Introduction

Diadenosine 5',5''-P1,P4-tetraphosphate (Ap4A) and other dinucleoside oligophosphates (DNOPs) occur in both prokaryotes and eukaryotes. Their synthesis, demonstrated first in vitro by Zamecnik et al. (1966), can be catalyzed by some aminoacyl-tRNA synthetases originating from various organisms as archaea, bacteria, fungi, plants or mammals (for review see McLennan, 1992). More recently, the synthesis of Ap4A and related compounds has been demonstrated in vitro for firefly luciferase (Guranowski et al., 1990).

Whereas the synthesis of Ap4A seems to be an unspecific process, there are known to be at least three different classes of specific degrading enzymes. In bacteria (Guranowski et al., 1983) and in the slime mould Physarum polycephalum (Barnes and Culver, 1982), Ap4A is cleaved by a symmetrically acting hydrolase. In mammals (Lobátón et al., 1975) and plants (Jakubowski and Guranowski, 1983), it is hydrolyzed asymmetrically to ATP and AMP. In the yeast Saccharomyces cerevisiae (Guranowski and Blanquet, 1985) and some protozoa,
Euglena gracilis and Acanthamoeba castellanii (Guranowski et al., 1995). Ap4A is cleaved by phosphorolysis to ADP plus ATP.

Several biological functions are suggested for DNOPs. First, because the concentration of Ap4A and other DNOPs increases dramatically in response to environmental stress, it has been suggested that they act as modulator of the cellular stress response (Johnstone and Farr, 1991). Secondly, binding sites for Ap4A at DNA polymerase α (Baxi et al., 1994; Grummt et al., 1979) and the observation that Ap4A may initiate replication eyes (Grummt, 1978) suggested a role in replication of DNA. This accords with the observation that proliferative activity of mammalian tissues (Rapaport and Zamecnik, 1976) and initiation of DNA replication during the S-phase of cell cycle (Weinmann-Dorsch et al., 1984) coincide with an enhanced level of Ap4A. Although this assumed function of Ap4A was supported by several experiments, a final proof for the involvement of Ap4A in DNA replication is still lacking, and parts of these results could not be confirmed by others (for review see Remy, 1992). Thirdly, Ap4A and related DNOPs have been shown to modulate activities of various enzymes of purine metabolism (Fernandez et al., 1984) and of ADP-ribosylation (Surowy and Berger, 1982). Alternatively, they act in some instances as transition state analogues e.g. for adenylate kinase (Purich and Fromm, 1972). In addition, Ap3A and Ap5A were found to regulate the vascular tonus (Schlüter et al., 1994). Finally, an Ap4A receptor has been demonstrated on the cell surface of mouse heart cells (Walker et al., 1993) which has a preference for binding Ap4A relative to Ap3A and Ap5A (Hilderman et al., 1994).

The supposition that Ap4A may play an important role in vascular plants is strengthened by the existence of a highly specific Ap4A hydrolase (Jakubowski and Guranowski, 1983). This Ap4A hydrolase from yellow lupin seedlings is practically the only enzyme of plant origin characterized so far. Our experience with plant cells grown in suspension as a convenient experimental model (Wasternack et al., 1985) has encouraged us to check if such cells contain Ap4A hydrolase. In preliminary trials we found that tomato cells are quite a good source of that enzyme. Here we present some of its properties and give first sequence information on an plant Ap4A hydrolase. After purification, studies on natural and artificial substrates were presented to categorize the type of this enzyme. To substantiate these data we determined a peptide sequence from the homogeneous protein, prepared a monospecific antibody against the peptide sequence and investigated the influence of stress on the intracellular level of Ap4A hydrolase.

