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METABOLISM OF DIADENOSINE TETRAPHOSPHATE (Ap_4A) AND RELATED NUCLEOTIDES IN PLANTS; REVIEW WITH HISTORICAL AND GENERAL PERSPECTIVE

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1. ABSTRACT

This review presents our knowledge of potential biochemical conversions of minor mononucleotides, such as adenosine-5′-tetraphosphate (p_4A) and adenosine-5′-pentaphosphate (p_5A), and dinucleotides, such as diadenosine-5′,5‴-P^1,P^3-triphosphate (Ap_3A) and diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), in plants. Although the occurrence of p_4A, Ap_3A and/or Ap_4A has been demonstrated in various bacteria, fungi and animals, identification of these compounds in plants has not been reported as yet. However, the ubiquity of both the compounds and enzymes that can synthesize them (certain ligases and transferases), the demonstration that certain plant ligases can synthesize p_nAs and Ap_nNs *in vitro*, and the existence in plants of specific and nonspecific degradative enzymes strongly suggest that these various p_nNs and Np_nN′s do indeed occur and play a biological role in plant cells. In fact, some of the plant enzymes involved in the synthesis and degradation of these minor mono- and dinucleotides have been studied even more thoroughly than their counterparts from other organisms.

2. INTRODUCTION

Two homologs of ATP, adenosine 5′-tetraphosphate (ppppA or p_4A) (Figure 1A) and adenosine 5′-pentaphosphate (pppppA or p_5A), represent naturally occurring nucleoside 5′-polyphosphates (p_nNs) while diadenosine-5′,5‴-P^1,P^3-triphosphate (ApppA or Ap_3A) and diadenosine-5′,5‴-P^1,P^4-tetraphosphate (AppppA or Ap_4A), (Figure 1B), are the most thoroughly investigated dinucleoside polyphosphates (Np_nN′s, where N and N′ are 5′-*O*-nucleosides and n represents the number of phosphate residues in the polyphosphate chain that links N with N′ esterifying the nucleosides in their 5′ positions). Although p_4A, the closest homolog of ATP, has been known for fifty years, it has not attracted much attention from biochemists. We know more about Np_nN′s than about p_nNs but our knowledge of the biochemistry and biological roles of these two groups of compounds is scarce. For earlier reviews see refs. 1-10.

As far as plants are concerned, although neither p_nNs nor Np_nN′s have yet been identified in such material, we do have quite a comprehensive understanding of plant enzymes that can synthesize $p_{4\text{-}5}A$s and $Ap_{3\text{-}5}A$s and of specific and non-specific degradative enzymes. This suggests indirectly that these compounds must occur in plant cells and that their levels are subjected to subtle regulation.

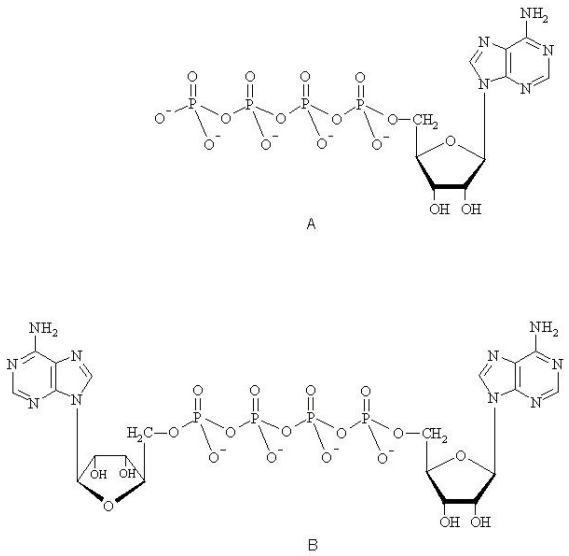

Figure 1. Structures of adenosine 5′-tetraphosphate (A) and diadenosine tetraphosphate (B).

3. MONONUCLEOSIDE POLYPHOSPHATES

3.1. Discovery of nucleoside-5′-polyphosphates

In his short notes, Marrian first expressed the belief that a compound found as a contaminant in bovine muscle ATP preparations was p_4A (11, 12). Subsequently, Sacks reported that, in addition to p_4A, commercial preparations of ATP obtained from brewer's yeast contained p_5A (13). These nucleotides comprised approximately 8% and 1%, respectively, of all adenine-containing compounds. In 1955, Lieberman confirmed Marrian's presumption, identifying p_4A in ATP preparations from horse muscle (14). Contamination of NTPs with the corresponding p_4Ns was then shown in preparations of ATP (15), GTP (16, 17), UTP (18) and CTP (19). The existence of p_4N and p_5N in batches of the corresponding NTPs can be easily demonstrated even on thin-layer chromatograms run in systems designed to separate nucleoside mono-, di- and triphosphates. For example, 3-5-µl aliquots of a 30-50 mM solution of commercially available (preferentially aged) ATP, chromatographed for 90 min in dioxane:ammonia:water (6:1:6, by volume) on silica gel containing a fluorescent indicator, show under short UV light spots of p_4A and p_5A migrating more slowly than ATP; the R_f values are 0.17, 0.11 and 0.31, respectively. (Such separations are shown in refs. 20 and 21). Presumably each NTP undergoes a spontaneous dismutation: NTP + NTP → p_4N + NDP. Such a suggestion has previously been made for ATP and p_4A (15), while the accelerated dismutation of ATP has been observed during a 4-day experiment involving repeated drying and redissolution of a solution containing 150 mM ATP and 50 mM $MgCl_2$ (8). Moreover, p_4A and p_5A have been shown to be minor products of the reaction of AMP-morpholidate with pyrophosphate in pyridine (22).

