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Accelerated Publications

Characterization of a Homogeneous Complex of Arginyl- and Lysyl-tRNA Synthetase: Zinc and Adenosine 5′-Phosphate Dependent Synthesis of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate†

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ABSTRACT: Zinc, adenosine 5′-phosphate (AMP), and pyrophosphatase greatly stimulate the synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) by rat liver lysyl-tRNA synthetase. The synthesis of Ap_4A does not require lysine; thus the lysyl–adenylate complex is not required. The substrates have been determined to be adenosine 5′-triphosphate (ATP) and AMP with apparent K_m values of 2.1 mM and 1.5 mM, respectively. A zinc-dependent hydrolysis of ATP and AMP has been shown to be associated with the synthetase. In the presence of zinc there is a direct correlation between both the amount of AMP formed and the amount of Ap_4A synthesized by lysyl-tRNA synthetase. Ap_4A acts as a competitive inhibitor for ATP in the aminoacylation reaction of lysyl-tRNA synthetase with a K_I of 2.5 μM. Concentrations of Ap_4A up to 12.5 μM do not inhibit the synthesis of Ap_4A by lysyl-tRNA synthetase. This suggests that there may be more than one binding site for ATP on the enzyme.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) has been detected in both procaryotic and eucaryotic species at concentrations of 10^{-8}–10^{-7} M (Zamecnik, 1969; Rapaport & Zamecnik, 1976). Several lines of evidence support the hypothesis that Ap_4A plays an essential role in controlling growth and cell division. The intracellular concentration of this dinucleotide fluctuates rapidly and is directly related to the proliferative activity of those cells (Rapaport & Zamecnik, 1976). In resting permeabilized baby hamster kidney cells, the initiation of DNA replication could be stimulated by Ap_4A (Grummt, 1978). At the molecular level Ap_4A binds noncovalently to DNA polymerase α from HeLa cells (Rapaport et al., 1981) and from calf thymus (Grummt et al., 1979). DNA polymerase catalyzes DNA synthesis with Ap_4A as a primer that is covalently linked to the first deoxynucleotide synthesized (Zamecnik et al., 1982).

Diadenosine tetraphosphatase has been described and purified from several different sources (Lobaton et al., 1975; Vallejo et al., 1976; Ogilvie & Antl, 1983). In general, these enzymes hydrolyze Ap_4A into adenosine 5′-triphosphate (ATP) and adenosine 5′-phosphate (AMP).

Ap_4A can be synthesized from ATP by lysyl- and phenylalanyl-tRNA synthetases isolated from *Escherichia coli*, yeast, and sheep liver (Zamecnik et al., 1966; Plateau et al., 1981; Goerlich et al., 1982; Brevet et al., 1982). Recently, Ap_4A formation has been shown to be dependent upon the presence of zinc, the cognate L-amino acid, and pyrophosphatase (Plateau et al., 1981; Brevet et al., 1982). Thus, Ap_4A was presumed to be made by the back-reaction of the aminoadenylate reaction.

By use of the homogeneous complex of arginyl- and lysyl-tRNA synthetase isolated from rat liver (Dang et al., 1982), evidence is presented that Ap_4A can be synthesized by lysyl-tRNA synthetase in the absence of lysine, provided an exogenous source of AMP is added. This study also demonstrates that $ZnCl_2$ greatly stimulates Ap_4A formation by lysyl-tRNA synthetase while inhibiting the aminoacylation reaction. In addition, Ap_4A acts as a competitive inhibitor for ATP for the aminoacylation activity but does not inhibit the ability of the enzyme to synthesize Ap_4A. These results suggest that both zinc ion and Ap_4A may be involved in the regulation of cellular growth.

Experimental Procedures

Materials. Female Long-Evans rats (120–150 g) were purchased from Charles River Breeding Laboratories, Inc. [γ-^{32}P]ATP was purchased from New England Nuclear. All

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other radioisotopes were purchased from Schwarz/Mann. Precoated TLC plastic sheets of poly(ethylenimine) (PEI)–cellulose F were purchased from Brinkmann Instruments Co. The Partisil PXS 10/25 SAX high-performance liquid chromatography (HPLC) column was purchased from Whatman. Aquasol-2 was purchased from New England Nuclear. All nucleotides were purchased from Sigma. All other chemicals and supplies were of analytical grade and were obtained from standard chemical sources.

