Binding of Zinc to *Escherichia coli* Phenylalanyl Transfer Ribonucleic Acid Synthetase. Comparison with Other Aminoacyl Transfer Ribonucleic Acid Synthetases†

Jean-François Mayaux and Sylvain Blanquet*

ABSTRACT: The zinc content and the effect of exogenous zinc on enzymatic activities have been explored in a series of aminoacyl-tRNA synthetases representing the various structural classes in this family of enzymes. The following situations can be distinguished. (1) Homogeneous *Escherichia coli* tyrosyl-tRNA synthetase, an α_2 enzyme of molecular weight 2 × 45K, does not contain zinc. Its tRNA aminoacylation activity is insensitive to the addition of metal complexing agents as well as to the addition of up to 60 μM Zn^{2+}. (2) Homogeneous *E. coli* isoleucyl-tRNA synthetase, an α enzyme (102K), has nearly 1 zinc atom strongly bound/polypeptide chain. The tRNA aminoacylation activity of this enzyme is inhibited by 1,10-phenanthroline but remains unaffected by the addition of 60 μM Zn^{2+} in the assay. (3) Homogeneous *E. coli* and *Bacillus stearothermophilus* methionyl-tRNA synthetases, dimers of molecular weight 2 × 85K, each contain 1 zinc atom strongly bound/enzyme subunit. In the case of either the *E. coli* or the *B. stearothermophilus* enzyme, the bound zinc is retained upon proteolytic modification. The tRNA aminoacylation activity of the *E. coli* enzyme is inhibited by 1,10-phenanthroline but is insensitive to the addition of Zn^{2+}. (4) Homogeneous *E. coli* phenylalanyl-tRNA synthetase, an $\alpha_2\beta_2$ enzyme, has no zinc strongly bound. However, its tRNA aminoacylation activity is impaired by the addition of Zn^{2+} to the assay. A similar effect of Zn^{2+} can be observed in the L-phenylalanine-dependent isotopic ATP–PP_i exchange reaction. The inhibition is fully reversible by the addition of zinc complexing agents. It is shown that the effect of zinc occurs through direct binding of the metal ion to the enzyme. A stoichiometry of 8 zinc atoms/$\alpha_2\beta_2$ enzyme has been determined by equilibrium dialysis and protein fluorescence measurements. The corresponding apparent affinity constant is equal to 0.8×10^6 M^{-1} (pH 8.0, 4 °C) in 150 mM KCl plus 7 mM $MgCl_2$. A parallel effect of zinc is observed on the tRNA aminoacylation activity of yeast phenylalanyl-tRNA synthetase. However, the aminoacylation activity of *E. coli* glycyl-tRNA synthetase, another $\alpha_2\beta_2$ enzyme, appears unaffected by the presence of up to 20 μM $ZnCl_2$. In the accompanying paper [Plateau, P., Mayaux, J. F., & Blanquet, S. (1981) *Biochemistry* (following paper in this issue), it is shown that important synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate by *E. coli* and yeast phenylalanyl-tRNA synthetase can be triggered in the presence of zinc.

A number of proteins involved in nucleic acid metabolism have been reported to be zinc metalloenzymes [Vallee, 1979; Rose et al. (1978) and references cited therein]. Zinc thus appears to play an important role at the level of the expression of the genome, via its association with key enzymes such as DNA[1] or RNA polymerases and other nucleotidyl-transferring enzymes. It is clear that metal ions may have a profound effect on the efficiency and stereoselectivity of template-directed reactions (Lohrmann et al., 1980). However, the precise nature of the role of zinc has proved difficult to define at a molecular level.

The search for zinc has been only recently extended to another class of enzymes recognizing nucleic acids, the class of aminoacyl-tRNA synthetases (Posorske et al., 1979). These enzymes catalyze the first step of protein biosynthesis

$$\text{amino acid} + \text{ATP} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP} + PP_i$$

and thus play a crucial role in genome expression since tRNA misacylation leads to erroneous proteins.

In the course of this study aimed at probing the zinc content of several prokaryotic aminoacyl-tRNA synthetases representing the various structural classes in this family of enzymes [for review see Schimmel & Söll (1979)], it was observed that, in contrast to the cases of methionyl- and isoleucyl-tRNA synthetases, *Escherichia coli* phenylalanyl- and tyrosyl-tRNA synthetases did not contain any firmly bound zinc atom. However, in the case of phenylalanyl-tRNA synthetase, reversible binding of the metal could be obtained provided that micromolar concentrations of exogeneous zinc were added to the solution containing the enzyme. A complete loss of activity of phenylalanyl-tRNA synthetase paralleled this complex formation.

This behavior, which markedly distinguishes phenylalanyl-tRNA synthetase from other studied synthetases, was further found to reflect a new catalytic activity of the enzyme, the production of diadenosine 5′,5‴-P^1,P^4-tetraphosphate. This unusual nucleotide, discovered in 1966 by Zamecnik and coworkers, was already presumed to be made by a back reaction of the first step in protein synthesis, namely, amino acid adenylation (Zamecnik et al., 1966; Randerath et al., 1966). In the accompanying paper (Plateau et al., 1981) it is shown that important synthesis of diadenosine tetraphosphate by *E. coli* as well as by yeast phenylalanyl-tRNA synthetases can be triggered in the presence of zinc.

Materials and Methods

Methionyl-tRNA synthetase from the *E. coli* EM 20031 strain carrying the F 32 episome was obtained and stored as

† From the Laboratoire de Biochimie, Ecole Polytechnique, Laboratoire Associé No. 240 du Centre National de la Recherche Scientifique, 91128 Palaiseau Cedex, France. *Received December 3, 1980.* This work has been supported in part by a grant from the Délégation Générale à la Recherche Scientifique et Technique (Décision d'Aide No. 79.7.0155).

[1] Abbreviations used: tRNA, transfer ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; PP_i, inorganic pyrophosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-4647$01.25/0 © 1981 American Chemical Society

previously described (Cassio & Waller, 1971a). This enzyme was estimated to be at least 95% homogeneous according to electrophoresis on a gel of polyacrylamide.

Trypsin-modified methionyl-tRNA synthetase was derived from the native enzyme according to Cassio & Waller (1971b). Isoleucyl-, phenylalanyl-, and tyrosyl-tRNA synthetases from the same *E. coli* strain, as well as tyrosyl-, native methionyl-, and subtilisin-modified methionyl-tRNA synthetases from *Bacillus stearothermophilus* strain ATCC 1518, were obtained to nearly 95% homogeneity according to purification procedures similar to that for methionyl-tRNA synthetase (Fayat et al., 1974, 1978; Kalogerakos et al., 1980). Baker's yeast phenylalanyl-tRNA synthetase was a gift from Dr. F. Fasiolo (Strasbourg). Unfractionnated tRNA from *E. coli* EM 20031 was obtained from the pilot facilities of the Institut des Substances Naturelles (Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France).