3.2. Occurrence of p_4A and p_5A in biological material

The first report of the presence of p_4A in biological material was published in 1965 by Heldt and Klingenberg (23) who found this compound in rat liver mitochondria. The following year, Small and Cooper reported p_4A in rabbit and horse muscle (24). Subsequently, p_4A has been detected in rat liver (25), bovine adrenal medulla (26-28), rabbit thrombocytes (29) and yeast (*Saccharomyces cerevisiae*) (30). It has been estimated that in animal cells the concentration of p_4A is between 3 and 4 orders of magnitude lower than the concentration of ATP (approximately 2 µM). Chromaffin granules from the adrenal medulla contain 2.2 ± 0.1 nmole p_4A per mg protein, which corresponds to almost 0.8 mM. This is 200-300 times lower than the concentration of ATP (28).

In an effort to identify signal nucleotides that could function during eukaryotic cell differentiation, Jakubowski showed that the highly phosphorylated nucleosides synthesized only during sporulation of the yeast *S. cerevisiae* were p_4A and p_5A, reaching 2% and 1.5% of the ATP concentration. In logarithmically growing yeast they did not exceed 0.1% of the ATP (30).

3.3. Enzymatic reactions that can lead to the accumulation of p_4A

Enzymes active in p_4A synthesis and the reactions catalyzed by them *in vitro* fall into three categories. The first comprises enzymes that catalyze transfer of a phosphate residue from a phosphate donor on to ATP: adenylate kinase (EC 2.7.4.3), which can transfer P_i from ADP (31), and phosphoglycerate kinase (EC 2.7.2.3), which has been reported to transfer P_i from position 1 of 1,3-diphosphoglycerate (24). (It is presumed that these two ubiquitous kinases function also *in planta*). To the same group belong a mutant succinyl-CoA synthetase (EC 6.2.1.5) from *Escherichia coli*, which can transfer P_i from the enzyme:phosphate complex (32), and the *E. coli* MurD synthetase (EC 6.3.2.9), which can transfer P_i from an enzyme-bound acyl~phosphate (33). The second category of enzymes includes those able to transfer an adenylate residue (-pA) on to tripolyphosphate (P_3). The pA residue comes either from a mixed anhydride such as aminoacyl~pA (lysyl-tRNA synthetase, EC 6.1.1.6) (25, 30), luciferyl~pA (firefly luciferase, EC 1.13.12.7) (34), acetyl~pA (acetyl-CoA ligase, EC 6.2.1.1, from yeast *S. cerevisiae*) (20), acyl~pA (acyl-CoA ligase, EC 6.2.1.3, from the bacterium *Pseudomonas fragi*) (35), aminoacyl~pAs, including derivatives of certain non-protein amino acids (nonribosomal peptide synthetases, the apo-form of tyrocidine synthetase 1, from *Bacillus brevis*) (36) and coumaryl~pA (coumaryl-CoA ligase, EC 6.2.1.12, from *Arabidopsis thaliana*) (21), or from an enzyme~pA complex, as in the case of the DNA- and RNA-ligases from T4 phage (37, 38). The third category includes enzymes that degrade Ap_5A or Ap_6A: (i) phosphodiesterase I (5′-exonuclease) (EC 3.1.4.1), a ubiquitous enzyme which hydrolyzes Ap_5A to p_4A and AMP (pA) (39, 40), (ii) symmetrically acting bacterial dinucleoside tetraphosphatase (EC 3.6.1.41), which will hydrolyze Ap_6A to p_4A and ADP (ppA) (41), (iii) dinucleoside tetraphosphate phosphorylase (EC 2.7.7.53), discovered in brewer's yeast (42) and protozoa (43), which catalyzes the phosphorolytic cleavage of Ap_5A to p_4A and ADP, (iv) dinucleoside triphosphatase (EC 3.6.1.29) occurring in

various phyla (9, 44) including plants (45), which can hydrolyze Ap_5A to p_4A and AMP, and (v) the recently discovered Ap_5A/Ap_6A hydrolases in budding and fission yeasts that split Ap_5A to p_4A and AMP and Ap_6A to p_4A and ADP (46, 47).

3.3.1. 4-Coumarate:coenzyme A ligase (4CL) from *Arabidopsis thaliana* as a "p_4A synthase"

So far, this ligase is the only enzyme thoroughly characterized as a p_4A synthase (21). Both the recombinant protein of the native 4CL2 isoform from *A. thaliana* (At4CL2 wild type) and the At4CL2 gain of function mutant M293P/K320L, which exhibits the capacity to use a broader range of phenolic substrates, catalyzed the synthesis of p_4A and p_5A when incubated with $MgATP^{2-}$ and tripolyphosphate or tetrapolyphosphate, respectively. Adenosine polyphosphate synthesis was either strictly dependent on or strongly stimulated by the presence of a cognate cinnamic acid derivative. Coumarate was the most effective cofactor for p_4A synthesis catalyzed by the wild-type ligase (k_{cat} = 0.42 s^{-1}) and ferulate for the mutant form (k_{cat} = 0.52 s^{-1}). For the wild-type ligase, ferulate was over 83-fold, cinnamate 50-fold and caffeate over 8-fold less effective than coumarate, while sinapate was inactive as a cofactor. The At4CL2 mutant M293P/K320L was stimulated by coumarate only slightly less than by ferulate (in 92%) with caffeate, cinnamate and sinapate being 69%, 45% and 4% as effective, respectively. Interestingly, the mutant form was able to support p_4A synthesis also in the absence of exogeneous phenolic acid, synthesis proceeding 25-fold more slowly than in the presence of ferulate. This suggests that there may also be an alternative mode of adenylate transfer from ATP on to tripolyphosphate *via* an enzyme~adenylate intermediate. In the presence of ferulate the mutant ligase synthesized p_5A at a rate 10-fold lower than p_4A. The maximum rate of p_4A synthesis was observed between pH 6 and 8 and the reaction was supported by the following cations: $Mg^{2+} > Mn^{2+} = Co^{2+} > Ni^{2+} > Zn^{2+}$. The K_m values estimated for wild-type 4CL2 for coumarate and caffeate were 22 μM and 1.4 μM, respectively while the values estimated for the mutant for ferulate, ATP and tripolyphosphate were 2.7 μM, 150 μM and 4.9 mM, respectively.