Methods. The aminoacylation assay conditions and purification for the complex of arginyl- and lysyl-tRNA synthetase have been described (Dang et al., 1982).

Ap_4A synthesis was monitored at 37 °C in a volume of 100 μL containing 25 μmol of Hepes, pH 8.0, 0.5 μmol of ATP, 0.5 μmol of AMP, 1.0 μmol of $MgCl_2$, 10 μg of bovine serum albumin, 0.02 μmol of ethylenediaminetetraacetic acid (EDTA), 0.5 μmol of KCl, 5 μmol of $ZnCl_2$, 5 μg of yeast pyrophosphatase, and radioactive ATP (3–10 cpm/nmol). The reactions were initiated by the addition of 1.6 μg of enzyme. After 60 min, 2- or 5-μL aliquots were spotted on PEI–cellulose thin-layer plates along with known standards. The plates were developed in 1 M LiCl as described (Randerath et al., 1966; Plateau & Blanquet, 1982). The Ap_4A spots were cut out and washed in 20 mL of methanol for 30 min to remove the LiCl. The PEI–celluloce strips were removed from the methanol, 1.0 mL of 4 M NH_4OH was added, and the samples were shaken for 30 min at room temperature. This procedure eluted >90% of the radioactive Ap_4A from the spots. The PEI–cellulose strips were removed and the samples left at room temperature overnight to evaporate the NH_4OH. Then 10 mL of Aquasol-2 was added, and the samples were counted in a Beckman LS-3100 liquid scintillation counter. Blanks were minus enzyme.

The products of Ap_4A synthesis were also detected by high-performance liquid chromatography. This apparatus assembled from an Altex pump, Model 110A, Rheodyne Model 7010 injector, equipped with a 20-μL capacity loop, and a variable wavelength absorbance detector (Micromeritic Instrument Corp.), Model 785, operated at 259 nm. The column was 4.6 mm by 25 cm packed with Partisil PXS 10/25 SAX and was protected with a Whatman precolumn. After incubation, Ap_4A and ADP were added to the reaction mixture as known standards. These samples were filtered with microfilters (Bioanalytical Systems, Inc.) to remove particulate contaminants in the reaction mixture. This mixture (20 μL) was injected, and isocratic elution using 0.5 M KCl and 0.25 M KH_2PO_4 (pH 5.0) was performed at a flow rate of 1.8 mL/min. Fractions of 0.9 mL were collected. Aliquots of 0.5 mL were removed, 10 mL of Aquasol-2 was added, and the samples were counted in a Beckman LS-3100 liquid scintillation counter. AMP, ADP, ATP, Ap_6A, Ap_5A, Ap_4A, Ap_3A, and Ap_2A were individually suspended in the assay mixture and individually injected and eluted from the HPLC column to determine their relative positions on the chromatographic profile.

The apparent K_m values for ATP and AMP were determined from initial velocity measurements as previously described (Dang et al., 1982).

The Ap_4A used in this paper was shown by high-performance liquid chromatography to be free of AMP, ADP, ATP, Ap_2A, Ap_3A, Ap_5A, and Ap_6A.

Results

The specific activities for Ap_4A synthesis by lysyl-tRNA synthetase are shown in Table I. The specific activity for Ap_4A formation in the presence of lysine is 1.21. However, the specific activity for Ap_4A formation is 134-fold greater when AMP replaces lysine. If both lysine and AMP are present, there is no further increase in the amount of Ap_4A synthesized. If arginine replaces lysine in the absence of AMP, no Ap_4A is synthesized (data not shown). These results demonstrate that the lysyl-tRNA synthetase portion of the complex synthesizes Ap_4A. The enzyme does not utilize a lysyl–adenylate complex because Ap_4A is synthesized very well in absence of lysine.

