

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

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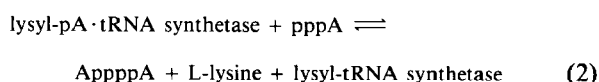
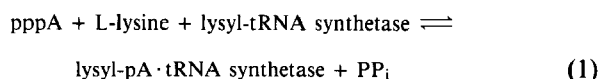
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Purified phenylalanyl-tRNA synthetases present in chloroplasts, mitochondria and cytoplasm of green and bleached *Euglena gracilis* strains, respectively, are able to synthesize diadenosine 5',5'''-P¹,P⁴-tetraphosphate (Ap₄A). Ap₄A synthesis is strictly dependent on zinc ions. This is the first evidence that chloroplasts should be able to synthesize Ap₄A. Synthesis of Ap₄A by phenylalanyl-tRNA synthetases of the three compartments of a plant cell or by other enzymes such as Ap₄A phosphorylase is discussed.

Ap₄A synthesis; Phenylalanyl-tRNA synthetase; Chloroplast; Mitochondria

1. INTRODUCTION

Ap₄A was discovered as a by-product of the aminoacylation reaction by aminoacyl-tRNA synthetase [1] according to the following equation



Meanwhile Ap₄A 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 Ap₄A and related dinucleotides, but recent reports showed that purified yeast Ap₄A phosphorylase likewise synthesizes Ap₄A 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 Ap₄A (cf. reviews in [2,7]): pleiotropic triggering of DNA replication [8,9], and Ap₄A as a signal molecule of oxidative and heat shock stress [10–12]. How and in which cell compartment Ap₄A is synthesized, is still an open question.

Starting from the observation that Ap₄A occurs in *Euglena gracilis*, we describe here that PRSs purified to homogeneity from chloroplasts, mitochondria and cytoplasm of this flagellate are able to synthesize Ap₄A in the presence of zinc ions.

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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Abbreviations: Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetraphosphate; PRS, phenylalanyl-tRNA synthetase

Göttingen, FRG) and an apoplastic mutant W₃BUL (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 Ap₄A synthesizing capacity

Cytoplasmic and mitochondrial PRSs were purified from dark-grown W₃BUL 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. Ap₄A synthesis was assayed in an incubation mixture that contained in a final volume of 25 μ l: 10 mM Hepes/KOH, pH 7.8, 10 mM MgCl₂, 1 mM L-phenylalanine, 12.5 μ g inorganic pyrophosphatase, 0.04 mM [¹⁴C]ATP, 2 mM ATP, 0.26–1.3 μ M of the equivalent enzyme and 0.5 mM ZnSO₄ (or without ZnSO₄). Incubations were performed at 30°C, and 2 μ l aliquots were taken at 0, 15, 30, 60 min, mixed with 2 μ l alkaline phosphatase (0.5 mg per ml 50 mM Tris-HCl, pH 8.0) and incubated for 8 min at 30°C. 2 μ l aliquots were transferred onto poly(ethylenimine)-cellulose sheets, the appropriate standards (Ap₄A, 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 Ap₄A spots were excised and measured by liquid scintillation counting in a Nuclear Chicago scintillation counter. The amount of Ap₄A formed was calculated from the specific activity of [¹⁴C]ATP per mol enzyme used in the incubation mixture.

2.3. Chemicals and radiochemicals

ATP, ADP, AMP, Ap₄A 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 [8-¹⁴C]ATP from Amersham (Buckinghamshire, England).

3. RESULTS AND DISCUSSION

[³²P]Ap₄A was found in photoorganotrophically grown wild-type cells of *E. gracilis* Z and dark-grown W₃BUL cells after ³²P labeling in a P_i-deficient medium. After removal of the bulk of nucleotides by alkaline phosphatase in the acid-soluble extracts [³²P]Ap₄A was identified by several enzymatic digestions (phosphodiesterase, yeast Ap₄A phosphorylase, AMP deaminase for AMP formation) and chromatographic procedures (results to be published). The occurrence of Ap₄A 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 W₃BUL strain and photoorganotrophically grown Z strain *E. gracilis* cells, respectively.

Ap₄A synthesis was checked in the presence or absence of 0.5 mM ZnSO₄. As shown in fig.1, all three PRSs were able to synthesize Ap₄A in the presence of Zn²⁺, although to different extents. This strict Zn²⁺ 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 Ap₄A was here confirmed for *Euglena* mitochondria, but our observation of Ap₄A synthesizing activity of chloroplast PRS is to our knowledge the first report that shows synthesis

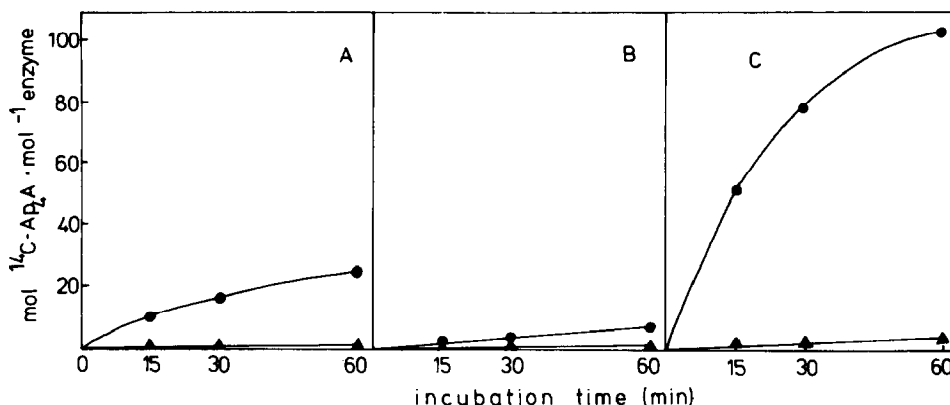


Fig.1. Synthesis of Ap₄A by purified phenylalanyl-tRNA synthetases of chloroplasts (A), mitochondria (B), and cytoplasm (C) of *E. gracilis*. Incubations were performed in the presence of 0.5 mM (●) or absence (▲) of Zn²⁺ as described in section 2.

of this dinucleotide in a characteristic plant organelle.

Besides the evidence for Ap₄A synthesis by aminoacyl-tRNA synthetases, yeast Ap₄A phosphorylase was shown to produce Ap₄A either in the reverse reaction [4,5] or by ATP transfer on-to AMP released after irreversible phosphorolysis of adenosine phosphosulfate [6]. So far it is unknown whether the Ap₄A 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 Ap₄A in this way in addition to the formation by PRSs as shown in this paper remains to be studied.

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