Coenzyme A, even at low micromolar concentrations, strongly inhibited the synthesis of p_4A catalyzed by At4CL (Guranowski and Pietrowska-Borek, unpublished observations).

3.4. Enzymes that can degrade p_4A

Enzymes that degrade p_4A can be classified either with respect to their substrate specificity or to the moiety in the p_4A substrate that is recognized by the active site. Phosphatases and apyrase are *exo*phosphatases that exhibit low substrate specificity and recognize the oligophosphate chain. Alkaline (EC 3.1.3.1) or acid (EC 3.1.3.2) phosphatases remove successive P_i residues, finally converting p_4A to $4P_i$ and adenosine. Apyrase (EC 3.6.1.5) initially catalyzes the relatively slow removal of the δ phosphate and the subsequent rapid removal of the γ and β phosphate residues, leaving AMP as the final product (48). Through recognition of the nucleotide moiety, non-specific phosphodiesterase I (EC 3.1.4.1) catalyzes hydrolysis of p_4A, generating AMP as one of the reaction products. Non-specific adenosine-phosphate deaminase (EC 3.5.4.17) from the snail *Helix pomatia* and fungus *Aspergillus oryzae* (49) is another enzyme that can act upon p_4A, converting it to inosine 5′-tetraphosphate (p_4I). Various hydrolases from the nudix protein family can also hydrolyze p_4A: asymmetrically acting Ap_4A hydrolase (EC 3.6.1.17) (45, 50-52) and the recently discovered Ap_5A/Ap_6A hydrolases (46, 47). Because these enzymes recognize the ATP moiety in their substrates (53), they degrade p_4A to ATP and P_i, acting as *endo*phosphatases. Cleavage of p_4A to ATP and P_i was first observed for the (*asymmetrical*) Ap_4A hydrolase from yellow lupin seeds (45) and confirmed in recent studies of the substrate specificity of the orthologous enzyme from the bacterium *Bartonella baciliformis* (50), the nematode *Caenorhabditis elegans* (51) and of a nudix hydrolase encoded by the g5R (D250) gene of African swine fiver virus (52).

Symmetrically acting dinucleoside tetraphosphate hydrolase (EC 3.6.1.41), which always liberates an NDP from its substrates (53), can degrade p_4A to ADP and presumably PP_i (41, 54, 55).

A much more specific enzyme that recognizes p_4A is the exopolyphosphatase (EC 3.6.1.11) from brewer's yeast (56) and bacteria (57). This enzyme prefers long-chain-polyphosphates as substrates but also hydrolyzes tetra- and tripolyphosphates and, as has been shown recently, p_4A and p_5N (58-61). However, the most specific p_4A-degrading enzyme is nucleoside tetraphosphate hydrolase (EC 3.6.1.14) found in mammals (62, 63) and higher plants (64). As has been shown for the homogeneous enzyme from yellow lupin seeds, it degrades p_4A to ATP and P_i. In contrast to the lupin (*asymmetrical*) Ap_4A hydrolase, the water molecule attacks the substrate within the oligophosphate chain and not at the ATP-site: p_4N hydrolase degraded p_5A to ATP and $2P_i$ whereas the Ap_4A hydrolase cleaved this compound to ATP and PP_i (64). Reactions that may be involved in the metabolism of p_4A in plant cells are shown in Figure 2.

3.4.1. Nucleoside tetraphosphate hydrolase from yellow lupin seeds

This plant enzyme is so far the only p_nN hydrolase purified to electrophoretic homogeneity. It has been purified from lupin seed meal by ammonium sulfate fractionation (50-70% saturation), ion-exchange chromatography on DEAE-Sephacel column, gel filtration (Sephadex G-200), chromatography on a dye-ligand column (Black C-2) and hydrophobic-interaction chromatography (Toyopearl butyl-650S). Lupin p_4N hydrolase is a single polypeptide chain of 25 ± 1 kDa. The following cations function as cofactors: $Mg^{2+} >> Co^{2+} > Ni^{2+} > Mn^{2+}$. Optimum pH is 8.2, the K_m for p_4A 3 ±0.6 μM and the k_{cat} is 8.5 s^{-1} [at 30°C, in 50 mM Hepes/KOH (pH 8.2), 5 mM $MgCl_2$ and 0.1 mM dithiothreitol]. Both p_4A and p_4G are hydrolyzed at the same rate and p_5A 200-fold more slowly, sequentially liberating two molecules of orthophosphate. Zn^{2+}, F^- and Ca^{2+} are inhibitory, with estimated I_{50} values of 0.1 mM, 0.12 mM and 0.2 mM, respectively. In addition to nucleoside polyphosphates, this enzyme hydrolyzes tripolyphosphates, but neither pyrophosphate nor tetraphosphate.