$tRNA_f^{Met}$ (1300–1500 pmol of methionine acceptance/A_{260} unit of tRNA) was purified according to Blanquet et al. (1973). Phenylalanine-specific tRNA from *E. coli* MRE 600 (1100–1300 pmol of phenylalanine acceptance/A_{260} unit) was purchased from Boehringer-Mannheim. $tRNA^{Phe}$ from yeast (1200 pmol of phenylalanine acceptance/A_{260} unit) was a gift from Dr. H. Tonetti (Palaiseau). [^{32}P]PP_i (55 Ci/mol) was from Amersham (United Kingdom). ^{14}C-Labeled amino acids (50–400 Ci/mol) were obtained from the Commissariat à l'Energie Atomique (Saclay, France). *p*- and *m*-Phenanthroline were purchased from G.F.S. Chemicals (Columbus, OH). All other chemicals were from Merck except ATP (Na_2 salt, Boehringer), EDTA (Na_2 salt, Prolabo), and L-phenylalanine (Schwarz/Mann). Sephadex G-50 and G-200 were purchased from Pharmacia; Chelex 100 (100–200 mesh) was from Bio-Rad. Membrane tubing for analytical dialysis (Spectrapor 2; cutoff 12 000–13 000 M_r) was from Spectra Industries. Before use, dialysis tubing was thoroughly rinsed several times with running metal-free water and equilibrated in the desired buffer.

When specificied, unfractionated or purified tRNAs were freed of metal ions (except magnesium) as follows: after filtration on a freshly prepared Chelex 100 column (3 × 0.7 cm) equilibrated in 20 mM Tris-HCl, pH 7.8, tRNA was dialyzed at 4 °C against 20 mM Tris-HCl, pH 7.8, containing 150 mM KCl and 7 mM $MgCl_2$. This treatment reduced by nearly 30-fold the initial contamination by zinc of tRNA samples. This initial contamination was estimated on the order of 0.2 g-atom of zinc/mol of unfractionated tRNA.

Enzyme and tRNA concentrations were determined at 280 and 260 nm, respectively, from their specific optical extinction coefficients. Zinc concentration measurements were performed by flame atomic absorption spectroscopy on a Varian AA 775 spectrophotometer equipped with an air–acetylene burner, a double-beam optical system, and a continuous background corrector.

Zinc atomic absorbancies in the peak height mode were measured at 213.9 nm with 0.1-mL injections and a 5-s integration time (lamp current 5 mA, detection bandwith 1 nm). Standard solutions were obtained by dilution from a Titrisol (Merck) solution containing 1.000 ± 0.002 g of zinc in 1 L of dilute HCl. Under these conditions, the detection limit of zinc was 0.01 μg/mL and the sensitivity was 0.5 $A_{213.9}$ unit·mg^{-1}·L according to Veillon & Vallee (1978). It must be emphasized that, in the concentration range used (0.1–1 mg/mL), the protein matrix did not interfere by more than 1–2% with the measurement.

Isotopic [^{32}P]PP_i–ATP Exchange and tRNA Aminoacylation. All buffers were stored in polyethylene, and the dilutions and reactions were carried out in plastic tubes. Prior to use, each container was carefully rinsed with metal-free water ($R \gg 5$ MΩ).

Enzymatic activities were measured at 25 °C in *standard buffer* (20 mM Tris-HCl, pH 7.8) by monitoring either the amino acid dependent isotopic [^{32}P]PP_i–ATP exchange [2 mM each of ATP and [^{32}P]PP_i (5 Ci/mol), 30 μM to 2 mM cognate amino acid and 7 mM $MgCl_2$] (Blanquet et al., 1974) or the tRNA aminoacylation reaction [2 mM ATP, 30–35 μM ^{14}C-labeled L-amino acid (50–150 Ci/mol), 75–100 μM unfractionated tRNA, or 2–4 μM pure tRNA, plus 7 mM $MgCl_2$ and 150 mM KCl] (Lawrence et al., 1973). The final volume of the reactants was 0.1 mL. In each reaction, initial velocity measurements were ensured by catalytic amounts of enzyme.

Prior to the reactions, stock solutions of enzymes were appropriately diluted (>1000-fold) in 20 mM Tris-HCl, pH 7.8, containing 200 μg/mL bovine serum albumin (Serva). The final concentration of albumin in the assay never exceeded 50 μg/mL.

Sephadex G-50 Filtration and Equilibrium Dialysis. The zinc content of the various enzymes under study was determined by atomic absorption spectrometry. Prior to the measurement, samples (0.2–1 mg of enzyme) were filtrated through Sephadex G-50 (superfine, 17 × 0.35 cm) equilibrated at 4 °C in Chelex-treated standard buffer containing 150 mM KCl plus the various magnesium and zinc concentrations specified under Results. Before equilibration, the column was extensively washed with standard buffer containing 1 mM EDTA. Fractions of 0.12 mL (flow rate of 2–3 mL/h) were collected in prerinsed plastic tubes already containing 0.15 mL of standard buffer and assayed successively for absorbancy at 280 nm and for atomic absorption at 213.9 nm (and occasionally for enzymatic activity, as well). In another set of experiments, enzyme or tRNA samples (0.5 mL) were dialyzed at 4 °C during 16 h in Spectrapor 2 against 1 L of standard buffer containing 150 mM KCl and various magnesium and zinc concentrations. Free and bound zinc concentrations were then determined by atomic absorption as described above.

Fluorescence at Equilibrium. E. coli phenylalanyl-tRNA synthetase intrinsic fluorescence (25 °C) was excited at 295 nm and registered at 322 nm with the apparatus described in Hyafil et al. (1976). Titration curves were systematically corrected for dilution. Prior to the experiments, concentrated enzyme (10–20 μM) was dialyzed overnight against standard buffer containing 150 mM KCl and 10 μM EDTA.

Sephadex G-200 Filtration. Chromatography of *E. coli* phenylalanyl-tRNA synthetase was performed at 4 °C on a column of Sephadex G-200 (superfine, 60 × 1 cm) equilibrated in standard buffer containing 150 mM KCl, 7 mM $MgCl_2$, and the EDTA or $ZnCl_2$ concentrations indicated under Results. Fractions of 0.4 mL were collected at a flow rate of 1.2 mL/h and assayed for [^{32}P]PP_i–ATP exchange and tRNA aminoacylation activities. Prior to the assay, the fractions were appropriately diluted in standard buffer containing 50 mM 2-mercaptoethanol and 0.2 mg/mL bovine serum albumin. The molecular weight of phenylalanyl-tRNA synthetase was estimated by comparison to the elution positions of *E. coli* isoleucyl-tRNA synthetase and of native and trypsin-modified methionyl-tRNA synthetases. Elution of these marker proteins was monitored in the presence of EDTA as well as $ZnCl_2$ in the equilibration buffer of the column.