Table I: Dependence of Ap_4A Formation in the Presence of Lysine and AMP[a]

assay	specific activity
plus lysine, minus AMP[b]	1.21
plus AMP, minus lysine[c]	161
plus AMP, plus lysine[d]	172

[a] Assay conditions were performed as discussed under Methods. The radioactively labeled substrate was 5 mM [γ-^{32}P]ATP (specific activity 2–5 cpm/nmol). Ap_4A was isolated and quantitated by the PEI–cellulose procedure described under Methods. A unit is defined as micromoles of Ap_4A formed per minute at 37 °C. [b] 0.1 mM lysine replaces AMP. [c] 5 mM AMP replaces lysine. [d] 5 mM AMP and 0.1 mM lysine were present.

Table II: Substrate Determination for Ap_4A Synthesis[a]

substrate	μmol
[γ-^{32}P]ATP	16.0
[^{3}H]AMP	16.2

[a] Assay conditions were as described under Methods with 5 mM [γ-^{32}P]ATP (specific activity 3.0 cpm/nmol) and 5 mM [^{3}H]AMP (specific activity 2.2 cpm/nmol). Ap_4A was isolated and quantitated by the PEI–cellulose procedure described under Methods.

Table III: Kinetic Constants for ATP and AMP for Ap_4A Formation[a]

	K_m (mM)
ATP[b]	2.1
AMP[c]	1.5

[a] Ap_4A was isolated and quantitated by the PEI–cellulose procedure described under Methods. For determination of the kinetic parameters, six points per enzyme preparation were taken in the linear velocity range. The apparent K_m values reported are an average obtained from two different enzyme preparations. [b] The assay procedure was as described under Methods except the ATP concentration was varied from 0.075 to 10 mM; 5 mM [^{3}H]-AMP (specific activity 2.2 cpm/nmol) was used. [c] The assay procedure was as described under Methods except the AMP concentration was varied from 0.075 to 10 mM; 10 mM [γ-^{32}P]ATP (specific activity 2.5 cpm/nmol) was used.

If AMP and ATP are the substrates for Ap_4A formation, then by use of [γ-^{32}P]ATP and [^{3}H]AMP, equal molar amounts of the two radioisotopes should be found in Ap_4A. The results in Table II show that indeed equal amounts of [γ-^{32}P]ATP and [^{3}H]AMP are incorporated into Ap_4A.

Table III summarizes the Michaelis–Menten constants for Ap_4A formation with respect to ATP and AMP. These are the first reported kinetic constants for the substrates involved in Ap_4A formation.

Other investigators have demonstrated the requirement for zinc and pyrophosphatase for Ap_4A synthesis (Plateau & Blanquet, 1982; Brevet et al., 1982). As shown in Table IV, the formation of Ap_4A is totally dependent upon the presence of zinc and pyrophosphatase. It makes no difference if lysine is present as long as an exogenous source of AMP is provided. The role of zinc in Ap_4A formation is not known. However, the requirement for pyrophosphatase demonstrates that Ap_4A

Table IV: Requirements for Ap_4A Formation[a]

conditions of assay	μmol of Ap_2A	rel %
complete	12.8	100
plus lysine	14.1	110
minus Zn^{2+}	0.3	2
minus AMP	0.4	3
minus pyrophosphatase	0.4	3

[a] Assay conditions were performed as described under Methods. The radioactively labeled substrate was 5 mM [γ-^{32}P]ATP (specific activity 2–5 cpm/nmol). Ap_4A was isolated and quantitated by the PEI–cellulose procedure described under Methods.

