates Synthesis of AppppA by E. coli Lysyl-tRNA Synthetase. The conversion of ATP into highly phosphorylated AppppA can be followed by ^{31}P or ^{1}H NMR spectroscopy (Plateau et al., 1981a,b). The kinetics of formation of AppppA was measured at 37 °C in 20 mM Tris-HCl (pH 7.8), 150 mM KCl, 10 mM $MgCl_2$, and 25% D_2O containing 5 mM ATP, 2 mM L-lysine, 130 μM $ZnCl_2$, catalytic amounts of *E. coli* lysyl-tRNA synthetase, and unlimiting yeast pyrophosphatase (0.01 mg/mL). Under these conditions, (56 nM enzyme, 15 h) the initial ATP (5 mM) was converted into 2.1 mM AppppA with the concomitant appearance of 4.9 mM P_i (Figure 2). Minor amounts of ADP and ApppA (0.3 and 0.1 mM, respectively) were observed in the ^{31}P NMR spectrum after the same period of incubation. AMP could not be distinguished in the spectra (<0.05 mM). Identical initial rates of AppppA appearance were measured (4.10 ± 0.25 s^{-1}) from ^{31}P or ^{1}H NMR spectra.

A control experiment with 10 μM EDTA instead of zinc

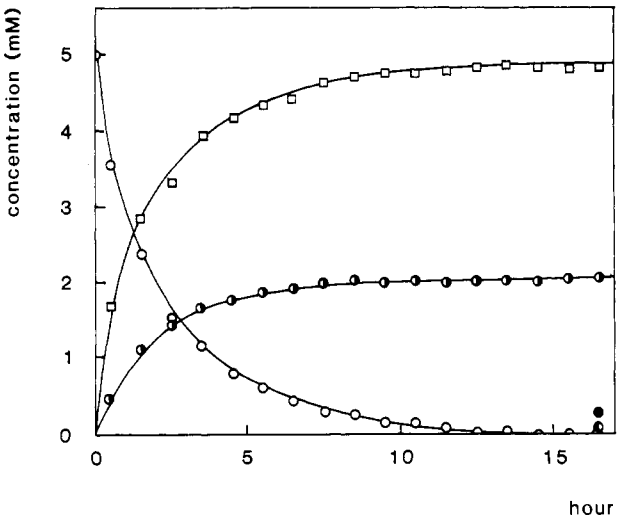

FIGURE 2: Kinetics of ATP consumption and of AppppA and P_i formation by *E. coli* lysyl-tRNA synthetase. Concentrations of ATP (○), AppppA (◑), and P_i (□) were calculated from a ^{31}P NMR experiment performed at 37 °C in 20 mM Tris-HCl, pH 7.8, containing 150 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 2 mM L-lysine, 130 μM $ZnCl_2$, 25% D_2O, 56 nM lysyl-tRNA synthetase, and 0.01 mg/mL yeast pyrophosphate. Sample volume was 1.5 mL. The production of ADP (●) and ApppA (◐) was negligible.

was performed. The initial rate of AppppA synthesis was reduced from 4.1 to 0.02 s^{-1} (Table I).

In the presence of Zn(II) and pyrophosphatase but in the absence of L-lysine, AppppA was undetectable (Table I). Also, in the presence of 0.2 mM of all of the L-amino acids, except L-lysine, AppppA was not formed.

In the presence of Zn(II) and L-lysine but in the absence of pyrophosphatase (plus 100 mM KF), AppppA could be initially formed at a rate greater than 0.7 s^{-1} with the concomitant appearance of PP_i instead of P_i. However, the reaction stopped when the PP_i concentration in the sample reached 450 μM.

Finally, the rate of AppppA formation in the presence of L-lysine, $ZnCl_2$, and pyrophosphatase was measured in the absence or presence of 150 μM unfractionated *E. coli* tRNA. Table I shows that the initial rate of AppppA formation (4.1 s^{-1}) in the presence of 5 mM ATP was not significantly changed upon addition of tRNA. Note that, in spite of the presence of 130 μM $ZnCl_2$, aminoacylation of $tRNA^{Lys}$ was completed within 3 min under the conditions of the NMR experiment.