Results

Sensitivity to Complexing Agents of the tRNA Amino-

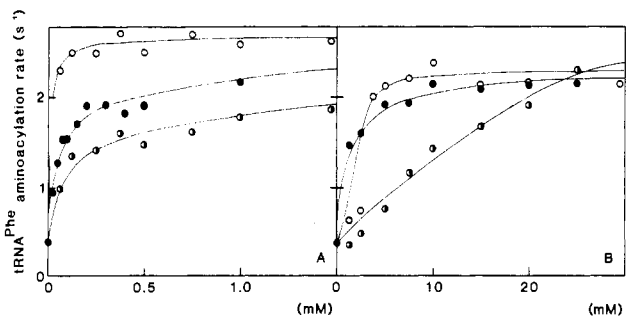

FIGURE 1: Effect of metal complexing agents on the tRNA aminoacylation activity of *E. coli* phenylalanyl-tRNA synthetase. Initial rates of aminoacylation by L-[^{14}C]phenylalanine of 80 μM unfractionated *E. coli* tRNA in the presence of 0.5 nM enzyme were monitored in the presence of various concentrations of (panel A) EDTA–Mg^{2+} (○), 1,10-phenanthroline (●), or 2,2′-bipyridine (◑) and of (panel B) dithioerythritol (●), 2-mercaptoethanol (◑), or L-cysteine (○). Unfractionated tRNA in this experiment was not treated for metal contamination.

acylation Activity of E. coli Phenylalanyl-tRNA Synthetase. During a systematic investigation of the effect of 1,10-phenanthroline upon the activity of various bacterial aminoacyl-tRNA synthetases, it was found that the tRNA aminoacylation activity of *E. coli* phenylalanyl-tRNA synthetase could be markedly stimulated by the addition of various complexing agents of metallic ions (Figure 1). In the case of 1,10-phenanthroline it was verified that this effect depended on the metal chelating property since neither 1,7- nor 4,7-phenanthroline, nonchelating isomers of 1,10-phenanthroline, affected the rate of the reaction catalyzed by phenylalanyl-tRNA synthetase.

It is noticeable that such a stimulation by 1,10-phenanthroline could not be detected in the case of *E. coli* isoleucyl-, methionyl-, and tyrosyl-tRNA synthetases as well as in the case of *B. stearothermophilus* tyrosyl-tRNA synthetase. Rather, an inhibition of the rate of tRNA aminoacylation was observed in the case of *E. coli* methionyl- and isoleucyl-tRNA synthetases. Recent work by Posorske et al. (1979) suggested that the inhibition of methionyl-tRNA synthetase by *o*-phenanthroline resulted from the reversible binding of the chelating agent to a zinc ion firmly bound to each subunit of the dimeric enzyme. On another hand, Takeda & Onishi (1975) have shown that EDTA could inhibit the spermine-stimulated isoleucyl-tRNA formation catalyzed by *E. coli* isoleucyl-tRNA synthetase. These authors concluded in favor of the presence of intrinsic metal ion(s) essential for the activity of this synthetase. In the case of phenylalanyl-tRNA synthetase, the observed stimulating effect of the various complexing agents could be equally well accounted for by (1) binding of the agent to the enzyme through a firmly bound metallic ion or (2) removal from the enzyme, or from the tRNA, of inhibitory metallic ion(s).

Since the above experiments were performed without attention to a possible metallic contamination of the reactants, the stimulation experiment by EDTA was repeated with the following precautions: contact with glassware (except pipets) was avoided; metallic contaminations in concentrated stock solutions were monitored by atomic absorption spectrometry; tRNA, the main source of contamination, was treated with Chelex as described under Materials and Methods. This treatment reduced from 15 μM to <0.3 μM the concentration of zinc contamination brought by unfractionated tRNA in the assay. The total zinc contamination in the reaction mixture was estimated to be <0.4 μM. Under these new conditions, it was found that (1) the rate of tRNA^{Phe} aminoacylation had increased from 0.45 to 2 s^{-1} and (2) the overall stimulation

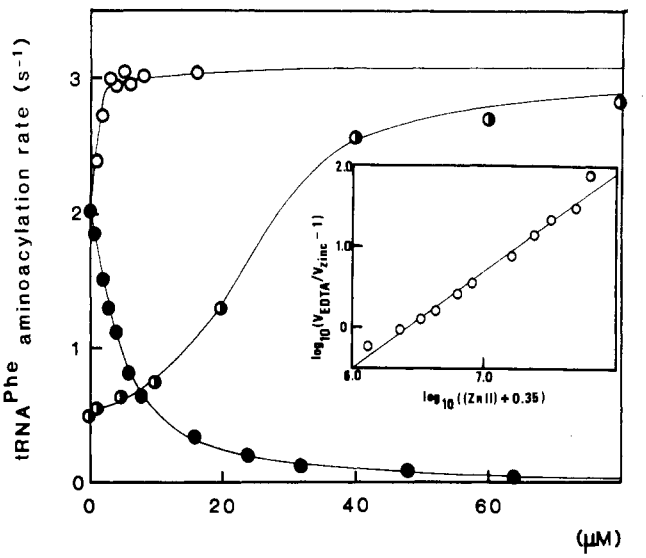

FIGURE 2: Effect of addition of $ZnCl_2$ or of EDTA on the tRNA aminoacylation activity of *E. coli* phenylalanyl-tRNA synthetase. Initial rates of aminoacylation by L-[^{14}C]phenylalanine of 2.5 μM pure *E. coli* tRNA^{Phe} (○, ●) or of 100 μM unfractionated *E. coli* tRNA (◑) were measured in the presence of 0.5 nM enzyme. The EDTA–Mg^{2+} (○, ◑) or $ZnCl_2$ (●) concentration was varied in the assay. Initial zinc contamination in the assay mixture is estimated to be on the order of 0.3–0.4 and 15 μM in the case of pure tRNA^{Phe} and unfractionated tRNA, respectively. In the inset to the figure, the effect of the addition of $ZnCl_2$ on the rate of aminoacylation of pure tRNA^{Phe} (V_{Zn}) is analyzed according to Kistiakowsky & Shaw (1953). The rate of aminoacylation in the rigorous absence of zinc is chosen equal to the rate measured in the presence of excess EDTA (V_{EDTA}). The actual zinc concentration is calculated by taking into account an initial metallic contamination of 0.35 μM. A linear plot of log ($V_{EDTA}/V_{Zn} - 1$) as a function of log ([Zn(II)] + 0.35) yields ordinate intercept and slope values of $K = (0.30 \pm 0.04) \times 10^6$ M^{-1} and $n = 1.2 \pm 0.1$, respectively.

by EDTA had decreased from 6- to 1.5-fold (Figure 2). In relation to the lower metallic contamination in the assay, the lower EDTA concentration performing a maximum stimulation could be noted. Finally, whatever the initial metallic contamination, identical rates of aminoacylation were reached at saturating EDTA in the assay. These results strongly suggested that the stimulation of phenylalanyl-tRNA synthetase proceeded from the sequestration in the assay of inhibitory metallic ion(s).