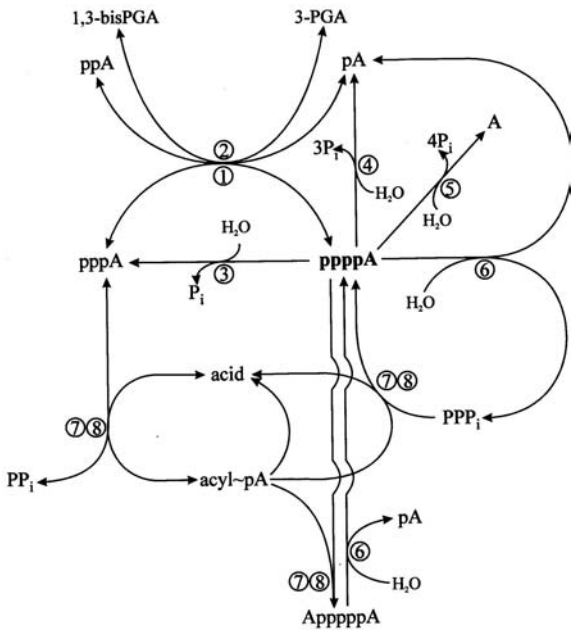

Figure 2. Reactions and relevant enzymes potentially involved in the synthesis and degradation of adenosine tetraphosphate in the plant cells. Numbers in circles represent: 1, adenylate kinase (EC 2.7.4.3); 2, phosphoglycerate kinase (EC 2.7.2.3); 3, nucleoside tetraphosphate hydrolase (EC 3.6.1.14); 4, apyrase (EC 3.6.1.5); 5, alkaline (EC 3.1.3.1) or acid phosphatese (EC 3.1.3.2); 6, phosphodiesterase I (EC 3.1.4.1); 7, phenylalanyl-tRNA synthetase (EC 6.1.1.20) or any other ligase of the EC 6.1.1. subsubclass; and 8, coumarate:CoA ligase (EC 6.2.1.12)

4. DINUCLEOSIDE POLYPHOSPHATES

4.1. Discovery of Ap_4A and other Np_nN's *in vitro*

Chronologically, Np_nN's were first discovered by chemists. In 1953, Todd's laboratory reported the detection of Ap_2A and Up_2U as by-products of reactions designed to synthesize ApU (65). Then in 1965-66, Moffatt and co-workers reported that various Ap_nAs (22, 66) and Gp_nGs (67) accumulated as highly stable end products of the dismutation of ATP and GTP in pyridine or pyridine-containing solvents. However, the most important finding for biochemists was that lysyl-tRNA synthetase is able to transfer the adenylate moiety from the lysyl~AMP intermediate onto various acceptors possessing a pyrophosphate moiety, including ADP and ATP (68). Since then, it has been shown that other aminoacyl-tRNA synthetases, other types of ligases and, certain transferases have the ability to catalyze the synthesis of Np_nN's (see below).

4.2. Occurrence of Ap_4A, Ap_3A and other Np_nN's in biological material

Two years after discovering Ap_4A *in vitro*, Zamecnik and co-workers first demonstrated the biosynthesis of Ap_4A in rat liver slices after incubating them with [^{14}C]adenine (25). Thereafter, Ap_4A and various other Ap_4Ns and Ap_3Ns were found at submicromolar to micromolar concentrations in virtually all cells and tissues examined, including bacteria, protozoa and animals (1). Ap_3A was first demonstrated in 1983 in the slime mold *Dictyostelium discoideum*, BCC-1 epithelial monkey cells and Ehrlich ascites tumor cells (69). In addition to Ap_4A, the presence of six non-adenylylated Np_4N's (Cp_4C, Cp_4G, Cp_4U, Gp_4G, Gp_4U and Up_4U) was reported in yeast *S. cerevisiae* and *E. coli* (70).

Embryonic cysts or eggs of crustaceans such as the brine shrimp *Artemia franciscana* (71) and *Daphnia magna* (72) contain abundant amounts of the guanine-containing Np_nN's, Gp_4G and Gp_3G, which probably serve as a reservoir of purine nucleotides for the developing organism (see 73 for review). Interestingly, the concentrations of Ap_4Ns and Ap_3Ns increase dramatically when cells are exposed to various kinds of stress (70, 74-80). Moreover, certain cells and cellular subfractions are particularly rich in Np_nN's. These include blood platelets (81, 82), granules within the chromaffin cells of the adrenal medulla (83, 84) and synaptic terminal secretory granules (85).

So far, the existence of Np_nN's in plant tissues has not been demonstrated. This may be partially explained by the high levels of various secondary metabolites in plants that interfere during estimation of Np_nN's when using procedures developed for the estimation of these compounds in extracts from bacteria, yeast and animals.

4.3. Enzymatic reactions that can lead to the accumulation of Np_nN's

Adenine-containing Np_nN's (Ap_nNs) can be synthesized by some ligases, transferases and firefly luciferase, the last being classified as an oxidoreductase but behaving in Ap_nN synthesis as a ligase (34). All enzymes able to produce p_4A (see section 3.3) can synthesize Ap_nNs, although some of them, e.g. the yeast acetyl-CoA ligase, do it at a very low rate (20). Moreover, not all can synthesize Ap_3A (Ap_3Ns). ADP was not an adenylate acceptor for the *Arabidopsis* coumarate:CoA ligase (21) and a very poor one for firefly luciferase (86). Among the aminoacyl-tRNA synthetases, lysyl- (EC 6.1.1.6), phenylalanyl- (EC 6.1.1.20), alanyl- (EC 6.1.1.7) and prolyl- (EC 6.1.1.15) tRNA synthetases are the most active in Ap_nN synthesis (2, 87) and all of them are strongly activated by Zn^{2+}, regardless of enzyme origin. Less active are histidyl-, leucyl-, isoleucyl-, seryl-, aspartyl- (88), glycyl- (89) and threonyl-(90) tRNA synthetases, while the tryptophanyl- and arginyl- ones proved to be inactive in this process (88). It should be mentioned here that not all kinds of aminoacyl-tRNA synthetase have been tested as potential "Ap_nN synthases".