Rate of AppppA Synthesis Depends on ATP and $ZnCl_2$ Concentrations. The initial rate of AppppA formation was measured by ^1H NMR (Plateau et al., 1981b) in the presence of 2 mM L-lysine, 150 μM $ZnCl_2$, 0.05 mg/mL pyrophosphatase, and various initial concentrations of ATP (free Mg^{2+} was held constant at 5 mM). The initial rate of AppppA formation increased with ATP concentration. The K_m for ATP in the reaction was 2.7 mM. At saturating ATP, the reaction rate reached 6.3 s^{-1}.

The dependence of the initial rate of synthesis of AppppA on the $ZnCl_2$ concentration was also followed by ^1H NMR in the presence of 2 mM L-lysine, 5 mM ATP, 10 mM $MgCl_2$, and 0.05 mg/mL pyrophosphatase. The initial rate increased with $ZnCl_2$ concentration. A plateau value of 4.5 s^{-1} was reached at zinc concentrations greater than 200 μM. The half-maximal rate corresponded to 52 ± 8 μM added $ZnCl_2$. This concentration of $ZnCl_2$ was in close agreement with that ensuring 50% inhibition of the $tRNA^{Lys}$ aminoacylation reaction as followed at 37 °C under identical buffer conditions.

Table II: Effect of Various Metals on the Rates of [γ-^{32}P]ATP Hydrolysis or of AppppA Synthesis by *E. coli* Lysyl-tRNA Synthetase[a]

metal ion present in the assay (150 μM each)	[γ-^{32}P]ATP hydrolysis (s^{-1})	AppppA synthesis (s^{-1})
no metal	<0.20	0.05
Zn^{2+}	4.20	4.20
Cd^{2+}	0.75	0.70
Mn^{2+}	0.24	0.19
Ca^{2+}	<0.20	0.08
Co^{2+}	<0.20	0.08
Cu^{2+}	<0.20	0.05
Ni^{2+}	<0.20	0.05
Fe^{2+}	<0.20	0.03

[a] The assay of [γ-^{32}P]ATP hydrolysis was performed as described. The rates of AppppA synthesis were measured as follows: (1) the assay mixtures (37 °C) contained 20 mM Tris-HCl (pH 7.8), 150 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 2 mM L-lysine, 25% D_2O, 0.05 mg/mL yeast pyrophosphatase, 150 μM metal ion, and 50–200 nM lysyl-tRNA synthetase, depending on the initial rate to be measured; (2) the reactions were stopped by rapid (less than 5 min) separation of the metal from the sample (0.2 mL) on Chelex 100 columns (φ 0.5 cm × 4 cm); (3) the metal(II)-free samples were complemented with 10 mM $MgCl_2$ and monitored by ^{31}P NMR to measure ATP and AppppA concentrations. In all cases, chloride salts of the metals were used except in the case of $CuSO_4$. It was verified in the presence of each metal that yeast pyrophosphatase activity was unlimiting with respect to the reaction of AppppA formation.

Cadmium Also Stimulates Synthesis of AppppA by E. coli Lysyl-tRNA Synthetase. The experiment shown in Figure 1 indicated that the $tRNA^{Lys}$ aminoacylation activity of *E. coli* lysyl-tRNA synthetase was sensitive to the presence of Zn(II) as well as to that of other divalent metals. The initial rate of AppppA formation was therefore measured in the presence of 150 μM amounts of these various metals (Table II). The reactions were performed at 37 °C. In preparation for NMR analysis, the samples were then cleaned of divalent ions by passage through a Chelex 100 column. This treatment was particularly necessary in the case of experiments involving the paramagnetic metal ions Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, and Mn^{2+}. The metal-free samples were complemented with 10 mM $MgCl_2$, which enabled us to clearly resolve the β phosphorus of ATP and AppppA on the ^{31}P NMR spectra. For each metal, ^{31}P NMR analysis of the sample was performed, and concentrations of ATP and AppppA was measured. Small quantities of ADP could also be observed. However, ADP appeared at rates at least 5-fold slower than the measured rates of AppppA formation.

In the presence of 150 μM $ZnCl_2$, the same rate of AppppA synthesis as directly measured by NMR (4.1 s^{-1}) was found with this method. Of the various metals assayed, only cadmium and, to a lesser extent, manganese clearly enhanced the rate of AppppA synthesis (0.7 and 0.19 s^{-1}, respectively, vs. 0.05 s^{-1} without metal). Note that the latter value was obtained in the absence of EDTA and was due to zinc contained in the buffer solution (1 μM, as determined by flame absorption spectroscopy).