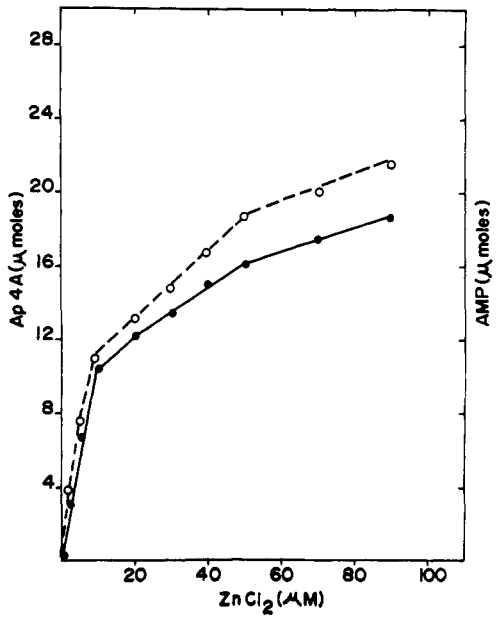

FIGURE 1: Effect of $ZnCl_2$ on formation of Ap_4A and AMP. The radioisotope was 5 mM [^{3}H]ATP (specific activity 2.2 cpm/nmol). Ap_4A and AMP were isolated and quantitated by the PEI–cellulose procedure described under Methods: Ap_4A formation (●); AMP formation (○).

formation requires the hydrolysis of ATP to AMP and inorganic pyrophosphate (PP_i). Zinc ion may be involved in the hydrolysis of ATP. Figure 1 demonstrates that in the presence of zinc both [^{3}H]Ap_4A and [^{3}H]AMP can be detected when [^{3}H]ATP and unlabeled AMP are the substrates. There is a direct correlation between the amount of labeled AMP and Ap_4A formed. The average ratio of labeled AMP to Ap_4A is 1.28 (data not shown). A small amount of Ap_3A is also synthesized (data not shown). Other investigators have also reported the formation of small amounts of Ap_3A (Zamecnik et al., 1966; Plateau et al., 1981; Plateau & Blanquet, 1982).

High-performance liquid chromatography was used to confirm the fact that lysyl-tRNA synthetase synthesizes AMP, Ap_4A, and a small amount of Ap_3A (Figure 2). Ap_2A, Ap_5A, and Ap_6A were not detected by HPLC. The ratio of AMP to Ap_4A plus Ap_3A determined by HPLC was 1.22, confirming that the formation of the dinucleotides is directly correlated with the amount of ATP hydrolyzed to AMP.

If there is a correlation between the amount of ATP hydrolyzed to AMP and PP_i and the amount of Ap_4A formed, then ATP should not be hydrolyzed if Ap_4A is not synthesized. When unlabeled AMP is omitted, lysyl-tRNA synthetase does not synthesize Ap_4A (Table IV) and [^{3}H]ATP is not hydrolyzed to [^{3}H]AMP and PP_i (data not shown).

The effect of diadenosine nucleotides on the aminoacylation activity of lysyl-tRNA synthetase was investigated. Figure 3 demonstrates that Ap_4A acts as a competitive inhibitor for

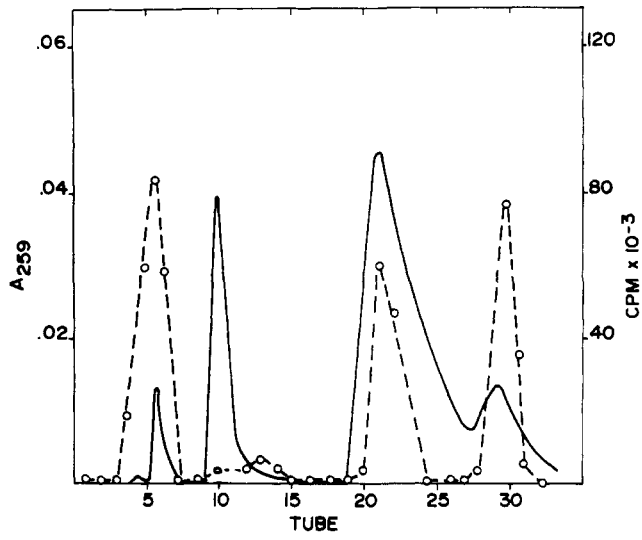

FIGURE 2: Detection of AMP and Ap_4A formed by lysyl-tRNA synthetase by high-performance liquid chromatography. The radioisotope was 3 mM [^{14}C]ATP (specific activity 1.4 cpm/nmol). Ap_4A and AMP were detected by HPLC as described under Methods: A_{259} (—); ^{14}C nucleotide (○). 95% of the cpm's applied to the column were recovered. Ap_3A was chromatographed independently and eluted at tube 13. Absorbance peaks left to right are AMP, ADP, Ap_4A, and ATP. Radioactive peaks left to right are AMP, Ap_3A, Ap_4A, and ATP.