Materials and Methods

Chemicals and radiochemicals

The unlabelled nucleotides were from Boehringer (Mannheim, Germany). Ap3A, Ap4A, Ap5A, Ap6A and Gp4G were from Sigma (Deisenhofen, Germany). Phosphonate analogues of Ap3A, Ap4A and Ap5A including the analogue bis-2,6-diaminopurine β-D-ribofuranoside P1,P4-tetraphosphate (Dp4D) were synthesized as previously described (Blackburn et al., 1992; Blackburn and Guo, 1991) and kindly provided by Drs. M.-J. Guo and G.M. Blackburn (University of Sheffield, U.K.). The chromatographic media as DEAE-Sephadex, Sephadex G-75 (superfine), and the FPLC-column MonoQ HR 5/5, were from Pharmacia (Freiburg, Germany). Epoxycertivated Sepharose and hydroxyapatite were from Sigma. TLC plastic sheets precoated with PEI-cellulose and a fluorescent indicator were from Merck (Darmstadt, Germany). Protein molecular weight markers were from Boehringer. Di[2,8-3H]-Ap4A (6.2 Ci/ mmol) was kindly provided by Dr. F. Grummt (University of Würzburg, Germany).

Plant material

Tomato (Lycopersicon esculentum cv. Lukullus) cells were grown in suspension as described by Tewes et al. (1984). Inoculum of 2 x 10⁷ mid-log phase cells per 15 ml were supplemented with 100 ml fresh nutrient medium containing 30 g/l sucrose, cultivated on a rotary shaker (150 rpm) at 29 °C without light, and collected at day 3 (mid-log phase, cell density of 0.7 - 1.0). The stock culture of the tomato cell suspension culture is deposited in the Department of Biochemistry at the University of Halle/Saale, Germany.
For heavy metal stress CdCl$_2$ was added to a final concentration of 100 μM on the second day of culture. For heat shock 3 days old tomato cell cultures were treated subsequently as follows: variant I: 1 h 37 °C; 2 h 38,5 °C; 1 h 40 °C; variant II: 15 min 40 °C; 2 h 25 °C; 2 h 40 °C. Phosphate deprivation was achieved by lack of KH$_2$PO$_4$ in the culture medium.

Enzyme assays

Activities of tomato Ap$_4$A hydrolase were determined at 37 °C using a standard incubation that contained in a final volume of 25 μl (during enzyme purification) or 50 μl (during quantitative measurements) 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 8.0, 5 mM MgCl$_2$, 100 μM dithiothreitol, 500 μM Ap$_4$A (or other DNOPs) and rate-limiting amounts of enzyme fraction. For quantitative measurements, incubation mixture contained radioactive substrate. The reaction was terminated, usually after 15, 30, 60 and 90 min by transferring 3 μl aliquots onto TLC sheets. During enzyme purification, the substrate Ap$_4$A and the products AMP, ADP and ATP were separated by TLC. Plates were precoated with silicicgel and fluorescent indicator. The TLC was developed in dioxane/H$_2$O/ammonia (v/v/v; 6:5:1) and the products were visualized under UV light. For quantitative measurements of Ap$_4$A cleavage, substrate and products were separated on PEI-cellulose plates and quantified as described by Jakurowski and Guranowski (1983). Quantification of the products of cleavage of DNOP analogues was analyzed by HPLC (Jasco, Groß Zimmern, Germany). Two weeks after third immunization rabbits were bleeded.

To detect Ap$_4$A hydrolase on Western blots the incubations followed a standard protocol (Harlow and Lane, 1988). The antiserum was used in a dilution of 1:1000 and the specific bound antibodies were visualized with a secondary antibody against rabbit IgG conjugated with alkaline phosphatase (Boehringer). The antiserum against HSP 17 was used in a dilution of 1: 1000 (Neumann et al., 1987).

Protein analysis and immunoblotting

Protein concentrations were determined according to Bradford (1976) during enzyme purification and according to Esen (1978) for SDS-PAGE- and IEF-PAGE-analysis. SDS-PAGE was performed according to Laemmli (1970) in a 15 % separation gel followed by Coomassie-staining or silver-staining (Blum et al., 1987). IEF under non-equilibrium conditions was performed in presence of 8 M urea in a pH gradient 3–10 according to Robertson et al. (1987). For Western blot analysis, the proteins were transferred onto nitrocellulose filters and stained with Fast Green (Serva, Heidelberg, Germany) in order to control transfer quality.