So far, only three plant enzymes have been shown to synthesize Ap_nNs *in vitro*: phenylalanyl- and seryl-tRNA synthetases from yellow lupin seeds (91) and the coumarate-CoA ligase from *A. thaliana* (21) (see below; sections 4.3.1. and 4.3.2).

There is also a brief report of Ap_4A synthesis by three phenylalanyl-tRNA synthetases purified from *Euglena gracilis* chloroplasts, mitochondria and cytosol.

The reaction catalyzed by these three (iso)enzymes is also strongly stimulated by Zn^{2+} (92).

In the yeast *S. cerevisiae* Ap_nNs can also be synthesized by Ap_4A phosphorylase (EC 2.7.7.53) which in the reverse reaction transfers adenylate from either ADP (42, 93) or adenosine 5′-phosphosulfate (94) onto ATP or other NTP *via* a covalent enzyme~AMP intermediate (95). In addition to Ap_4G, which can be formed by the enzymes mentioned above, this and other Gp_nNs can be synthesized by a unique enzyme, GTP:GTP guanylyltransferase (EC 2.7.7.45), demonstrated first in *Artemia* (96). It catalyzes transfer of GMP- *via* an enzyme~GMP intermediate onto GTP, GDP or other acceptors, yielding Gp_4G, Gp_3G or Gp_nN, respectively (97). Recently, another transferase, the uridine triphosphate:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) from *S. cerevisiae* has been found to synthesize Up_nNs, including Ap_4U and Ap_5U, by transferring the UMP-moiety from UDP-glucose onto ATP and p_4A, respectively (98). This and similar transferases that form NDP-sugars may be responsible for the accumulation of pyrimidine-containing Np_nN′s in the cells demonstrated earlier (70).

4.3.1. Phenylalanyl- and seryl-tRNA synthetases from yellow lupin seeds as "Ap_nN synthases"

For the two decades since 1983, these two synthetases have been the only plant enzymes known to be able to produce Ap_nNs (91). This short section summarizes the data concerning that catalytic ability. After prolonged (16 h) incubation of 40 nM homogeneous lupin phenylalanyl-tRNA synthetase at 25°C in a reaction mixture containing 50 mM Hepes (pH 8.0), 0.1 mM phenylalanine, 0.1 mM $ZnCl_2$ and 10 mM [^{14}C]ATPMg, 71% of the radioactivity was transformed into [^{14}C]Ap_4A, 14% into [^{14}C]Ap_3A, 5% into [^{14}C]ADP with 8% remaining as [^{14}C]ATP. Exogenous inorganic pyrophosphatase enhanced the rate of Ap_4A formation 3-4-fold and tRNA inhibited the reaction. Periodate-oxidized tRNA was a much poorer inhibitor, indicating that the intact 3′-terminus of the tRNA is required for inhibition. $ZnCl_2$ was absolutely required for Ap_4A synthase activity, however it did not affect the rate of Ap_4A formation catalyzed by seryl-tRNA synthetase. Kinetic parameters of the Ap_4A synthase activities were as follows: With phenylalanyl-tRNA synthetase, K_m for ATP was 5 mM, for phenylalanine < 1 μM and for $ZnCl_2$ 15 μM. At a saturating concentration of ATP the rate constant of Ap_4A formation was 1.1 s^{-1} (pH 8.0, 25°C, 10 mM $MgCl_2$, 10 μg/ml inorganic pyrophosphatase). The rate constant of phenylalanyl-adenylate formation was 45 s^{-1}. With seryl-tRNA synthetase, the K_m values for ATP and serine were 3 mM and 15 μM, respectively and k_{cat} 0.06 s^{-1}. In addition to Ap_4A and Ap_3A, both lupin enzymes were able to synthesize Ap_4Ns and Ap_3Ns. Four other lupin enzymes, the alanyl-, arginyl-, tryptophanyl- and tyrosyl-tRNA synthetases did not promote synthesis of Ap_4A.

4.3.2. 4-Coumarate:coeanyzme A ligase (4CL) from *A. thaliana* as an "Ap_4N synthase"

The same variants of At4CL2 that could produce p_4A and p_5A (see section 3.3.1.) were also found to act as an Ap_4N synthase (21). When the At4CL2 mutant M293P/K320L was incubated in the absence of CoA with ATP, which served as both adenylate donor and acceptor, 5 mM $MgCl_2$ and 62 μM ferulate, accumulation of Ap_4A was observed. Maximum rates of Ap_4A synthesis were obtained between pH 6 and 7. The K_m for ATP was 4.1 mM and V_{max} 1.2 nkat mg^{-1}. The enzyme tolerated replacement of ATP with dATP, in which case dAp_4dA accumulated at a comparable rate. With p_4A as the only nucleotide present in the reaction mixture, the enzyme additionally supported the synthesis of Ap_5A. The At4CL2 mutant also synthesized Ap_4C, Ap_4G and Ap_4U when the reaction mixture contained the corresponding NTP in addition to the standard ingredients (ATP, $MgCl_2$ and ferulate) (Guranowski and Pietrowska-Borek, unpublished observations). However, as mentioned above, no Ap_3A synthesis could be detected in a reaction mixture containing ADP as potential adenylate acceptor. The At4CL2 wild-type also catalyzed the synthesis of Ap_4A, albeit at a rate 10-fold lower than the At4CL2 double mutant. The specific activity determined in the presence of 0.1 mM coumarate as activator was 0.12 nkat mg^{-1}.