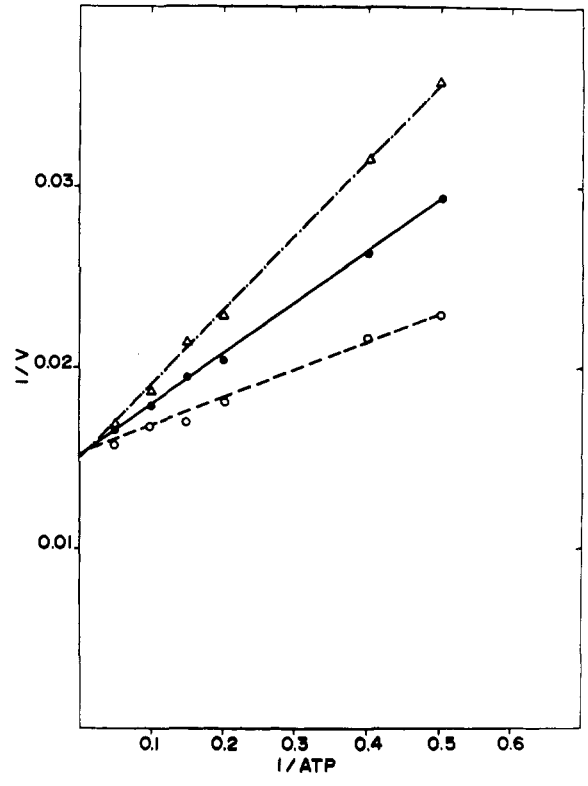

FIGURE 3: Inhibition of lysyl-tRNA synthetase aminoacylation activity by Ap_4A. Aminoacylation reactions were performed as described (Dang et al., 1982). The concentration of ATP was varied as indicated in the presence of saturating tRNA (12 μM) and lysine (0.25 mM with a specific activity of 20–30 cpm/pmol). The concentration of Ap_4A was (○) 0, (●) 12, and (△) 18.75 μM. $1/V$ is in minutes per picomole. ATP concentrations are millimolar.

ATP in the lysyl-tRNA synthetase aminoacylation activity. The K_I was calculated to be 2.5 μM. The extent of inhibition by other diadenosine nucleotides on the aminoacylation activity depended on the length of the polyphosphate bridge linking the adenosine moieties. Ap_5A inhibited the synthetase activity

Table V: Effect of Ap_4A on Lysyl-tRNA Synthetase Ap_4A Synthesis Activity[a]

assay	μmol
complete	13.2
plus 0.5 μM Ap_4A	12.5
plus 1.0 μM Ap_4A	12.9
plus 5 μM Ap_4A	14.0
plus 12.5 μM Ap_4A	13.6

[a] Assay conditions were performed as described under Methods. The radioactive substrate was 5 mM [γ-^{32}P]ATP (specific activity 2–5 cpm/nmol). Ap_4A was isolated and quantitated by the PEI-cellulose procedure described under Methods.

essentially the same as Ap_4A, whereas Ap_3A and Ap_6A were much less effective and Ap_2A had no effect. Also, ADP, dATP, and GTP did not inhibit the aminoacylation activity (data not shown).

Ap_4A, up to a concentration of 12.5 μM, does not inhibit lysyl-tRNA synthetase from the new synthesis of Ap_4A (Table V). This observation suggests that there may be at least two binding sites: one site involved in aminoacylation and the second site involved in Ap_4A formation.