Sensitivity to Zinc of the tRNA Aminoacylation Activity of E. coli Phenylalanyl-tRNA Synthetase. The rate of tRNA^{Phe} aminoacylation was observed to sharply decrease upon the addition of micromolar zinc concentrations in the assay (Figure 2). It could be observed that the rate at 15 μM added $ZnCl_2$ reached nearly the same value as that measured with nontreated unfractionated tRNA in which the zinc concentration was determined to be on the order of 15 μM. The inhibition effect could be reversed by dilution of zinc or by the addition of excess EDTA. Also, the inhibition did not depend on up to 30 min of incubation of the enzyme in the presence of 100 μM $ZnCl_2$ prior to the assay. Analysis (Kistiakowsky & Shaw, 1953) of the inhibition curve obtained with 3 μM tRNA^{Phe} (inset to Figure 2) gave the following parameters: $n = 1.2 \pm 0.1$ associated to an affinity constant of $(0.30 \pm 0.04) \times 10^6$ M^{-1}. It is noticeable that slightly different parameters were obtained in the presence of unfractionated tRNA (120 μM metal-treated unfractionated tRNA instead of 3 μM pure tRNA^{Phe}). Equilibrium dialysis experiments described further below indicated that this difference resulted from sequestration of zinc by the large amount of unfractionated tRNA present in the reaction mixture.

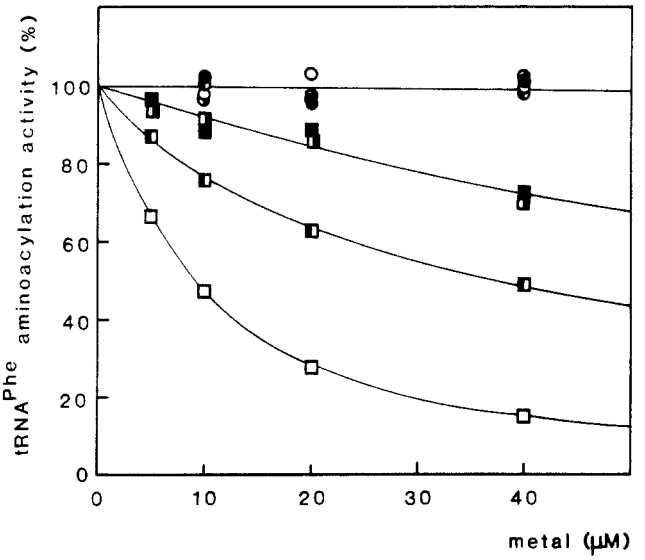

FIGURE 3: Effect of various metals on the tRNA aminoacylation activity of *E. coli* phenylalanyl-tRNA synthetase. Initial rates of aminoacylation by L-[^{14}C]phenylalanine of 120 μM unfractionated tRNA were measured in the presence of various concentrations of the following metals: $ZnCl_2$ (□), $CdCl_2$ (◧), $CuSO_4$ (◨), $NiCl_2$ (■), $CoCl_2$ (○), $CaCl_2$ (◐), $MnCl_2$ (◑), and $FeCl_2$ (●). Initial rates measured 5 min at 25 °C in the presence of 0.4 nM enzyme are expressed as percentages of the rate in the absence of any added metal. Unfractionated tRNA in this experiment was treated for metal contamination.

Other metallic ions were assayed in the phenylalanyl-tRNA synthetase aminoacylation assay. Figure 3 clearly shows that Zn(II) was the strongest inhibitor. Weaker inhibitions were obtained with Cd(II), Cu(II), and Ni(II) while inhibition could not be observed with up to 40 μM Co(II), Ca(II), Mn(II), and Fe(II).

Sensitivity to Zinc of the Isotopic [^{32}P]PP_i-ATP Exchange Reaction Catalyzed by E. coli Phenylalanyl-tRNA Synthetase. The effect of Zn(II) was also investigated in the phenylalanine-dependent isotopic [^{32}P]PP_i–ATP exchange reaction. In standard buffer containing 7 mM $MgCl_2$ and 2 mM of each phenylalanine, ATP, and PP_i, the addition of up to 50 μM $ZnCl_2$ did not affect the rate of exchange. Under ionic conditions indentical with those of the aminoacylation assay and in the presence of a reduced phenylalanine concentration (30 μM), a weak but significant inhibition of the isotopic exchange by zinc could be shown. Since pyrophosphate was susceptible to complex zinc in spite of 3 mM free magnesium in the assay (Sillen & Martell, 1971), the effect of zinc on the reaction was monitored at various PP_i concentrations. The inhibition by zinc was observed to increase with the decreasing of the PP_i concentration in the assay. Extrapolation at zero pyrophosphate concentration of the apparent inhibition constant of zinc gave a value (3 ± 2 μM) very similar to that measured in the tRNA aminoacylation reaction (3 μM) under identical conditions of KCl, $MgCl_2$, ATP, and amino acid. At this stage, it could be concluded that also in the absence of tRNA, the activity of phenylalanyl-tRNA synthetase was markedly sensitive to the presence of Zn(II).

Effect of Zinc on Activities of Other Aminoacyl-tRNA Synthetases. The tRNA aminoacylation activities of homogeneous *E. coli* isoleucyl-, methionyl-, and tyrosyl-tRNA synthetases remained unaffected by the addition to the assay of up to 60 μM Zn(II) (Figure 4). This result agreed with the observation made above that the activity of these enzymes could not be stimulated by the addition of chelating agents.

In the case of yeast phenylalanyl-tRNA synthetase, an inhibition by zinc of the $tRNA^{Phe}$ aminoacylation reaction was found which ressembled that observed in the case of the *E. coli* enzyme (Figure 4). In contrast with the majority of aminoacyl-tRNA synthetases, *E. coli* and *yeast* phenylalanyl-tRNA synthetases possess an $\alpha_2\beta_2$ oligomeric structure (Fayat et al., 1974; Hanke et al., 1974; Fasiolo et al., 1970; Schmidt et al., 1971). It was therefore of interest to investigate the possible effect of Zn(II) on the tRNA aminoacylation activity of *E. coli* glycyl-tRNA synthetase, another $\alpha_2\beta_2$ oligomeric enzyme (Ostrem & Berg, 1974; McDonald et al., 1980). The experiment, as described in Table I, was performed with the help of a fresh *E. coli* crude extract. The effects of 20 μM Zn(II) and of 100 μM EDTA on the $tRNA^{Met}$, $tRNA^{Phe}$, and $tRNA^{Gly}$ aminoacylation activities of this crude extract were compared.