4.4. Enzymes that can degrade Np_nN′s

4.4.1. Nonspecific enzymes that hydrolyze Np_nN′s

Higher eukaryotes, both animals and plants, possess type I phosphodiesterase, a 5′-exonucleotidase that liberates 5′-NMP from its substrates and for which Np_nN′s are just one group among a much wider range of possible substrates. This non-specific enzyme is probably predominantly responsible for the hydrolysis of Np_nN′s in crude extracts of higher eukaryote tissues. Snake venom phosphodiesterase was originally used to characterize the newly discovered Np_nN′s, both by biochemists (68, 71) and chemists (66). Characterization of phosphodiesterases as enzymes that catabolize Np_nN′s has been carried out using preparations obtained from yellow lupin seeds (45), rat liver (99), human plasma (100) and serum (101), the slime mold *Physarum polycephalum* (102) and bovine adrenal medullary plasma membranes (103). At least in mammals, much of the phosphodiesterase activity is associated with membranes from where it can be extracted with Triton X-100 (99, 103). Also, analysis of three heterologously expressed mammalian members of the ectonucleotide pyrophosphatase/phosphodiesterase family, NPP1, NPP2 and NPP3, as Ap_4A-hydrolyzing enzymes has led to the suggestion that these enzymes are major candidates for the hydrolysis of extracellular Ap_nAs in vertebrate tissues (104).

The yellow lupin phosphodiesterase is a single polypeptide chain of 56 kDa. It does not require Mg^{2+} for activity and was inhibited neither by other divalent cations nor by EDTA (45). The K_m values estimated for Ap_3A and Ap_4A were around 2 μM. Activities called nucleotide pyrophosphatase (EC 3.6.1.9) which hydrolyze Ap_nAs have been demonstrated in potato tuber (105) and *Ph. polycephalum* (102). The potato enzyme did not require divalent metal cations for activity. The molecular mass of the native enzyme (tetramer of 74 kDa subunits) was 343 kDa. The K_m values for Ap_3A and Ap_4A were 50 μM and 200 μM, respectively.

Non-specific adenosine-phosphate deaminases (EC 3.5.4.17) that occur in the snail *H. pomatia* and fungus *A. oryzae* are able to convert adenosine(5′)oligophospho(5′)

adenosines via inosine(5′) oligophospho(5′) adenosines in to inosine(5′) oligophospho(5′) inosines (49).

As has been reported very recently (106), Ap_nAs can be substrates of the ATP *N*-glycosidase from a marine sponge *Axilla polypoides*. This unusual enzyme catalyzes hydrolysis of the *N*-glycosidic bond in any compound containing an adenosine-5′-diphosphoryl- moiety. Thus, Ap_nAs can be depurinated via adenosine(5′) oligophospho(5′) riboses to the ribose(5′) oligophospho(5′) riboses.

4.4.2. Specific Np_nN'-degrading enzymes

Although *in vitro* the Np_nN'-synthesizing enzymes can catalyze the reverse reactions leading to the degradation of Np_nN's in the presence of the cognate organic acid (21, 91), there is no evidence that these reactions can proceed *in vivo* at physiological levels of pyrophosphate. Therefore those ligases will not be considered in this section, which deals exclusively with degradative enzymes. We know of two classes of Np_nN'-degrading enzymes: hydrolases and phosphorylases. The hydrolases discriminate between Np_nN's on the basis of oligophosphate chain length. Thus there are (i) dinucleoside triphosphate hydrolases (EC 3.6.1.29), (ii) two subclasses of dinucleoside tetraphosphate hydrolases, one comprising asymmetrically acting enzymes (EC 3.6.1.17) that catalyze splitting of Ap_4A to ATP and AMP, and the second comprising symmetrically acting hydrolases (EC 3.6.1.41) that split Ap_4A to 2ADPs, and (iii) the recently discovered diadenosine penta- and hexaphosphate hydrolases (for which an EC number has not yet been assigned). The dinucleoside polyphosphate phosphorylases can be divided only with respect to their specificity towards Ap_3A. The yeast (*S. cerevisiae*) enzyme does not split Ap_3A but only Ap_4A and its higher homologs while the phosphorylase from *Euglena gracilis* cleaves both Ap_3A and Ap_4A (107).

Np_3N' hydrolases occur in different organisms — mammals (44, 108), invertebrates (109), bacteria (110), yeast (111) and plants (45, 112). They prefer Np_3N's but can hydrolyze also higher homologs liberating always an NMP as one of the reaction products. As demonstrated for the Np_3N' hydrolase from yellow lupin seeds using $H_2{}^{18}O$, water attacks substrates only at P^{α} , yielding [^{18}O]NMP and [^{16}O]ADP in the case of Ap_3A and [^{16}O]ATP in the case of Ap_4A hydrolysis (53).

(*Asymmetrical*) Np_4N' hydrolases prefer Np_4N's as substrates. They can cleave the higher homologs but do not recognize Np_3N's. An NTP always appears as one of the reaction products and in this case the water molecule attacks the δ phosphate which yields [^{18}O]AMP and unlabeled ATP in case of Ap_4A, and [^{18}O]ADP and unlabeled ATP in case of Ap_5A (53). These hydrolases occur in eubacteria and higher eukaryotes, both animals and plants, and are the most thoroughly investigated Np_nN'-degrading enzymes. The first enzyme reported to specifically degrade Np_nN's was Gp_4G (*asymmetrical*) pyrophosphohydrolase from embryonic cysts of *Artemia franciscana* (113). The same enzyme was then found in rat liver extracts (114). Homogeneous preparations of (*asymmetrical*) Np_4N' hydrolases have now been obtained from human leukemia cells, human red blood cells, human placenta, yellow lupin seeds, encysted *Artemia* embryos, rat liver, the green alga *Scenedesmus obliquus*, firefly (*Photinus pyralis*) tails, tomato cells and narrow-leafed lupin (*Lupinus angustifolius*) seeds (for corresponding references see review, 9). More recently, highly purified or electrophoretically homogeneous preparations of the recombinant (*asymmetrical*) Np_4N' hydrolases have been obtained from a monocotyledonous plant, barley (*Hordeum vulgare*) (115), *C. elegans* (51) and the bacteria *B. bacilliformis* (50, 116), *Salmonella typhimurium* (encoded by the *ygdP* gene) (117) and *Helicobacter pylori* (118).