AppppA synthesis in the presence of the various metals was also determined by [γ-^{32}P]ATP hydrolysis (Plateau et al., 1981a). The rates of hydrolysis are in agreement with those derived from the ^{31}P NMR measurements (Table II). The same buffers were used, except that the NMR buffer contained 25% D_2O.

Zinc Stimulates Formation of Mixed Dinucleoside Tri- or Tetraphosphates (ApppX or AppppX) by E. coli Lysyl-tRNA Synthetase. The unusual reactivity of enzyme-bound amin-

Table III: Effect of the Presence of Zinc on the Rates of Formation of Dinucleoside 5',5'''-P^1,P^3-Tri- or 5',5'''-P^1,P^4-Tetraphosphates by *E. coli* Lysyl-tRNA Synthetase[a]

nucleotide present in the assay (5 mM each)	10 μM EDTA		130 μM $ZnCl_2$	
	AppppA		AppppA	
ATP	0.020		4.1	
		AppppX		AppppX
ATP + GTP	0.020	0.005	2.3	0.90
ATP + CTP	0.021	0.007	3.1	0.72
ATP + UTP	0.022	0.006	3.3	0.31
ATP + dGTP	0.024	0.009	2.4	1.20
		ApppX		ApppX
ATP + ADP	0.021	0.064	2.0	0.94
ATP + GDP	0.018	0.180	1.6	0.69
ATP + CDP	0.018	0.120	2.8	0.35
ATP + UDP	0.026	0.046	2.9	0.50
ATP + dGDP	0.017	0.086	1.1	0.90
ATP + dTDP	0.021	0.027	2.4	0.35
		ApppGpp		ApppGpp
ATP + ppGpp	0.019	0.045	1.9	<0.10
	dAppppdA		dAppppdA	
dATP	0.015		4.9	

[a] Initial rates (s^{-1}) of AppppA, dAppppdA, AppppX, ApppX, and ApppGpp synthesis were measured at 37 °C (1) from ^{31}P NMR spectra in the case of the reactions involving the XDP species, ppGpp, and ATP or dATP alone and (2) from ^1H NMR spectra in the case of the reactions involving XTP. The reaction mixture, buffered with 20 mM Tris-HCl (pH 7.8), contained catalytic amounts of lysyl-tRNA synthetase, 150 mM KCl, 2 mM L-lysine, 5 mM ATP (or dATP), and 5 mM of the indicated XDP, XTP, and ppGpp species. $MgCl_2$ was 20 mM, except in the reactions involving ATP or dATP alone where $MgCl_2$ was 10 mM. In all cases, yeast pyrophosphatase (0.01–0.05 mg/mL) was present in the assay, with 10 μM EDTA or 130 μM $ZnCl_2$.

oacyl adenylate can theoretically lead to a variety of complex nucleotides. The only requirement for reaction would be a free 5'-diphosphoryl end (Zamecnik & Stephenson, 1968; Rapaport et al., 1975). We have therefore searched for the effect of zinc on the reaction of various 5' ribo- or deoxyribonucleotides (XDP or XTP) with lysyl adenylate to form ApppX or AppppX.

As already discussed in Plateau et al. (1981b), the ^{31}P NMR method enables us to distinguish between AppppA and ApppX. The mixture contained ATP and XDP (5 mM each), 20 mM Tris-HCl (pH 7.8), 150 mM KCl, 20 mM $MgCl_2$, 25% D_2O, 2 mM L-lysine, 56 nM lysyl-tRNA synthetase, unlimiting pyrophosphatase (0.01 mg/mL), and 10 μM EDTA or 130 μM $ZnCl_2$.

From the ^{31}P NMR spectra, for each studied XDP, both the initial rates of AppppA and ApppX formation were measured, in the absence or presence of $ZnCl_2$ (Table III). Zinc stimulated the production of ApppX by 3–13-fold. The stimulation was observed with 5' ribonucleotides as well as with 5' deoxyribonucleotides.

We also used the ^{31}P NMR method to verify that 5'-AMP did not significantly react with lysyl adenylate to form AppA. The reaction mixture, as above, contained ATP and AMP (5 mM each). After 15 h, ATP was totally converted into AppppA, but the AMP concentration remained unchanged. Moreover, regardless of the $ZnCl_2$ concentration, the rate of AppppA formation was not affected by the presence of 5 mM AMP. This behavior contrasts with that observed with the various XDP, where the competition between ATP and XDP to form AppppA and ApppX systematically decreases the initial rate of AppppA synthesis (Table III).