As expected, the phenylalanyl-tRNA synthetase activity was inhibited 5-fold by 20 μM zinc, whereas the methionyl-tRNA synthetase activity remained unaffected. Glycyl-tRNA

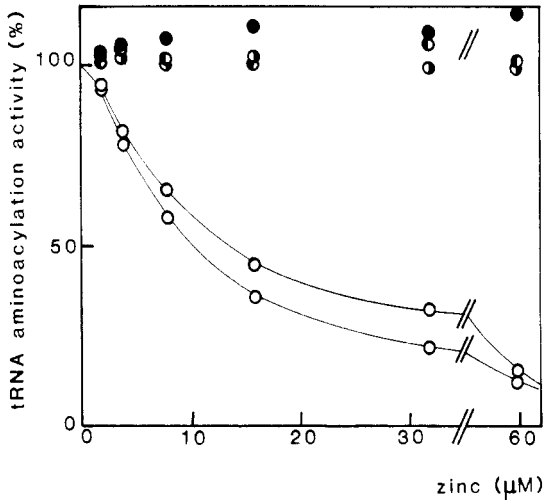

FIGURE 4: Comparison of effects of added $ZnCl_2$ on the tRNA aminoacylation activities of *E. coli* (○, upper curve) phenylalanyl, (◐) methionyl-, (◑) tyrosyl-, and (●) isoleucyl-tRNA synthetases and of yeast (○, lower curve) phenylalanyl-tRNA synthetase. Initial rates of tRNA aminoacylation are expressed as percentages of the rates in the absence of $ZnCl_2$. Unfractionated *E. coli* tRNA (85 μM) and metal-contaminated yeast $tRNA^{Phe}$ (4 μM) were used in this experiment. The assay mixtures contained in addition 5 μM EDTA. Under this condition, in the absence of added $ZnCl_2$, the rate of yeast $tRNA^{Phe}$ aminoacylation by its homologous cognate synthetase was of 2 s^{-1}. This rate could be increased up to 5 s^{-1} upon the addition of 12 μM EDTA in the assay.

Table I: Comparison of the Effect of Zinc on tRNA Aminoacylation Activities of *E. coli* Glycyl-, Methionyl-, and Phenylalanyl-tRNA Synthetases[a]

	specific activity ($pmol \cdot mg^{-1} \cdot s^{-1}$)	
	100 μM EDTA	20 μM $ZnCl_2$
Gly	5.7 ± 0.1	5.5 ± 0.1
Met	55.5 ± 1.4	54.5 ± 2.0
Phe	8.5 ± 0.1	1.9 ± 0.1

[a] 1.4 g of wet cells (*E. coli* EM 20031) were suspended and washed twice in 3 mL of Tris-HCl, 20 mM, pH 7.8, containing 10 mM 2-mercaptoethanol at 0 °C. Cells were disrupted by sonication, and the extract was centrifuged for 60 min at 105000*g*. The supernatant was assayed for protein content and for glycyl-, methionyl-, and phenylalanyl-tRNA synthetases activities in the presence of added EDTA or $ZnCl_2$. The values and standard errors in the table for initial velocity of tRNA aminoacylation were obtained by linear least-squares regression of rates as a function of various dilutions of the supernatant in the assay: 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 7 mM $MgCl_2$, 30 μM ^{14}C-labeled amino acid, 2 mM ATP, and 150 μM unfractionated *E. coli* tRNA, for 10 min at 25 °C.

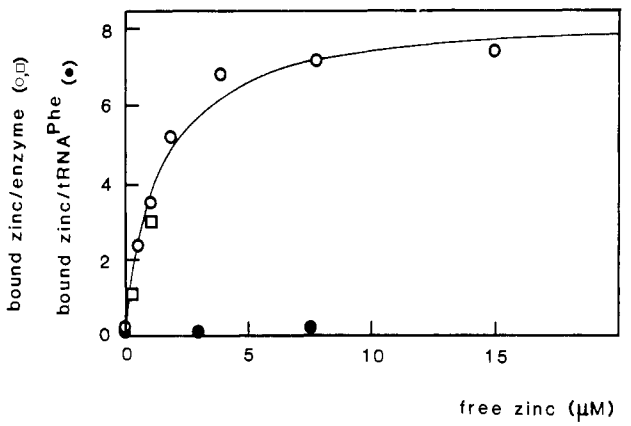

FIGURE 5: Binding of zinc to *E. coli* phenylalanyl-tRNA synthetase (equilibrium dialysis). The enzyme (3.3–4 μM) was dialyzed at 4 °C against standard buffer containing 150 mM KCl, 7 mM $MgCl_2$, and the free $ZnCl_2$ concentrations given in abscissa on the figure. Free and free plus bound zinc concentrations were measured by flame atomic absorption. Enzyme concentrations in the dialysis tubing were determined by absorbancy at 280 nm. The experimental values (○) were fitted by nonlinear regression procedures to a reaction scheme involving the independent binding of n zinc atoms/mol of enzyme. The binding isotherm corresponding to $n = 8.37 \pm 0.33$ associated to an affinity constant of $(0.78 \pm 0.11) \times 10^6$ M^{-1} is superimposed to the experimental values on the figure. Values measured at 4 °C by filtration on Sephadex G-50 under the same buffer condition (□) are also indicated on the figure. A control dialysis experiment with 2 μM *E. coli* tRNA^{Phe} (●) instead of enzyme is shown on the figure.

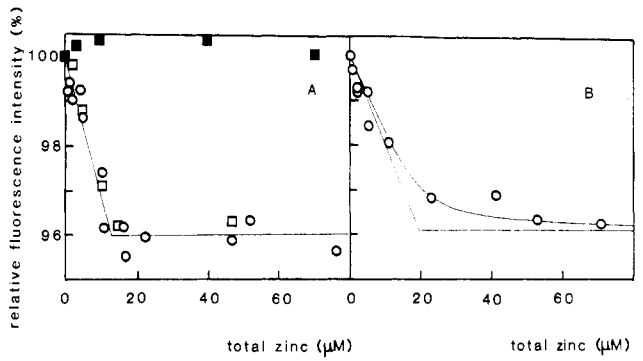

FIGURE 6: Binding of zinc to *E. coli* phenylalanyl-tRNA synthetase (fluorescence at equilibrium). At low ionic strength (panel A), the enzyme (1.8 μM) in 20 mM Tris-HCl, pH 7.8, containing 7 mM KCl and <1 μM EDTA was titrated at 25 °C by $ZnCl_2$ in the presence (□) or absence (○) of 100 μM $MgCl_2$. Titration of the enzyme by $MgCl_2$ (■) is shown on the same figure. The line on the figure corresponds to the binding of 7.7 ± 0.5 $ZnCl_2$/mol of enzyme associated with a decreasing of the initial fluorescence of the enzyme of 4.0 ± 0.2%. At high ionic strength (panel B), the enzyme (2.5 μM) in 20 mM Tris-HCl, pH 7.8, containing 150 mM KCl, 7 mM $MgCl_2$, and <1 μM EDTA was titrated at 25 °C by $ZnCl_2$. The isotherm on the figure corresponds to the independent binding of eight zinc atoms (fixed into the calculation) with an associated affinity constant of $(0.6 \pm 0.3) \times 10^6$ M^{-1}.

synthetase activity also remained unchanged in the presence of 20 μM zinc or 100 μM EDTA. This preliminary experiment tended therefore to make unique in the class of aminoacyl-tRNA synthetases the sensitivity to zinc of phenylalanyl-tRNA synthetase.