For antibody formation, a synthetic peptide (cys-pro-glu-gly-val-asp-asp-gly-asp-pro) derived from the partial peptide sequence of tomato Ap$_4$A hydrolase (see Results) was used. The peptide was coupled to the high molecular carrier proteins bovine serum albumin and keyhole limpet hemocyanine (Calbiochem, Frankfurt, Germany) with the coupling reagent m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce, Rockford, USA) as described previously (Harlow and Lane, 1988).

Two New Zealand rabbits were immunized subcutaneously on the first, 7th and 22nd day with each 1 mg peptide coupled to bovine serum albumin and to keyhole limpet hemocyanine, supplemented with 0.2 mg adjuvants peptide and 0.5 ml Freund incomplete adjuvant (Sigma, Deisenhofen, Germany). Two weeks after third immunization rabbits were bleeded.

To prepare Ap$_4$A-Sepharose column

To prepare Ap$_4$A-Sepharose, 330 mg freeze-dried epoxy-activated Sepharose was swollen in 10 ml bidistilled H$_2$O, washed three times with 10 ml H$_2$O and suspended in 2 ml 0.4 M potassium bicarbonate, pH 11.0, containing 64 mg Ap$_4$A. After overnight incubation at 37 °C, the column was formed with this gel. Non-covalent bound Ap$_4$A was washed by alternate use of 0.1 M ammonium bicarbonate, pH 8.6, and 0.1 M sodium acetate, pH 4.5. Unoccupied free epoxy groups were blocked by incubation with 1 M ethanolamine. Before using the column the Ap$_4$A-Sepharose gel was washed with binding buffer (20 mM 2-[N-morpholinol]ethanesulfonic acid, pH 6.0, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol, 10 % (v/v) glycerol; A. G. McLennan, Liverpool, U.K., pers. communication).
**Purification of Ap₄A hydrolase**

Lyophilized tomato cells were suspended in buffer C (20 mM potassium phosphate, pH 6.8, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 10 % glycerol) placed on ice and disrupted by sonication. The homogenate was centrifuged for 30 min at 15,000×g. The supernatant, referred to as a crude extract, was subjected to ammonium sulphate fractionation. Ap₄A hydrolase was precipitated in the range of 30-50 % saturation, resolubilized with buffer D (as buffer C but 50 mM potassium phosphate, pH 6.8), dialyzed against this buffer and centrifuged for 15 min at 10,000×g. The clear supernatant was loaded onto a Sephadex G-75 (superfine) column, equilibrated with buffer D, and was eluted with buffer D. The pooled active fractions of Ap₄A hydrolase which appeared at Vₑ/Vₒ = 1.9 were free of phosphodiesterase activity. They were applied onto a DEAE Sephacel column (equilibrated with buffer D). After a wash, elution was performed with a linear gradient of 0–500 mM KCl in buffer D. Fractions containing Ap₄A hydrolase appeared at 200 mM KCl and were pooled and dialyzed against buffer D. Up to this step, the procedure was performed three times to get a sufficient amount of the protein. Next, the active fractions were loaded onto a second DEAE Sephacel column and eluted as described for the first one. The active fractions were pooled and dialyzed overnight against buffer E (as buffer C but containing 10 mM potassium phosphate, pH 6.8) and then applied onto a hydroxypatite column, equilibrated with buffer E. The break-through fractions containing Ap₄A hydrolase activity were free of adenylyl kinase activity.

Further purification was performed by FPLC (Pharmacia) on a MonoQ HR 5/5 column equilibrated with buffer D. After loading of pooled Ap₄A hydrolase-containing fractions, the column was washed with buffer B and eluted with a linear gradient of 0–350 mM KCl in buffer D. Ap₄A hydrolase activity appeared at 140 mM KCl as a sharp peak. After dialyzing the Ap₄A-hydrolase against buffer E, aliquots of the pooled MonoQ fractions were loaded onto a Ap₄A-Sepharose column. The column was washed first with buffer E and then with the buffer E containing 50 mM KCl. Desorption of Ap₄A hydrolase was performed with the buffer E containing, in addition to 50 mM KCl, 100 μM Ap₄A. Due to the fact that the coupled Ap₄A was degraded by Ap₄A hydrolase, the Ap₄A-Sepharose column could be used only once.