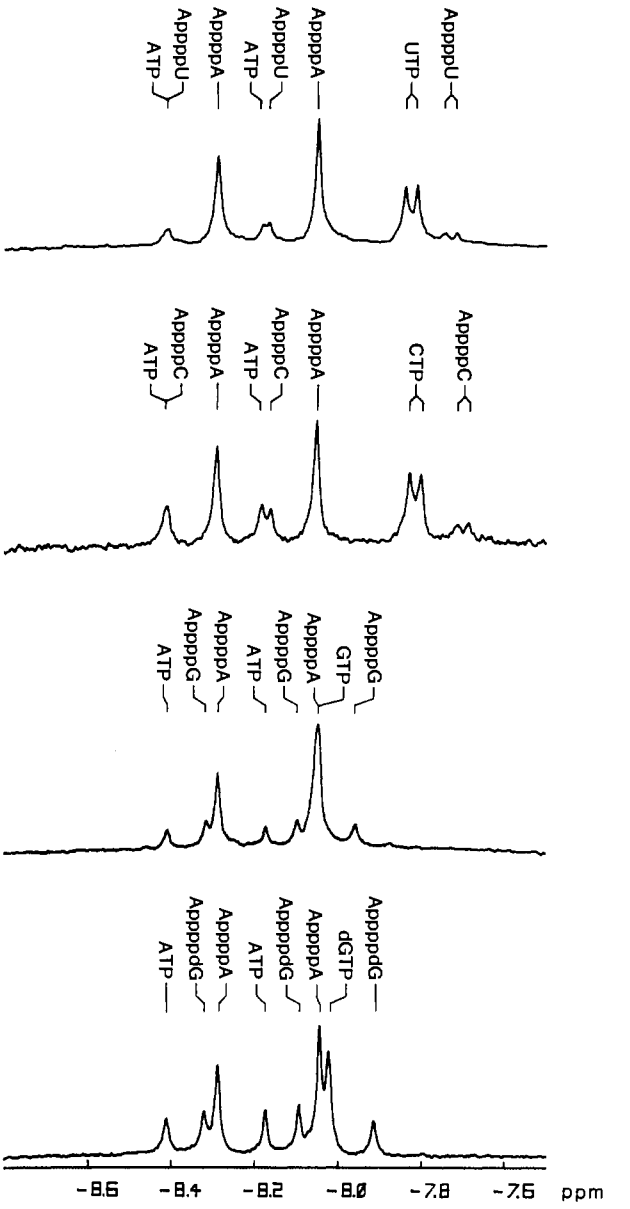

FIGURE 3: Evidence by ^1H NMR spectroscopy for synthesis of various dinucleoside 5,5'''-P^1,P^4-tetraphosphates by *E. coli* lysyl-tRNA synthetase. The sample (0.2 mL) was buffered with 20 mM Tris-HCl, pH 7.8, 150 mM KCl, and 20 mM $MgCl_2$ and included 25% D_2O. It contained 5 mM ATP, 5 mM XTP, 2 mM L-lysine, 130 μM $ZnCl_2$, and 0.05 mg/mL yeast pyrophosphatase. Each reaction (37 °C) was initiated by the addition of lysyl-tRNA synthetase, for concentrations of 620 nM (for the reaction involving dGTP), 420 nM (GTP), 600 nM (CTP), and 470 nM (UTP). Each spectrum was obtained from the sum of 150 free-induction decays, spanning the following time intervals: 15–25 min (for the reaction involving dGTP), 45–55 min (GTP), 35–40 min (CTP), and 60–75 min (UTP). The repetition time was 8 s; the line broadening was 1 Hz.