Demonstration That Zinc Binds to E. coli Phenylalanyl-tRNA Synthetase. (i) Equilibrium Dialysis. Binding of zinc to phenylalanyl-tRNA synthetase was followed at 4 °C as a function of free zinc concentration in the dialysis buffer. Under the conditions of the aminoacylation assay (standard buffer containing 150 mM KCl and 7 mM $MgCl_2$) it was found that 1 mol of $\alpha_2\beta_2$ enzyme could bind up to eight zinc atoms with an apparent affinity constant of 0.78×10^6 M^{-1} (the best fit in Figure 5 was calculated for a single class of independent binding sites). The binding of zinc to unfractionated tRNA or to pure tRNA^{Phe} was measured under the same conditions. With up to 8 μM free zinc, binding of the metal to tRNA^{Phe} (2 μM) was not detectable. With unfractionated tRNA (120 μM), 0.2 zinc atoms bound/mol of tRNA was measured in the presence of 8 μM free zinc.

This result indicated that the effect of zinc on the activity of the enzyme (Figure 2) originated from the binding of zinc to the enzyme and not to the tRNA^{Phe}. Binding of zinc to the enzyme was also evidenced by filtration at 4 °C on Sephadex G-50. In the presence of 0.5 μM free zinc in the elution buffer of the column, one zinc was found associated to each enzyme molecule. This partial stoechiometry was in close agreement with the equilibrium parameters as determined above by equilibrium dialysis.

The possibility of a zinc-induced aggregation of the enzyme under the conditions of the equilibrium dialysis experiment was explored by chromatography at 4 °C on Sephadex G-200. It was found that phenylalanyl-tRNA synthetase migrated on the column with a normal molecular weight of 250K ± 10K, independent of the presence of 100 μM EDTA or of 50 μM $ZnCl_2$ in the elution buffer. It was also verified that the elution position of the enzyme remained unchanged even if applied on the column at a concentration lower than 50 nM. This allowed us to conclude that the inhibition of the enzymatic activity observed in this study could not be accounted for by simple aggregation or dissociation of the oligomeric synthetase.

(ii) Tryptophan Fluorescence. Upon titration by Zn(II), a reproducible decrease of 4% of the tryptophan fluorescence of phenylalanyl-tRNA synthetase was measured (Figure 6). The significance of this effect was supported by the observation that the enzyme fluorescence remained unchanged upon the addition of up to 100 μM $MgCl_2$.

Titrations by zinc of the enzyme were monitored at 25 °C at low (standard buffer containing 7 mM KCl) or high (150 mM KCl and 7 mM $MgCl_2$) ionic strengths. In low ionic strength, a stoichiometry of 7.7 ± 0.5 Zn(II)/mol of phenylalanyl-tRNA synthetase could be firmly established (Figure 6, panel A). The titration curve was not modified by the presence of 100 μM $MgCl_2$. In high ionic strength, with the assumption of a single class of 8 independent binding sites/enzyme molecule, the titration curve by zinc could be fitted with an affinity constant of $(0.6 \pm 0.3) \times 10^6$ M^{-1} (Figure 6B).

Zinc Content of Various Prokaryotic Aminoacyl-tRNA Synthetases. A series of homogeneous *E. coli* aminoacyl-tRNA synthetases with various structures were chosen for investigating their zinc content. Enzyme samples were prepared either by filtration on Sephadex G-50 or by dialysis, according to Material and Methods. As expected from the results of Posorske et al. (1979), native dimeric methionyl-tRNA synthetase and its monomeric tryptic derivative (Cassio & Waller, 1971b) were found to contain tightly bound zinc (2 zinc ions/native enzyme and 1 zinc ion/modified enzyme). This zinc was not removed by chromatography on Sephadex G-50 in standard buffer containing 100 μM EDTA or by overnight dialysis against standard buffer containing 2% Chelex 100. Similar zinc stoichiometries were estimated from Figure 7 for native and subtilisin-modified *B. stearothermophilus* methionyl-tRNA synthetases (Kalogerakos et al., 1980). *E. coli* isoleucyl-tRNA synthetase, a single-chain enzyme of molecular weight 110K (Arndt & Berg, 1970), was also found associated with zinc at a molar ratio close to unity (1.3 zinc ion/enzyme). These stoichiometries were not modified by overnight dialysis against standard buffer containing 100 mM KCl plus 0.1 mM EDTA or plus 1% Chelex 100. Finally,

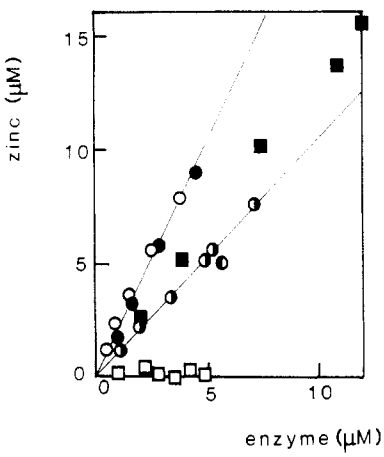

FIGURE 7: Zinc content of various prokaryotic aminoacyl-tRNA synthetases. Enzyme samples were chromatographied at 4 °C on a column of Sephadex G-50 equilibrated in 20 mM Tris-HCl, pH 7.8, containing 150 mM KCl. Fractions containing enzyme were each measured for enzyme and zinc concentrations (absorbancy at 280 nm and atomic absorption at 213.9 nm, respectively). Data for each fraction are plotted on the figure. Studied enzymes were as follows: (upper line) native methionyl-tRNA synthetase from *E. coli* (○) and from *B. stearothermophilus* (●); (lower line) trypsin-modified methionyl-tRNA synthetase from *E. coli* (◑) and subtilisin-modified methionyl-tRNA synthetase from *B. stearothermophilus* (◐); *E. coli* isoleucyl-tRNA synthetase (■); *E coli* tyrosyl-tRNA synthetase (□). The various slopes on the figure gave the number of zinc atoms associated to each enzyme molecule. Free zinc in the equilibration buffer of the column was <0.2 μM.

samples of *E. coli* tyrosyl-tRNA synthetase, a dimer of molecular weight 90K (Chousterman & Chapeville, 1973), contained on the order of 0.1 zinc ion/mol of enzyme. These findings were in agreement with the observation that the tRNA aminoacylation activities of *E. coli* methionyl- and isoleucyl-tRNA synthetases, not that of tyrosyl-tRNA synthetase, were sensitive to the addition of 1,10-phenanthroline.