Discussion

This study indicates that in the presence of AMP an exogenous source of lysine is not required for lysyl-tRNA synthetase to form Ap_4A. The specific activity of Ap_4A formation in the presence of AMP is 134-fold higher than that of Ap_4A formed in presence of lysine without exogenous AMP (Table I). The lysine-dependent reaction probably requires a critical concentration of AMP to be generated before Ap_4A can be synthesized. There is a time lag in the formation of Ap_4A by the lysine-dependent activity, but no time lag is seen in the AMP-dependent activity (data not shown). The AMP is generated in the lysine-dependent reaction through the hydrolysis of the lysyl–adenylate complex. Thus the synthesis of Ap_4A requires the consumption of a second molecule of ATP:

$$2ATP + AMP \xrightarrow{E} Ap_4A + AMP + PP_i$$

The role of zinc ion in Ap_4A formation is not known. Zinc inhibits the aminoacylation activity of a number of aminoacyl-tRNA synthetases (Mayaux & Blanquet, 1981). Upon the addition of $ZnCl_2$, the aminoacylation activity of lysyl-tRNA synthetase is inhibited (50% inhibition at 80 μM $ZnCl_2$) (unpublished observation) while 42-fold stimulation of Ap_4A synthesis is reached by the addition of 50 μM $ZnCl_2$ to the reaction (Table IV). The effect of $ZnCl_2$ on *E. coli* lysyl-tRNA synthetase activities (Plateau & Blanquet, 1982) is in good agreement with the information reported in this paper. However, in the presence of $ZnCl_2$, Ap_4A synthesis by sheep liver lysyl-tRNA synthetase is stimulated, but the aminoacylation activity is not inhibited (Brevet et al., 1982). The reason for this discrepancy is not known. The only other aminoacyl-tRNA synthetase that is known to synthesize Ap_4A is phenylalanyl-tRNA synthetase. Phenylalanyl-tRNA synthetase has been purified from *E. coli*, yeast, and sheep liver. In all three cases $ZnCl_2$ inhibits phenylalanyl-tRNA synthetase aminoacylation activity and stimulates Ap_4A formation. The sheep liver lysyl-tRNA synthetase was isolated from an aminoacyl-tRNA synthetase complex by controlled proteolysis (Brevet et al., 1982). Since a homogeneous aminoacyl-tRNA synthetase complex (Dang et al., 1982) was used to perform the experiments in this paper, it would be of interest to see the effect of controlled proteolysis on the lysyl-tRNA synthetase activities.

Zinc ion stimulates not only the synthesis of Ap_4A but also the hydrolysis of ATP to AMP (Figure 1). Igloi and coworkers (Igloi et al., 1980) have demonstrated that in the case of yeast phenylalanyl-tRNA zinc induces the enzymatic hydrolysis of ATP to AMP in the presence of 1–5 mM amino acid. Plateau et al. (1981) have shown that in the presence of phenylalanine zinc triggered the production of AMP and PP_i by yeast phenylalanyl-tRNA synthetase at the expense of ATP. Rat liver lysyl-tRNA synthetase zinc-dependent ATP hydrolysis does not require amino acids. It is possible that the rat liver enzyme has a small amount of lysine associated with it; however, there is no increase in the lysyl-tRNA synthetase zinc-dependent ATP hydrolysis activity if varying amounts of lysine up to 5 mM are added (data not shown). This shows that the reaction is not dependent upon the concentration of lysine. There is a direct correlation between the amount of Ap_4A synthesized and the amount of AMP formed in the presence of zinc (Figures 1 and 2). These results suggest that the zinc-dependent ATP hydrolysis supplies the energy required for Ap_4A formation.

It is of interest that Ap_4A acts as a competitive inhibitor for ATP in the aminoacylation reaction (Figure 3) while an exogenous source of Ap_4A does not inhibit the ability of the enzyme to synthesize Ap_4A (Table V). These results suggest the interesting possibility that there may be two ATP binding sites. Inhibition by diadenosine nucleotides appears to be characteristic of enzymes that catalyze reactions involving more than a single nucleotide site (Powers et al., 1977; Boettcher & Meister, 1980; Lienhard & Secemski, 1973; Tanaka et al., 1981; Ono et al., 1980) but not of enzymes that have one nucleotide binding site (Lienhard & Secemski, 1973).