To follow the competition between ATP and various XTP to form AppppA and AppppX, we have taken advantage of the differences in the ^1H NMR spectra of these species (Plateau et al., 1981b), due to both the differences between A and X and the shifts presumably induced by stacking effects (Scott & Zamecnik, 1969; Kolodny et al., 1979). This approach is summarized in Figure 3, where typical ^1H NMR spectra of mixtures of ATP, XTP, AppppA, and AppppX are shown. From such spectra acquired in the block mode during the enzymatic reaction, both of the rates of AppppA and AppppX synthesis were measured in the presence or absence of $ZnCl_2$ (Table III). In all cases, the addition of 130 μM

$ZnCl_2$ markedly stimulated (50–180-fold) the synthesis of the mixed AppppX molecule. Here again, the effect of zinc did not depend on the nature of the nucleotide sugar moiety: identical effects were obtained on the production of AppppG or AppppdG, starting from GTP or dGTP, respectively.

The case of dATP was particularly interesting since this nucleotide was reported by Randerath et al. (1966) to be converted into dAppppdA by *E. coli* lysyl-tRNA synthetase. This would seem to imply the capability of dATP to form lysyl deoxyadenylate. We showed that this is indeed the case, with the help of the L-lysine-dependent isotopic $^{32}PP_i$–ATP exchange. The buffer was 20 mM imidazole–HCl, pH 7.6 (25 °C), containing 2 mM lysine, 2 mM dATP, 2 mM ^{32}P-labeled PP_i, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.05 mg/mL bovine serum albumin (Blanquet et al., 1974). A rate of 10 s^{-1} was measured in the presence of 2 mM dATP, compared to 75 s^{-1} when ATP was used instead of dATP. Secondly, we followed the conversion of dATP into dAppppdA by ^{31}P NMR in the absence or presence of $ZnCl_2$ (Table III). In the absence of zinc, dAppppdA appeared at a rate of 0.015 s^{-1}. The synthesis of dAppppdA was paralleled by a noticeable production of dAMP (0.26 s^{-1}). The reaction stopped when 50% of the initial dATP (5 mM) was hydrolyzed. In the presence of 130 μM $ZnCl_2$, we observed a dramatic stimulation of the rate of appearance of dAppppdA (4.9 s^{-1}). The rate of production of dAMP was not significantly changed (<0.30 s^{-1}).

Zinc Does Not Stimulate Production of ApppGpp from ATP and ppGpp by E. coli Lysyl-tRNA Synthetase. Finally, the ^{31}P NMR method was used to measure the rate of formation of ApppGpp from the reaction of ppGpp with enzyme-bound lysyl adenylate. The resonances of the various phosphoruses of ppGpp were resolved from those of the phosphoruses of ATP in the ^{31}P NMR spectrum (Figure 4). The possible formation of ApppGpp could therefore be followed from (1) the evolution of the area of the phosphorus resonance peaks of ppGpp and (2) the appearance of a new resonance at 22.1 ppm, corresponding to the position of the β phosphorus of an ApppX species. Under the conditions of Figure 4 (i.e., 10 μM EDTA, without added zinc), a species with the characteristics of ApppX appeared in the NMR spectrum at a rate of 0.045 s^{-1} (Table III). The resonance peak at 9.66 ppm decreased at an identical rate. This resonance corresponds to that of the 5′-α phosphorus of ppGpp. Upon formation of ApppGpp, this resonance must be shifted upfield at 11.0 ppm, the position of the α phosphorus of an ApppX or AppppX species (Plateau et al., 1981a). At this stage, it must be remarked that the characteristic resonances of an ApppX species (β phosphorus at 22.1 ppm and α phosphorus at 11.0 ppm) could reflect the appearance of ApppGpp as well as that of ApppG. Hydrolysis of ppGpp into 5′-GDP or 3′-GDP, followed by the formation of ApppG instead of the expected ApppGpp, could be ruled out, however, for the following reasons: (1) the spontaneous hydrolysis of ppGpp under our experimental conditions was measured at 37 °C in 20 mM Tris-HCl (pH 7.8), 150 mM KCl, 10 mM $MgCl_2$. After 15 h of incubation, 95% of the initial ppGpp remained. (2) Under the conditions of Figure 4, the relative concentration of formed P_i in the last spectrum represented 22 ± 2% of the total phosphorus in the NMR spectrum. The relative concentration of the α phosphorus of ApppX plus AppppA was 23 ± 2%. These values were those expected if the formation of each ApppX and AppppX molecule was accompanied by the liberation of two P_i molecules. If the ApppX species had been formed through the hydrolysis of ppGpp into GDP, the stoichiometry should have been four liberated phosphates per synthesized ApppG molecule. It was therefore concluded that the species appearing in the NMR spectrum with the ApppX characteristics was indeed mainly ApppGpp.