Discussion

The zinc content and the effect of exogeneous zinc on enzymatic activities have been explored in a series of prokaryotic aminoacyl-tRNA synthetases representing the various structural classes in this family of enzymes. The encountered situations can be distinguished as follows. (1) Homogeneous *E. coli* tyrosyl-tRNA synthetase, an α_2 enzyme (2 × 45K), does not appear to contain zinc tightly bound. Its activity of tRNA aminoacylation remains insensitive to the addition of zinc chelators as well as to the addition of up to 60 μM zinc in the assay. (2) Homogeneous *E. coli* isoleucyl-tRNA synthetase, an α enzyme (110K) containing repeating sequences (Kula, 1973), is found associated to nearly 1 zinc ion/polypeptide chain. The tRNA aminoacylation activity of this enzyme appears sensitive to the addition of *o*-phenanthroline but remains unaffected by the addition of up to 60 μM zinc in the aminoacylation assay. (3) Homogeneous *E. coli* and *B. stearothermophilus* methionyl-tRNA synthetases, dimers of molecular weight 2 × 85K which possess repeating sequences (Koch & Bruton 1974; Koch et al., 1974), each contain 1 zinc ion/enzyme subunit. In the case of either the *E. coli* or the *B. stearothermophilus* enzyme, the bound zinc is retained upon proteolytic modification (Cassio & Waller, 1971b; Kalogerakos et al., 1980). Also the crystalline form of *E. coli* trypsin-modified methionyl-tRNA synthetase (Waller et al., 1971; Zelwer et al., 1976) contains 1 zinc ion/polypeptide chain, despite its growth in 1 M citrate (results not shown). In agreement with the results of Posorske et al., (1979), the tRNA aminoacylation of *E. coli* methionyl-tRNA synthetase is reversibly inhibited by the addition of *o*-phenanthroline in the aminoacylation assay. On another hand, the enzyme remains insensitive to the addition of 60 μM zinc in the aminoacylation assay. It was observed, however, that, upon prolonged incubation of the enzyme in the presence of zinc (10–100 μM) and in the absence of substrates, aggregates of methionyl-tRNA synthetase appeared with a concomitant loss of activity of the enzyme. The activity could be recovered by the addition of excess EDTA or of 50 mM thiol-containing agents (result not shown). (4) Homogeneous *E. coli* phenylalanyl-tRNA synthetase, an $\alpha_2\beta_2$ enzyme (Fayat et al., 1974) has no zinc strongly bound. However, its tRNA aminoacylation activity can be totally inhibited by the addition of 40 μM zinc in the assay. Under the same conditions, an important inhibition is also observed in the phenylalanine-dependent isotopic ATP–PP_i exchange reaction, provided the amino acid and PP_i concentrations in the assay are sufficiently decreased. The inhibition sustained by the presence of zinc is fully and instantaneously reversed by dilution or by the addition of chelating or complexing agents. It is shown that the effect of zinc on the activity of phenylalanyl-tRNA synthetase occurs through direct binding of the metal ion to the enzyme. However, it appears that zinc does not induce a change of the enzyme quaternary structure such as an aggregation or a (partial) dissociation.

A stoichiometry of 8 zinc ions/$\alpha_2\beta_2$ enzyme has been determined with an apparent equilibrium constant of 0.6×10^6 M^{-1} in 150 mM KCl and 7 mM $MgCl_2$ (pH 7.8, 25 °C). This constant is well on the order of magnitude of the constant derived from the analysis of the inhibition by zinc of the $tRNA^{Phe}$ aminoacylation reaction (0.3×10^6 M^{-1}). An *n* value close to 1 may reflect the binding of 1 zinc ion/metallic site responsible for the inhibition. At this stage, owing to the apparent equivalence of the binding of the eight metal ions to the enzyme, the precise number of zinc ions actually involved in the inhibition process remains unknown. It can only be presumed, in view of the $\alpha_2\beta_2$ structure, that at least two bound zinc ions may play an essential role in inhibiting the enzyme activity.

Hennecke & Böck (1974) have shown by chemical modification studies the importance of histidine residues for the *E. coli* phenylalanyl-tRNA synthetase activity. Among 50 accessible residues under nondenaturing conditions, the loss of aminoacylation activity was correlated with the modification of ~15 residues in the absence of substrates. In the presence of phenylalanyl adenylate, this number was reduced to two to four. It is possible that some of these residues are involved in the binding of the zinc ions since imidazole nitrogens are often found to chelate Zn(II) in metalloproteins (Chlebowski & Coleman, 1976).

On another hand, Pimmer & Holler (1979) have shown, with a fluorescent reporter group, that magnesium could bind to the enzyme following a biphasic concentration dependence (K_d = 0.16 and 4.1 mM). Also, in agreement with the present study, these authors have reported that magnesium binding did not affect the tryptophan fluorescence of phenylalanyl-tRNA synthetase. Considering the fact that the binding of zinc is not affected by the presence of magnesium, it can be concluded that the two cations have different binding sites as well as different roles on the enzyme activity.

In the case of fructose 1,6-biphosphatase from rat liver, a reversible binding of Zn^{2+}, distinct from that of Mg^{2+}, has been reported (Tejwani et al., 1976), which resembles that described here. This tetrameric enzyme is specifically and totally inhibited through the binding of eight Zn^{2+}, with a K_i of ~0.4

μM (Pedrosa et al., 1977). Full activity of the enzyme is recovered in the presence of a variety of complexing agents.

It has been recently reported in the case of yeast phenylalanyl-tRNA synthetase (Igloi et al., 1980) that zinc induced the enzymatic hydrolysis of ATP into AMP, provided the amino acid was present. With respect to the low rate of this hydrolytic activity (<1 s^{-1}), it is likely that no significant accumulation of AMP occurs during the aminoacylation assay under our experimental conditions (0.4 nM enzyme, 5 min). This was, however, verified in the case of the *E. coli* enzyme by registering the ^{31}P NMR spectrum of the aminoacylation mixture prior to and after 30 min of incubation at 25 °C in the presence of 0.5 nM enzyme (not shown). Independent of the incubation, the spectra only displayed the expected ATP and tRNA resonances and were virtually superimposable. It will be shown in the following paper that, beyond its effect on the tRNA aminoacylation activity, zinc triggers the enzymatic production of modified nucleotides at the expense of ATP. The possibility that such nucleotides, not detectable on the above ^{31}P NMR spectra, could have been produced in small amounts in the course of the enzymatic assay and be responsible for the inhibition is excluded by the observation that the initial rate of tRNA aminoacylation measured in the presence of 0.1 mM EDTA was not affected by a previous incubation of the assay mixture in the absence of EDTA but in the presence of inhibiting amounts of zinc and also that the rate measured in the presence of 3 μM $ZnCl_2$ was not sensitive to incubation of the reagents prior to the addition of $tRNA^{Phe}$. Also, the possibility that the presence of zinc could have increased the sensitivity of the tRNA aminoacylation reaction to its AMP product was ruled out by comparing the effects of added AMP on the initial rate of aminoacylation in the presence or absence of 3 μM $ZnCl_2$ in the assay (results not shown). In each experiment 50% inhibition of the reaction was reached for the same AMP concentration (4 mM). At this stage, it can be concluded that it is the reversible binding of zinc to the enzyme which turns off the tRNA aminoacylation activity, at the benefit of a new activity: the synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate.