**Analysis of partial amino acid sequences**

The purified Ap₄A hydrolase was cleaved with CNBr. The peptides were separated on SDS-PAGE according to Schägger and von Jagow (1987) and blotted onto PVDF membrane. The dominant fragment was submitted to a pulse liquid phase sequencing system (Applied Biosystems, Weiterstadt, Germany). Phenylthiohydantoin amino acids were identified by the on-line-HPLC-systems 120 A (Applied Biosystems; Weiterstadt) by their specific retention times.

**Results and Discussion**

**Purification of Ap₄A hydrolase**

In the present paper we describe the occurrence of a specific Ap₄A hydrolase in tomato cells grown in suspension and a procedure which yields homo-

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**Fig. 1.** SDS-PAGE (A), and IEF-PAGE (B) of tomato Ap₄A hydrolase. In (A) purification of hydrolase to homogeneity by affinity chromatography on Ap₄A-Sepharose is documented. Only one band (arrow in lane 2) appeared in the elute fraction, whereas the break-through fraction (lane 1) still contained other proteins. Molecular weight markers are given on the right margin. In (B) IEP was determined by IEF-PAGE under non-equilibrium conditions in the presence of 8 M urea and a pH gradient from 3 to 10. For lane 1 0.1 μg homogeneous Ap₄A hydrolase was used and compared with markers (M) whose IEPs are given on the right margin. Ap₄A hydrolase is indicated by an arrow.
Table I. Purification of Ap4A hydrolase of tomato cells grown in suspension.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein [mg]</th>
<th>Specific activitya [nmol/mg protein×min]</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1,400</td>
<td>104b</td>
<td>1</td>
</tr>
<tr>
<td>Dialysed ammonium</td>
<td>216</td>
<td>298b</td>
<td>2.9</td>
</tr>
<tr>
<td>sulphate (30–50%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>precipitate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-75 (superfine)</td>
<td>22.4</td>
<td>2,410</td>
<td>23.2</td>
</tr>
<tr>
<td>DEAE-Sephacel I</td>
<td>1.56</td>
<td>11,384</td>
<td>109.5</td>
</tr>
<tr>
<td>DEAE-Sephacel II</td>
<td>1.07</td>
<td>19,833</td>
<td>190.7</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.214</td>
<td>42,584</td>
<td>407.4</td>
</tr>
<tr>
<td>FPLC (MonoQ)</td>
<td>0.002</td>
<td>468,750</td>
<td>4,507.0</td>
</tr>
</tbody>
</table>

a Activity was assayed in a mixture containing of 50 mM HEPES/KOH buffer (pH 8.0), 100 µM dithiothreitol, 5 mM MgCl₂, 500 µM d[2,8-3H]-Ap4A at 37 °C; b activity of all Ap4A degrading enzymes (nmol/mg protein×min).

Homoegeneous enzyme. The purification procedure of Ap4A hydrolase from tomato cells is summarized in Table I. After FPLC purification step at a MonoQ column, a 4,500-fold purification was achieved. For getting homogeneous protein a purification step at a Ap4A-Sepharose column was added. Since Ap4A was used as the eluent in this affinity chromatography step, quantitative determination of Ap4A hydrolase activity could not exactly be performed at this stage without further loss of homogeneous protein. However, homogeneity of the protein has been demonstrated by SDS-PAGE (Fig. 1A, lane 2). Additionally, after two-dimensional electrophoresis using denaturing IEF (pH 4.5 to 7.5) followed by SDS-PAGE, only a single spot was observed (data not shown). The achieved Ap4A hydrolase of different steps of purification was stable at -20 °C for several month.

Properties of Ap4A hydrolase

Homogeneous Ap4A hydrolase of tomato, obtained after affinity chromatography on Ap4A-Sepharose was subjected to one-dimensional SDS-PAGE and approximate molecular mass of 20 kDa was determined (Fig. 1A, lane 2). Due to the binding of Ap4A hydrolase at pH 6.8 on DEAE-Sepharose, an acidic IEP was indicated. Using IEF-PAGE an IEP of 4.5 was determined (Fig. 1B). With Ap4A as a substrate, the hydrolase exhibited an apparent K_m value of 0.8 µM.