Ap_4A also acts as a competitive inhibitor for ATP for $pp60^{src}$ protein kinase purified from Rous sarcoma virus transformed tumor cells (Maness et al., 1983). The protein phosphorylated by this kinase has been implicated in the mechanism of RSV-induced cell transformation (Sefton et al., 1980). Since Ap_4A could be modulator of $pp60^{src}$ activity, it would be of interest to determine if Ap_4A is a competitive inhibitor for ATP for all aminoacyl-tRNA synthetases or is specific for a few synthetases. Preliminary results show that in the presence of 12.5 μM Ap_4A only lysyl-, aspartyl-, and seryl-tRNA synthetases of the 18 partially purified aminoacyl-tRNA synthetases assayed were as much as 50% inhibited by the dinucleotide (unpublished observations).

It is becoming increasing apparent that Ap_4A is a very important cellular modulator that is involved in different aspects of cell proliferation. Therefore, it is of utmost importance to determine what triggers aminoacyl-tRNA synthetases to increase the cellular level of Ap_4A.

Registry No. Ap_4A, 5542-28-9; ATP, 56-65-5; AMP, 61-19-8; Zn, 7440-66-6; pyrophosphatase, 9024-82-2; lysyl-tRNA synthetase, 9031-26-9.

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Reconstitution of Catecholamine-Stimulated Guanosinetriphosphatase Activity†

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ABSTRACT: β-Adrenergic receptors were partially purified from turkey erythrocyte membranes by alprenolol–agarose chromatography to 0.25–2 nmol/mg of protein, and the stimulatory guanosine 5′-triphosphate (GTP) binding protein of adenylate cyclase (G_s) was purified from rabbit liver. These proteins were reconstituted into phospholipid vesicles by addition of phospholipids and removal of detergent by gel filtration. This preparation hydrolyzes GTP to guanosine 5′-diphosphate (GDP) plus inorganic phosphate (P_i) in response to β-adrenergic agonists. The initial rate of isoproterenol-stimulated hydrolysis is approximately 1 mol of GTP hydrolyzed·min^{-1}·mol^{-1} of G_s. This low rate may be limited by the hormone-stimulated binding of substrate, since it is roughly equal to the rate of binding of the GTP analogue guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) to G_s in the vesicles. Activity in the absence of agonist, or in the presence of agonist plus a β-adrenergic antagonist, is 8–25% of the hormone-stimulated activity. Guanosinetriphosphatase (GTPase) is not saturated at 10 μM GTP, and the response to GTP is formally consistent either with the existence of multiple K_m's or of a separate stimulatory site for GTP. The GTPase activity of G_s in vesicles is also stimulated by 50 mM $MgCl_2$ in the presence or absence of receptor. Significant GTPase activity is not observed with Lubrol-solubilized G_s, although [^{35}S]-GTPγS binding is increased by Lubrol solubilization.

Hormonal regulation of adenylate cyclase requires the presence of guanosine 5′-triphosphate (GTP)[1] or some closely related nucleotide, and poorly hydrolyzable analogues of GTP cause the persistent activation of the enzyme. These and related findings in many laboratories led to the speculation that hydrolysis of GTP might be involved in the regulation of adenylate cyclase activity [see Ross & Gilman (1980) for review]. This hormone-stimulated guanosinetriphosphatase (GTPase) activity was discovered by Cassel & Selinger (1976), who proposed an explicit model for the stimulation of adenylate cyclase by GTP and the termination of activation by hydrolysis. In this scheme, the receptor–hormone complex was proposed to act by facilitating release of guanosine 5′-diphosphate (GDP), thereby allowing another GTP molecule to bind and activate the enzyme. Considerable data consistent with this model have been obtained [Cassel & Selinger, 1976, 1977a,b; see Ross & Gilman (1980) for review], and it is likely that GTP hydrolysis is central to hormonal control of adenylate cyclase. In at least some cases, however, release of GDP is probably not the rate-limiting, hormone-stimulated step

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[1] Abbreviations: G_s, stimulatory GTP-binding protein of adenylate cyclase; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTPase, guanosinetriphosphatase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); P_i, inorganic phosphate; NaHepes, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DMPC, dimyristoylphosphatidylcholine; PEI, poly(ethylenimine); Gpp(NH)p, guanyl-5′-yl imidodiphosphate; ITP, inosine 5′-triphosphate; ATP, adenosine 5′-triphosphate.

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