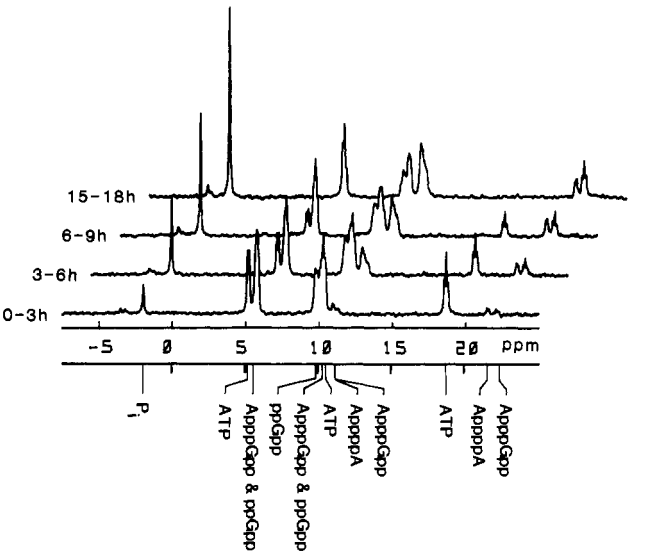

FIGURE 4: Evidence by ^{31}P NMR spectroscopy for synthesis of ApppGpp by *E. coli* lysyl-tRNA synthetase. The sample (0.45 mL) was buffered with 20 mM Tris-HCl, pH 7.8, 150 mM KCl, and 20 mM $MgCl_2$ and included 25% D_2O. It contained 5 mM ATP, 5 mM ppGpp, 2 mM L-lysine, 10 μM EDTA, and 0.01 mg/mL yeast pyrophosphatase. The reaction (37 °C) was initiated by the addition of 1.2 μM enzyme. Each spectrum was obtained from the sum of 2160 free-induction decays, spanning the indicated time interval. The repetition time was 5 s; the line broadening was 7.5 Hz. The origin of chemical shifts was 85% H_3PO_4, whose frequency is computed from that of the lock signal provided by D_2O (Caron et al., 1980). The 5′-α phosphorus resonance of pp(5′)G(3′)pp (9.66 ppm) was identified by comparison with that of the 5′-α phosphorus of 5′-GDP (9.66 ppm) in the same buffer conditions. Upon spontaneous hydrolysis, the resonance of the 5′-α phosphorus of pp(5′)G(3′)pp was not modified, while that of the 3′-α phosphorus at 10.35 ppm disappeared completely. New resonances appeared at −19.82 and −3.79 ppm. These resonances are likely to reflect a cyclic 2′,3′-phosphate and a 3′- (or 2′-) monophosphate, respectively (Cozzone & Jardetzky, 1976). The product, guanosine 5′-diphosphate cyclic 2′,3′-monophosphate has already been noted in Hecht & Hawrelak (1975). The 3′-β and 5′-β phosphorus resonances of ppGpp at 5.64 ppm are unresolved in the spectrum.

Lastly, at the end of the experiment shown in Figure 4, 60% of the initial ppGpp had reacted to form ApppGpp. On the other hand, the area of the resonance at 10.35 ppm [corresponding to the 3′-α phosphorus of ppGpp and very likely to that of A(5′)ppp(5′)G(3′)pp] had remained at least 90% intact. This demonstrates directly that it is the 5′-diphosphoryl moiety of ppGpp that reacts with the aminoacyl adenylate to give A(5′)ppp(5′)G(3′)pp, in agreement with Rapaport et al. (1975).

It is worth noting that, in the absence of zinc, ApppGpp appeared on the ^{31}P NMR spectrum at a somewhat faster rate than AppppA. In the presence of 130 μM $ZnCl_2$, the production of ApppGpp from ATP and ppGpp was not significantly accelerated (Table III). The effect of 5 mM ppGpp on the measured rate of AppppA formation (1.9 s^{-1} instead of 4.1 s^{-1}) argued, however, that ppGpp inhibits the reaction of ATP to form AppppA.

Discussion

This study confirms the previous finding by Zamecnik and collaborators (Zamecnik et al., 1966) that *E. coli* lysyl-tRNA synthetase is able to sustain a noticeable synthesis of AppppA, provided amino acid, ATP–Mg^{2+}, and pyrophosphatase are

present. Our data, however, clearly indicate the importance of zinc in promoting this synthesis.