An Ap_4A hydrolase that catalyzed a symmetrical mode of cleavage was first found in extracts of *P. polycephalum* (54) and, independently, a Co^{2+}-stimulated activity was identified in bacteria (55). (*Symmetrical*) Np_4N' hydrolases prefer Np_4N's but can also hydrolyze Np_3N's and their higher homologs. The enzyme from *E. coli* splits its substrates in such a way as to always produce an NDP as one of the reaction products. Experiments in $H_2{}^{18}O$ showed that it is β phosphate that is attacked during the catalytic process (53). When the enzyme hydrolyzed Ap_3A, Ap_4A, Ap_5A and $Ap_4A\alpha S$ (Ap_spppA), [^{18}O]ADP was always produced with unlabeled AMP, ADP, ATP and ADPαS (p_spA), respectively (53).

For the diadenosine hexaphosphatase from *S. cerevisiae,* Ap_6A was an 8-fold better substrate than Ap_5A (46) and Ap_4A and Ap_3A were not substrates. This yeast enzyme prefers to generate p_4A from both Ap_6A and Ap_5A. However, the substrate "wobbles" in the active site during hydrolysis — Ap_6A yielded p_4A + ADP (76%) and p_5A + AMP (34%). Similarly, Ap_5A yielded predominantly p_4A + AMP (96%) and a small amount of ATP + ADP (4%). Hydrolysis of Ap_6A in $H_2{}^{18}O$ showed that both the AMP and ADP products were ^{18}O-labeled; thus the attack by water was directed either at $P^{\alpha'}$ or, preferentially, at $P^{\beta'}$, respectively. This indicates that the yeast Ap_6A hydrolase preferentially accommodates a p_4A moiety in the substrate-binding site. In this respect the Ap_6A hydrolase from fission yeast *Schizosaccharomyces pombe* differs. The major reaction products were ADP + p_4A. The *S. pombe* enzyme showed only limited activity with Ap_4A and no detectable activity with Ap_3A (47). Finally, an orthologous human Ap_6A hydrolase showed a predominant (80%) route of Ap_6A hydrolysis to AMP + p_5A, with the formation of ADP + p_4A as a more minor reaction (20%). Ap_5A was primarily (at least 96%) hydrolyzed to p_4A and AMP and much less efficiently (4%) to ATP + ADP. Interestingly, all these Ap_6A/Ap_5A hydrolases also exhibit hydrolytic activity towards diphosphoinositol polyphosphates (119). Moreover, the catalytic site of these and some other $Np_{4\text{-}6}N$ hydrolases have been demonstrated to possess pyrophosphatase activity that removes the β phosphate residue from the pyrophosphate moiety in 5-phosphoribosyl-1-pyrophosphate, generating the glycolytic activator ribose 1,5-bisphosphate (120).

The (*asymmetrical*) Np_4N' hydrolases and the Ap_6A/Ap_5A hydrolases belong to nudix (formerly MutT)

protein family while the Np_3N' hydrolases (at least from humans and yeast) are members of the HIT protein family; the latter have a catalytic histidine triad in their active sites. To this latter group also belongs the dinucleoside polyphosphate hydrolase from *S. pombe*, which can hydrolyze various Np_nN's (n = 3-5) (121). Although it prefers Ap_4A as a substrate, structurally it is a member of the HIT family.

Plant enzymes that specifically degrade Ap_3A and/or Ap_4A will now be presented in more detail.

4.4.2.1. Dinucleoside triphosphate hydrolase from yellow lupin seeds

So far, this is the only plant enzyme with this specificity that has been purified to electrophoretic homogeneity and characterized (45, 112). It has been purified from yellow lupin seed meal by ammonium sulfate fractionation (50-70%), ion-exchange chromatography (DEAE-Sephacel), gel filtration (Sephadex G-200) and elution from AMP-agarose (the resin with AMP attached to the matrix at *N*-6 *via* an eight-atom-spacer; Sigma Cat. No. A 3019) with 0.1 mM Ap_3A in 50 mM potassium phosphate (pH 6.8) containing 1 mM $MgCl_2$ (112). The enzyme is a single polypeptide chain of 41 kDa. It exhibits a broad pH optimum for activity (from 5-8), requires Mg^{2+} and is inhibited by Zn^{2+}. In addition to various Ap_3Ns, it hydrolyzes Ap_4A (10-fold more slowly than Ap_3A) and various 5′-mRNA cap analogs. $m^7Gppp(m^7G)$ was hydrolyzed 4.5-fold and m^7GpppC 3-fold faster than Ap_3A. Of four different $m^7GpppNs$, only m^7GpppG was degraded randomly, yielding equal amounts of m^7GDP + GMP and m^7GMP + GDP. Other cap analogs were split with a preference towards generation of m^7GMP. For instance, m^7GpppU was hydrolyzed almost exclusively to m^7GMP + UDP (98-99%) and UMP + m^7GDP (1-2%) and m^7GpppA to m^7GMP + ADP (75-80%) and AMP + m^7GDP (20-25%).