By recording initial velocities of Ap4A hydrolase activity at various concentrations of Mg²⁺, Mn²⁺ or Co²⁺, maximal activity was found with Mg²⁺ above 5 mM, whereas with 5 mM Mn²⁺ only half of full enzyme activity was found. With Co²⁺, a weak stimulation of Ap4A hydrolase activity (about 35% of that found with Mg²⁺) was observed at 1 mM. Without divalent cations no Ap4A hydrolase activity was detectable.

To determine pH dependence standard incubation mixture was used with varying pH range between 5.7 and 11. The pH optimum for Ap4A hydrolase was broad between pH 6.5 to 9.0 with a maximum at 6.8.

Among the naturally occurring DNOPs, Ap4A, Ap5A, Ap6A and Gp4G were found to be substrates of the tomato Ap4A hydrolase. Corresponding nucleoside triphosphates appeared always as one of the reaction products. The tomato enzyme was unable to degrade Ap3A but exhibited activity towards p4A.

Fluoride anion (F⁻) proved to be a strong inhibitor of tomato Ap4A hydrolase. Fifty per cent inhibition was found at 6.25 µM. Similar values (2 - 3 µM) were estimated for other Ap4A hydrolases such as from yellow lupin, sunflower and marrow seeds as well (Guranowski, 1990). For the lupin Ap4A hydrolase a noncompetitive inhibition by NaF was indicated (Guranowski, 1990).

All the properties of the tomato Ap4A hydrolase described here are similar to those found for the enzyme from yellow lupin seeds (Guranowski, 1990; Jakubowski and Guranowski, 1983). The pH optima in the range of 6.5–9.0 was somewhat broader than that found for the lupin enzyme (pH 7.5–9.0).
Fig. 2. HPLC analysis of the hydrolysis of AppCH2ppA by tomato Ap4A hydrolase, obtained after the hydroxyapatite purification step. Incubations were performed without (A) or with the enzyme (B) at 37 °C for 60 min. Aliquots were separated by HPLC on a Partisil 10-SAX column using a linear gradient of 5 to 60 % of buffer B to A (buffer A: 50 mM ammonium phosphate, pH 5.2; buffer B: 1 mM ammonium phosphate, pH 5.7) at a flow rate of 1 ml per min during 20 min. After injecting 20 samples onto a Partisil 10 SAX column (Merck, Darmstadt, Germany) elution profile was recorded at 254 nm and quantities were calculated using software GINA (Nuclear Interface, Münster, Germany).

Phosphonate analogues of DNOPs as substrates and inhibitors of Ap4A hydrolase

ββ'-Substituted or αβ',β'-disubstituted phosphonate analogues of Ap4A (n = 3–5) were used to get some further insight on the active center of the enzyme (Guranowski et al., 1994; Guranowski et al., 1989; McLennan et al., 1989). We used compounds with substitution of the bridging oxygen(s) by methylene or halomethylene group(s) (e.g. AppCH2ppA and ApCHClppCHClpA), and by ethylene group (e.g. AppCH2CH2ppA). In addition to that, we tested a compound with modified base moiety (DppCH2ppD, D = diaminopurine) and ones which differed in the length of the oligophosphate chain (ApCH3CH2pPA, AppCH2pCH2ppA). As an example of the hydrolysis of Ap4A analogues by tomato Ap4A hydrolase, cleavage of the methylene group-containing analogue, AppCH2ppA, analyzed by HPLC, is shown in Fig. 2. The products, AMP and pCH2ppA were clearly detectable after 60 min of incubation, but the velocity of cleavage was 70 % lower than that of Ap4A (Table II). The αβ',αβ'-disubstituted phosphonate analogue of Ap4A, which does not contain the oxygens between the αβ- and α'β'-phosphates, was not hydrolyzed (Table II). Interestingly, AppCH2pCH2ppA, an analogue of Ap5A was not cleaved by the enzyme, too (Table II). With respect to the interaction with DppCH2ppD, and AppCH2ppA versus ApCHCHClpCpA, the tomato Ap4A hydrolase behaved as other eukaryotic Ap4A hydrolases (Guranowski et al., 1994; McLennan et al., 1989). Ap4A is not a substrate of the tomato Ap4A hydrolase. Interestingly, however, its analogue ApCH2CH2ppA is hydrolyzed to some extent (Table II).