The specificity of phenylalanyl-tRNA synthetase with respect to isoleucyl- and methionyl-tRNA synthetases for this zinc-induced synthesis of diadenosine tetraphosphate will be documented in the following paper. However, this specificity has to be compared to the unique behavior of phenylalanyl-tRNA synthetase in the presence of zinc. This behavior does not relate to the peculiar oligomeric structure of the enzyme since the activity of *E. coli* glycyl-tRNA synthetase, another $\alpha_2\beta_2$ enzyme, appears insensitive to a zinc concentration which otherwise reduces 4-fold the rate of $tRNA^{Phe}$ aminoacylation by *E. coli* phenylalanyl-tRNA synthetase.

In conclusion, the various examined aminoacyl-tRNA synthetases display strikingly different features in terms of zinc content and of sensitivity to the addition of zinc. These various behaviors could be unexpected in view of the common function ensured by this family of enzymes. However, aminoacyl-tRNA synthetases are also characterized by an intriguing variety of molecular weights and of oligomeric structures which might relate with the variety of their zinc content. In this context, it will be of the utmost importance to establish the role of the metal when it is present tightly bound to the synthetase. An essential functional role of zinc for the specificity and/or for the catalysis of tRNA aminoacylation would raise the possibility that in other synthetases, this role is naturally ensured by some other undiscovered metallic ion. On the other hand, a structural role of zinc might be restricted to particular enzymes of the class of aminoacyl-tRNA synthetases exhibiting particular structural features such as, for instance, the larger molecular weights associated to the existence of repeating sequences. Such possibilities are presently under investigation in our laboratory.

References

Arndt, D. J., & Berg, P. (1970) *J. Biol. Chem. 245*, 665.

Blanquet, S., Iwatsubo, M., & Waller, J. P. (1973) *Eur. J. Biochem. 36*, 213.

Blanquet, S., Fayat, G., & Waller, J. P. (1974) *Eur. J. Biochem. 44*, 343.

Cassio, D., & Waller, J. P. (1971a) *Eur. J. Biochem. 20*, 283.

Cassio, D., & Waller, J. P. (1971b) *FEBS Lett. 12*, 309.

Chlebowski, J. F., & Coleman, J. E. (1976) *Met. Ions Biol. Syst. 5*, 1.

Chousterman, S., & Chapeville, F. (1973) *Eur. J. Biochem. 33*, 51.

Fasiolo, F., Befort, N., Boulanger, Y., & Ebel, J. P. (1970) *Biochim. Biophys. Acta 217*, 305.

Fayat, G., Blanquet, S., Dessen, P., Batelier, G., & Waller, J. P. (1974) *Biochimie 56*, 35.

Fayat, G., Fromant, M., & Blanquet, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2088.

Hanke, T., Bartmann, P., Hennecke, H., Kosakowski, H. M., Jaenicke, R., Holler, E., & Böck, A. (1974) *Eur. J. Biochem. 43*, 601.

Hennecke, H., & Böck, A. (1974) *Eur. J. Biochem. 50*, 157.

Hyafil, F., Jacques, Y., Fayat, G., Fromant, M., Dessen, P., & Blanquet, S. (1976) *Biochemistry 15*, 3678.

Igloi, G. L., Von der Haar, F., & Cramer, F. (1980) *Biochemistry 19*, 1676.

Kalogerakos, T., Dessen, P., Fayat, G., & Blanquet, S. (1980) *Biochemistry 19*, 3712.

Kistiakowsky, G. B., & Shaw, W. M. R. (1953) *J. Am. Chem. Soc. 75*, 2751.

Koch, G. L. E., & Bruton, C. J. (1974) *FEBS Lett. 40*, 180.

Koch, G. L. E., Boulanger, Y., & Hartley, B. S. (1974) *Nature (London) 249*, 316.

Kula, M. R. (1973) *FEBS Lett. 35*, 299.

Lawrence, F., Blanquet, S., Poiret, M., Robert-Gero, M., & Waller, J. P. (1973) *Eur. J. Biochem. 36*, 234.

Lohrmann, P., Bridson, P. K., & Orgel, L. E. (1980) *Science (Washington, D.C.) 208*, 1464.

McDonald, T., Breite, L., Pangburn, K. L. W., Hom, S., Manser, J., & Nagel, G. M. (1980) *Biochemistry 19*, 1402.

Ostrem, D. L., & Berg, P. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1967.

Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2742.

Pimmer, J., & Holler, E. (1979) *Biochemistry 18*, 3714.

Plateau, P., Mayaux, J. F., & Blanquet, S. (1981) *Biochemistry* (following paper in this issue).

Posorske, L. H., Cohn, M., Yanagisawa, N., & Auld, D. S. (1979) *Biochim. Biophys. Acta 576*, 128.

Randerath, K., Janeway, C. M., Stephenson, M. L., & Zamecnik, P. C. (1966) *Biochem. Biophys. Res. Commun. 24*, 98.

Rose, K. M., Allen, M. S., Crawford, I. L., & Jacob, S. T. (1978) *Eur. J. Biochem. 88*, 29.

Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601.

Schmidt, J., Wang, R., Stanfield, S., & Reid, B. R. (1971) *Biochemistry 10*, 3264.

Sillen, L. G., & Martell, A. E. (1971) *Spec. Publ. Chem. Soc. No. 25*, 112.

Takeda, Y., & Ohnishi, T. (1975) *J. Biol. Chem. 250*, 3878.
Tejwani, G. A., Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2692.
Vallee, B. L. (1979) in *New Trends in Bio-Inorganic Chemistry*, p 11, Academic Press, New York.
Veillon, C., & Vallee, B. L. (1978) *Methods Enzymol. 54*, 446.
Waller, J. P., Risler, J. L., Monteilhet, C., & Zelwer, C. (1971) *FEBS Lett. 84*, 57.
Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., & Randerath, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 91.
Zelwer, C., Risler, J. L., & Monteilhet, C. (1976) *J. Mol. Biol. 102*, 93.

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

Pierre Plateau,‡ Jean-François Mayaux, and Sylvain Blanquet*

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

† From the Laboratoire de Biochimie, Laboratoire Associé No. 240 du Centre National de la Recherche Scientifique, Ecole Polytechnique, 91128 Palaiseau Cedex, France. *Received December 3, 1980.* This work has been supported in part by grants from the Délégation Générale à la Recherche Scientifique et Technique (Décision d'Aide No. 79.7.0155).

‡ Permanent address: Groupe de Biophysique, Laboratoire de Physique de la Matière Condensée, Ecole Polytechnique, G.R. No. 05.0038 du Centre National de la Recherche Scientifique, 91128 Palaiseau Cedex, France.

[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).

0006-2960/81/0420-4654$01.25/0 © 1981 American Chemical Society