The zinc-dependent AppppA-synthetase activity of phenylalanyl-tRNA synthetase was established in the case of the *E. coli* as well as in the case of the yeast enzyme (Mayaux & Blanquet, 1981; Plateau et al., 1981a). In the context of this study, we have verified that homogeneous yeast lysyl-tRNA synthetase (a gift of B. Çirakoğlu, Ecole Polytechnique) was also able to sustain a fast conversion of ATP into AppppA provided that zinc was present in the reaction mixture. The production of AppppA by this enzyme was enhanced at least 100-fold by the addition of 150 μM zinc (results not shown). These data suggest the occurrence of a zinc-dependent AppppA synthesis in eukaryotic cells as well as in prokaryotic cells.

Of all of the other assayed metals, only cadmium and, to a lesser extent, manganese were able to substitute for zinc in triggering the AppppA-synthetase activity of *E. coli* lysyl-tRNA synthetase. At a given conentration, zinc had, however, a more pronounced effect than cadmium (4.2 and 0.7 s^{-1}, respectively).

As already noted in the case of *E. coli* and yeast phenylalanyl-tRNA synthetases (Mayaux & Blanquet, 1981; Plateau et al., 1981a), the zinc dependence of the AppppA-synthetase activity of lysyl-tRNA synthetase was paralleled by an inhibition of the reaction of $tRNA^{Lys}$ aminoacylation. Upon addition of 400 μM $ZnCl_2$, the initial rate of $tRNA^{Lys}$ aminoacylation decreased from 4.6 to 0.4 s^{-1}, while the initial rate of AppppA synthesis was stimulated from 0.02 to 4.6 s^{-1}. Note that upon addition of the more reasonable concentration of 10 μM $ZnCl_2$, the rate of AppppA synthesis reached a value of nearly 0.5 s^{-1} (i.e., a 25-fold stimulation).

At this stage, it can be remarked that there exists a good correlation between the relative inhibitory effects of zinc, cadmium, and manganese on $tRNA^{Lys}$ aminoacylation activity and their ability to promote AppppA synthesis. This is not true, however, with copper and nickel, which markedly affected the aminoacylation reaction and did not sustain AppppA formation.

It is worth noting that the metal specificity of the AppppA-synthetase activity of lysyl-tRNA synthetase resembles that of metallothioneins (Margoshes & Vallee, 1957; Kägi & Vallee, 1960, 1961). The biological significance of these unusual metal-binding proteins is only gradually being unravelled [for a review, see Kojima & Kägi (1978)]. They are ubiquitous among eukaryotes and have been reported in microorganisms. They bind Zn^{2+} and Cd^{2+} with high affinity and are synthesized only during exposure of the cells to Cd^{2+} or to excess Zn^{2+}. Current evidence sustains the view that this effect on gene expression is mediated at the transcriptional level (Hildebrand & Enger, 1980; Griffith et al., 1981; Ohi et al., 1981; Durnam & Palmiter, 1981). Such properties emphasize the importance of metallothioneins in tightly controlling free zinc concentration in vivo. In turn, this control might be part of the regulation of AppppA synthesis during the cell cycle. Another possibility is that AppppA itself contributes to the regulation of metallothionein gene expression.

It is believed that in the attack of aminoacyl adenylate to form AppppA, the β- and γ-phosphates of ATP mimic pyrophosphate. This suggests that, in a similar fashion, a variety of AppppX or ApppX species can be obtained from XTP or XDP nucleotides. The only requirement for reaction would be a 5′-diphosphoryl end. The first evidences for such a general mechanism were provided by Randerath et al. (1966), Zamecnik et al. (1967), Żamecnik & Stephenson (1968), and Rapaport et al. (1975).