Results of previous studies on the interaction of various Ap4A analogues with (asymmetrical) Ap4A hydrolases concerning the regiospecificity of hydrolysis allowed to propose a model of action of the eukaryotic Ap4A hydrolases (Lazewskia and Guranowski, 1990; McLennan et al., 1989). The most important feature of this enzyme action is that it recognizes four phosphate residues and cleaves by addition of water at the “fourth” phosphate (P4) from the more strongly bound nucleotide residue with scission of the P4-O-P3 bond both in Ap4A and Ap5A (Guranowski et al., 1994). Moreover, the enzyme site which recognizes the NTP moiety of a substrate tolerates some modifications in the oligophosphate chain of that moiety. Lack of cleavage observed by us for AppCH2pCH2ppA is in line with that model. Neither of the two “fourth” phosphorus atoms of this symmetrical analogue can be attacked by a water oxygen because they have a CH2 bridge to the adjacent phosphorus whose properties deny this mode of cleavage. The much slower hydrolysis observed for the ethylene analogue of Ap3A, ApCH2CH2ppA, suggests that the tomato Ap2A hydrolase can also operate by a “frame shift” mechanism as proposed for the Ap4A hydrolases acting on various analogues by McLennan et al. (1989). It is possible that the spatially extensive ethylene group may bridge the binding site for the “second” phosphorus in the NTP site of the hydrolase.

We have also examined inhibition of Ap4A hydrolase activity by DNOP analogues described above, and estimated inhibition constants using the Ez-Fit software (Perella Scientific Inc., USA). All these compounds are competitive inhibitors.
Table II. Kinetic constants of purified tomato \( \text{Ap}_4\text{A} \) hydrolase for \( \text{Ap}_4\text{A} \) and its analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( V_{rel} ) (%)</th>
<th>( K_i(\mu\text{M}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ap}_{\text{ppppA}} )</td>
<td>100</td>
<td>0.8 (( K_m ))</td>
</tr>
<tr>
<td>( \text{Ap}_4\text{A}-\text{analogue}: )</td>
<td>29.3</td>
<td>0.6 (+/-0.11)</td>
</tr>
<tr>
<td>( \text{AppCH}_2\text{ppA} ) (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{ApCHClppCHClpA} ) (2)</td>
<td>0</td>
<td>6.9 (+/-2.1)</td>
</tr>
<tr>
<td>( \text{DppCH}_2\text{ppD} ) (3)</td>
<td>5.3</td>
<td>0.4 (+/-0.08)</td>
</tr>
<tr>
<td>( \text{Ap}_3\text{A}-\text{analogue}: )</td>
<td>0.5</td>
<td>735 (+/-188)</td>
</tr>
<tr>
<td>( \text{ApCH}_2\text{CH}_2\text{pppA} ) (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Ap}_5\text{A}-\text{analogue}: )</td>
<td>0</td>
<td>0.8 (+/-0.22)</td>
</tr>
<tr>
<td>( \text{AppCH}_2\text{pCH}_2\text{ppA} ) (5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Most of them show \( K_i \) value in the range of the \( K_m \) value for \( \text{Ap}_4\text{A} \), whereas for the \( \text{Ap}_3\text{A} \) analogue, \( \text{ApCH}_2\text{CH}_2\text{ppA} \), the \( K_i \) value is two orders of magnitude higher (Table II).

