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Synthesis of diadenosine 5′,5‴-P¹,P⁴-tetraphosphate by organellar and cytoplasmic phenylalanyl-tRNA synthetases of *Euglena gracilis*

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Purified phenylalanyl-tRNA synthetases present in chloroplasts, mitochondria and cytoplasm of green and bleached *Euglena gracilis* strains, respectively, are able to synthesize diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A). Ap_4A synthesis is strictly dependent on zinc ions. This is the first evidence that chloroplasts should be able to synthesize Ap_4A. Synthesis of Ap_4A by phenylalanyl-tRNA synthetases of the three compartments of a plant cell or by other enzymes such as Ap_4A phosphorylase is discussed.

Ap_4A synthesis; Phenylalanyl-tRNA synthetase; Chloroplast; Mitochondria

1. INTRODUCTION

Ap_4A was discovered as a by-product of the aminoacylation reaction by aminoacyl-tRNA synthetase [1] according to the following equation

$$\text{pppA + L-lysine + lysyl-tRNA synthetase} \rightleftharpoons \text{lysyl-pA·tRNA synthetase + } PP_i \quad (1)$$

$$\text{lysyl-pA·tRNA synthetase + pppA} \rightleftharpoons \text{AppppA + L-lysine + lysyl-tRNA synthetase} \quad (2)$$

Meanwhile Ap_4A was shown to be synthesized in vitro by 13 different aminoacyl-tRNA synthetases [2], provided additional ATP was given after the cleavage of PP_i by pyrophosphatase [3]. Thus aminoacyl-tRNA synthetases were thought to be the only enzyme group capable of synthesizing Ap_4A and related dinucleotides, but recent reports showed that purified yeast Ap_4A phosphorylase likewise synthesizes Ap_4A either by the reverse reaction [4,5] or by catalyzing a transfer of ATP onto AMP, which is formed in the irreversible phosphorolysis of adenosine phosphosulfate [6].

At present two hypotheses are discussed concerning the function of Ap_4A (cf. reviews in [2,7]): pleiotropic triggering of DNA replication [8,9], and Ap_4A as a signal molecule of oxidative and heat shock stress [10–12]. How and in which cell compartment Ap_4A is synthesized, is still an open question.

Starting from the observation that Ap_4A occurs in *Euglena gracilis*, we describe here that PRSs purified to homogeneity from chloroplasts, mitochondria and cytoplasm of this flagellate are able to synthesize Ap_4A in the presence of zinc ions.

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Abbreviations: Ap_4A, diadenosine 5′,5‴-P¹,P⁴-tetraphosphate; PRS, phenylalanyl-tRNA synthetase

2. MATERIALS AND METHODS

2.1. *Cells and cultivation*

The flagellate *Euglena gracilis* Klebs var. Z Pringsheim (no.1224-5/25 from the Algal Collection of the University of

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Göttingen, FRG) and an apoplastic mutant W_3BUL (kindly received from J. Diamond, Gif-sur-Yvette) were used in the experiments. Cultivation was performed photoorganotrophically as described previously [13].

2.2. *Purification of PRSs and assay of Ap_4A synthesizing capacity*

Cytoplasmic and mitochondrial PRSs were purified from dark-grown W_3BUL cells, and the chloroplast-specific PRS was purified from photoorganotrophically grown wild-type cells of *E. gracilis* Z as described elsewhere [14]. The amount (mol of enzyme) of each purified PRS was calculated using a molecular mass of 260 kDa for cytoplasmic PRS and 80 kDa for PRS from chloroplasts and mitochondria. Ap_4A synthesis was assayed in an incubation mixture that contained in a final volume of 25 μl: 10 mM Hepes/KOH, pH 7.8, 10 mM $MgCl_2$, 1 mM L-phenylalanine, 12.5 μg inorganic pyrophosphatase, 0.04 mM [^{14}C]ATP, 2 mM ATP, 0.26–1.3 μM of the equivalent enzyme and 0.5 mM $ZnSO_4$ (or without $ZnSO_4$). Incubations were performed at 30°C, and 2 μl aliquots were taken at 0, 15, 30, 60 min, mixed with 2 μl alkaline phosphatase (0.5 mg per ml 50 mM Tris-HCl, pH 8.0) and incubated for 8 min at 30°C. 2 μl aliquots were transferred onto poly-(ethylenimine)-cellulose sheets, the appropriate standards (Ap_4A, ATP, ADP, AMP) were added, and after a prerun in 75% methanol for 10 min the chromatograms were developed in 0.85 M LiCl for 1 h. Spots were visualized by UV light and radioautography using X-ray film ORWO HS 11. Labeled Ap_4A spots were excised and measured by liquid scintillation counting in a Nuclear Chicago scintillation counter. The amount of Ap_4A formed was calculated from the specific activity of [^{14}C]ATP per mol enzyme used in the incubation mixture.