It was therefore of interest to compare the effect of zinc on the rates of synthesis of AppppA and of any AppppX or ApppX species (including ApppGpp). Our results showed that zinc was able to stimulate all of the assayed synthesis, except that of ApppGpp (Table III). The stimulation, however, was more pronounced in the case of the reaction of an XTP to form AppppX than in the case of the reaction of an XDP (to form an ApppX). This might reflect a more accessible 5′-diphosphoryl end of XTP than of XDP in attacking the enzyme-bound adenylate. When compared to the rate of AppppA synthesis, the various rates of AppppX synthesis were found to be of the same order of magnitude although systematically smaller. For instance, in conditions where AppppA was formed at a rate of 2.3 s^{-1}, AppppG and AppppdG appeared at rates of 0.9 and 1.2 s^{-1}, respectively. A small advantage of ADP compared to the other XDP was also observed in the reactions of formation of ApppA and ApppX, respectively. In this context, it should be noted that dATP (which is able to sustain the synthesis of enzyme-bound lysyl deoxyadenylate) is converted into dAppppdA at a rate (4.9 s^{-1}) very similar to that observed for AppppA synthesis (4.1 s^{-1}). This observation supports the conclusion that the presence of a deoxyribose instead of a ribose had no effect on the specificity of the reaction. The same conclusion can be reached when comparing the respective reactivities of GTP and dGTP or of GDP and dGDP (Table III).

In the case of ppGpp, we were not able to demonstrate a stimulating effect of zinc in the production of ApppGpp. Nevertheless, in the absence of zinc, ApppGpp appeared faster (0.045 s^{-1}) than AppppA (0.019 s^{-1}). Note that, in the absence of zinc, ApppG appeared 10 times faster (0.18 s^{-1}) than AppppA (0.018 s^{-1}), when identical concentrations of ATP and GDP were allowed to compete for the attack of the adenylate. In fact, in the absence of zinc, all of the studied XDPs reacted with the adenylate better than ATP. This behavior markedly contrasted with that observed in the presence of added zinc.

Rapaport et al. have proposed in 1975 that the formation of ApppGpp might constitute a step in the metabolism of ppGpp. Recently, the product of the *spoT* gene, which governs the major degradation pathway of ppGpp (Laffler & Gallant, 1974), was characterized as a Mn^{2+}-requiring enzyme hydrolyzing ppGpp into 5′-GDP and PP_i (Sy, 1977; Heinemeyer & Richter, 1977). The *relA*$^+$ *E. coli* strains accumulate high levels of ppGpp, in the vicinity of 1 mM intracellular concentration, during the stringent response (Gallant, 1979). In *relA*$^+$ *spoT*$^+$ *E. coli* cells, upon blocking ppGpp synthesis, ppGpp decayed with a $t_{1/2}$ of 20–30 s, while in *relA*$^+$ *spoT*$^-$ cells, the $t_{1/2}$ was of 9–15 min (Voellmy & Goldberg, 1980). It remains possible that in the absence of a functional *spoT* gene product, the conversion of ppGpp into ApppGpp contributes to the turnover of ppGpp. The rate of 0.045 s^{-1}, which was measured in this study in the presence of 5 mM competing ATP, would account for the modification of 0.5 mM ppGpp in 15 min, provided that a 10 μM concentration of lysyl-tRNA synthetase plus any other involved aminoacyl-tRNA synthetase would be present in the *E. coli* cell. Such a value is compatible with the measured number of the various *E. coli* aminoacyl-tRNA synthetases (300–900 molecules of each synthetase per cell of about 1-μm^3 volumes (Neidhardt et al., 1977; Blanquet et al., 1973)).

On the other hand, the rate values as determined in this study raise the possibility that, besides the observed major

synthesis of AppppA, other dinucleoside tri- or tetraphosphates might be significantly produced in vivo. For instance, with zinc present, AppppG and AppppA were synthesized in vitro at rate values of 0.9 and 2.3 s^{-1}, respectively. In turn, the in vivo pools of ATP and GTP in normally growing *E. coli* cells were determined to be of the order of 3 and 1.5 mM, respectively (Friesen et al., 1975; A. Danchin, personal communication). It should also be noted that in the absence of zinc (Table III), the production of ApppG by lysyl-tRNA synthetase dominates over that of any of the other assayed dinucleoside tri- or tetraphosphates including AppppA [note that ApppG has been identified by Gilmour & Warner (1978) in acid extracts of *Artemia salina* eggs].

It cannot be excluded that some of these compounds may play a role in vivo, even if present in very small quantities. This is illustrated, for instance, by the case of the interferon-induced inhibitor of protein synthesis pppA(2′)p(5′)A(2′)p(5′)A, which is effective at concentrations below 1 nM (Kerr et al., 1977). Clearly, new methods should be developed in order to address this question and to, primarily, determine the various unusual dinucleoside tri- or tetraphosphates in biological material.

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