Partial amino acid sequence of \( \text{Ap}_4\text{A} \) hydrolase

For the first time a peptide sequence of an asymmetrical cleaving \( \text{Ap}_4\text{A} \) hydrolase of plants was estimated by microsequencing of a dominant fragment obtained after CNBr cleavage. The sequence of 17 amino acids length is shown in Fig. 3A. Interestingly, a part of this sequence is in accordance with the consensus sequence for one part of a trimeric nucleotide binding motif, a so-called kinase II motif (Traut, 1994). This motif is characterised by an invariant aspartate which coordinates a divalent metal ion (e.g. \( \text{Mg}^{2+} \) of Mg-ATP) in order to fix the phosphate residue of NTP. The motif was already found within the protein sequences of AMP kinase, ras p21 and actin (Traut, 1994). The detection of the kinase II motif in the partial sequence of \( \text{Ap}_4\text{A} \) hydrolase supports the above described model of its catalysis, in which the NTP moiety of a DNOP molecule is fixed in a pocket as part of the active center (McLennan et al., 1989). In Fig. 3B a sequence comparison with other representative kinase II sequence motifs from different NTP-binding proteins is shown.

During preparation of this manuscript the first cDNA sequence of an asymmetrically cleaving \( \text{Ap}_4\text{A} \) hydrolase (human) was described (Thorne et al., 1995). A comparison between the tomato peptide sequence and the deduced protein sequence of the human \( \text{Ap}_4\text{A} \) hydrolase shows 38.9 % identity (exact correspondence) and 72.2 % similarity (conservative substitution; Fig. 3C).
Our data related to the kinetic and molecular characteristics and the relation at amino acid level confirm the assumption, that Ap₄A hydrolases belong to a conserved protein family in higher eukaryotes.

Generation of an antiserum directed against an internal Ap₄A hydrolase peptide sequence

A polyclonal antiserum was raised against an internal peptide sequence of tomato Ap₄A hydrolase (see Material and Methods). The specificity of this antiserum was tested by Western blot analysis using probes of different purification steps of Ap₄A hydrolase. The antiserum recognized Ap₄A hydrolase (indicated by arrow) monospecifically in the crude extract as well as in the following steps of purification (Fig. 4, lane 1–6).

Influence of various environmental stresses on the intracellular level of tomato Ap₄A hydrolase

A significant increase of endogenous Ap₄A and related DNOPs upon environmental stress like heat shock or oxidative stress was reported several times (Brevet et al., 1985; Lee et al., 1983). This occurs in various prokaryotic and eukaryotic organisms and is thought to be a modulator in cellular responses to stress (Johnstone and Farr, 1991). Since Ap₄A is synthesized unspecifically by aminoacyl tRNA synthetases (for review see McLennan, 1992) but is cleaved by specific enzymes, its cellular level should be regulated mainly via Ap₄A degrading activities. Therefore, we studied Ap₄A hydrolase under conditions which are known to change dramatically intracellular level of Ap₄A. Using an antiserum directed against an internal peptide sequence of tomato Ap₄A hydrolase this...
Fig. 5. Influence of environmental stress on the intracellular content of tomato Ap4A hydrolase. Tomato cells grown in suspension were stressed according to different protocols. (A) Heavy metal stress was achieved by adding CdCl2 to the final concentration of 100 µM to the culture medium at day 2 of culturing. At times indicated cells were harvested. (B) Heat shock was given subsequently to 3 days old tomato cell cultures using the following two types of treatment: hs I: 1 h 37 °C; 2 h 38,5 °C; 1 h 40 °C; hs II: 15 min 40 °C; 2.5 h 25 °C; 2 h 40 °C. (C) Phosphate deprivation (-P) was achieved by lack of KH2PO4 in the culture medium. Cells were harvested after 3 days of culturing. Western blot analysis was performed with an antiserum directed against an internal peptide of tomato Ap4A hydrolase (anti-Ap4A-H) or with a HSP 17-specific antiserum (anti-HSP 17), respectively. Equal amounts of protein (15 µg) were subjected to SDS-PAGE and blotted onto nitrocellulose.

Acknowledgments

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Lazewski D. and Guranowski A. (1990), P·chiral phosphorothioate analogues of bis(5'-adenosyl)tetraphosphate (Ap4A); their enzymatic synthesis and degradation. Nucleic Acids Res. 18, 6083–6088.