2.3. *Chemicals and radiochemicals*

ATP, ADP, AMP, Ap_4A and the enzymes, alkaline phosphatase and inorganic pyrophosphatase, were purchased from Boehringer (Mannheim, FRG). Poly(ethylenimine)-cellulose sheets (type F) were from Merck (Darmstadt, FRG) and [8-^{14}C]ATP from Amersham (Buckinghamshire, England).

3. RESULTS AND DISCUSSION

[^{32}P]Ap_4A was found in photoorganotrophically grown wild-type cells of *E. gracilis* Z and dark-grown W_3BUL cells after ^{32}P labeling in a P_i-deficient medium. After removal of the bulk of nucleotides by alkaline phosphatase in the acid-soluble extracts [^{32}P]Ap_4A was identified by several enzymatic digestions (phosphodiesterase, yeast Ap_4A phosphorylase, AMP deaminase for AMP formation) and chromatographic procedures (results to be published). The occurrence of Ap_4A in *E. gracilis* raised the question about the intracellular site of synthesis of this dinucleotide. On the basis of our work on organellar phenylalanyl-tRNA synthetases in this organism [14] we purified cytoplasmic, mitochondrial and chloroplast PRS to homogeneity using dark-grown W_3BUL strain and photoorganotrophically grown Z strain *E. gracilis* cells, respectively.

Ap_4A synthesis was checked in the presence or absence of 0.5 mM $ZnSO_4$. As shown in fig.1, all three PRSs were able to synthesize Ap_4A in the presence of Zn^{2+}, although to different extents. This strict Zn^{2+} dependence of the enzyme activity corresponds with similar observations on PRS of *Escherichia coli* [3,15–17] and *Methanosarcina barkeri* [18]. The ability of yeast mitochondrial PRS to form Ap_4A was here confirmed for *Euglena* mitochondria, but our observation of Ap_4A synthesizing activity of chloroplast PRS is to our knowledge the first report that shows synthesis

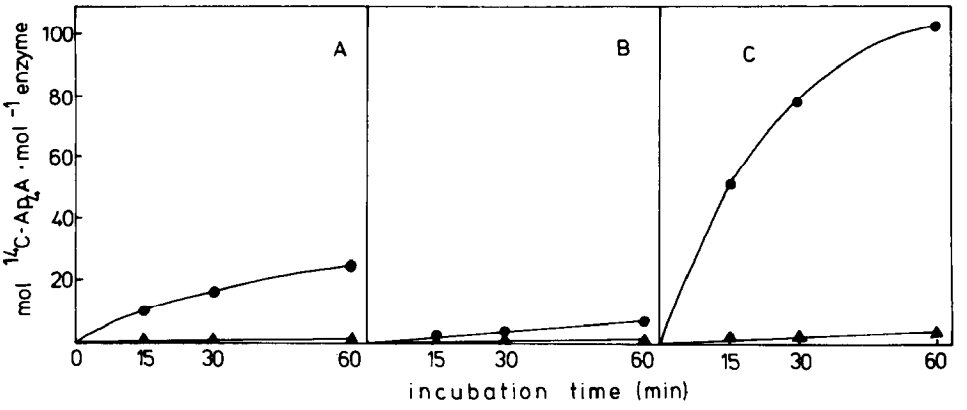

Fig.1. Synthesis of Ap_4A by purified phenylalanyl-tRNA synthetases of chloroplasts (A), mitochondria (B), and cytoplasm (C) of *E. gracilis*. Incubations were performed in the presence of 0.5 mM (●) or absence (▲) of Zn^{2+} as described in section 2.

of this dinucleotide in a characteristic plant organelle.

Besides the evidence for Ap_4A synthesis by aminoacyl-tRNA synthetases, yeast Ap_4A phosphorylase was shown to produce Ap_4A either in the reverse reaction [4,5] or by ATP transfer onto AMP released after irreversible phosphorolysis of adenosine phosphosulfate [6]. So far it is unknown whether the Ap_4A phosphorylase recently found in *E. gracilis* with properties similar to that of the yeast enzyme [19] does the same. Whether *E. gracilis* cells can synthesize Ap_4A in this way in addition to the formation by PRSs as shown in this paper remains to be studied.

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