4.4.2.2. Dinucleoside tetraphosphate hydrolases from higher plants

Regardless of their origin, the asymmetrically acting Np_4N hydrolases are the most well known of the specific Np_nN'-degrading enzymes. Among the plant enzymes, the (*asymmetrical*) Ap_4A hydrolase was first identified in extracts of yellow lupin (*Lupinus luteus*) seeds (45). This activity was then demonstrated in the extracts of sunflower and marrow seeds (122), tomato cells grown in suspension (123) and seeds of the narrow-leafed lupin (*L. angustifolius*) (124). The yellow lupin enzyme is a single polypeptide chain of 18-18.5 kDa, the narrow-leafed lupin 19 kDa (125) and the tomato enzyme 20 kDa (123). The cDNA obtained from *L. angustifolius* cotyledons encodes a protein of 199 amino acids and molecular mass 22,982 Da (124). In addition to substrate specificity and low molecular mass, another common feature of the (*asymmetrical*) Ap_4A hydrolases is their susceptibility to fluoride. This anion is a strong and specific noncompetitive inhibitor of all these enzymes and the plant hydrolases are inhibited particularly strongly. The estimated I_{50} values of 2-6 μM (122-124) are among the lowest ones reported for fluoride in any enzymatic system; these are usually in the mM range (122). Very recently, it has been shown that an $ATP{\cdot}MgF_x$ complex can bind strongly to the lupin Ap_4A hydrolase, mimicking the substrate (126). The three-dimensional structure of the lupin Ap_4A hydrolase complexed with $ATP{\cdot}MgF_x$ has been solved and comparative studies showed that the majority of residues involved in substrate binding by the lupin enzyme are conserved in (*asymmetrical*) Ap_4A hydrolases from pathogenic bacteria (50, 116) but are absent in their human counterpart (127).

The three-dimensional structure of the narrow-leafed lupin (*asymmetrical*) Ap_4A hydrolase was the first high-resolution solution structure of an (*asymmetrical*) Ap_4A hydrolase to be determined (128). Although the structure is similar to that of *E. coli* MutT, (129), clear differences were observed. Studies have been also conducted of the active-site residues of this lupin Ap_4A hydrolase (130). Site-directed mutagenesis has been used to characterize the functions of key amino acid residues in the catalytic site of this nudix hydrolase. The results revealed a high degree of functional conservation between lupin Ap_4A hydrolase and the MutT 8-oxo-dGTP hydrolase from *E. coli* (129). E55, E59 and E125 all contributed to catalysis. Mutations of these residues to Q reduced k_{cat} markedly, whereas mutations R54Q, E58Q and E122Q had lesser effects. None of the mutations substantially changed the K_m for Ap_4A but modified the pH-dependence, sensitivity to fluoride and preference of bond cleavage of the asymmetrical substrate analog, 2′-deoxyadenylated(Ap_4A) (130).

Previously, the stereochemical course of hydrolysis of Ap_4A catalyzed by the yellow lupin Ap_4A hydrolase obtained from my laboratory had been established (131). In their elegant studies, Dixon and Lowe showed that the reaction proceeds with inversion of configuration at the phosphorus indicating that the enzyme-catalyzed displacement by water occurs by a direct "in-line" mechanism. The yellow lupin enzyme exhibits optimal activity in the pH range from 7.5-9, requires Mg^{2+} for activity and is inhibited by Ca^{2+}(45). The lupin enzymes also hydrolyze certain Ap_4A analogs and derivatives. In addition to various hybrid Ap_4Ns, and Ap_4A homologs, Ap_5A and Ap_6A (45), they were shown to hydrolyze methylene and halomethylene analogs, such as $AppCH_2ppA$ or $AppCF_2ppA$ (132, 133), P^{α}-phosphorothioate analogs, such as $Ap_4A\alpha S$ (134), cap-analogs, such as $m^{2,7}GppppG$ (135), the aforementioned 2′-deoxyadenylylated(Ap_4A) (130) and 3′-adenylylated(Ap_4A) (136). Among different Ap_4A analogs tested as inhibitors the strongest ones appeared to be the adenosine-5′-*O*-phosphorothioylated polyols (137). Particularly effective for the *L. angustifolius* Ap_4A hydrolase was the non-hydrolyzable di(adenosine-5′-*O*-phosphorothio) erythritol (K_i = 0.15 μM).

Studies on substrate specificity and inhibition shed some light on the substrate requirements and one of the nonhydrolyzable analogs, P^1,P^4-dithio-P^2,P^3-monochloromethylene diadenosine-5′,5‴-P^1,P^4-tetraphosphate that formed an enzyme:ligand complex has been used in studies of the three-dimensional structure of the Ap_4A hydrolase from *L. angustifolius* (128).

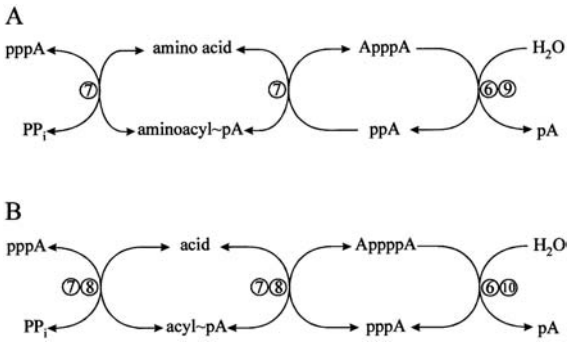

Figure 3. Reactions and enzymes that can be involved in the metabolism of diadenosine triphosphate (ApppA) (A) and/or diadenosine tetraphosphate (AppppA) (B). Numbers in the circles represent: 6, phosphodiesterase I (EC 3.1.4.1); 7, phenylalanyl-tRNA synthetase (EC 6.1.1.20); 8, coumarate:CoA ligase (EC 6.2.1.12); 9, dinucleoside triphosphate hydrolase (EC 3.6.1.29); 10, dinucleoside tetraphosphate (*asymmetrical*) hydrolase (EC 3.6.1.17)

Partial sequencing of the tomato Ap_4A hydrolase and generation of antibodies to particular peptide fragments (123) allowed a monospecific polyclonal antibody to be raised. This was used to show a predominant nuclear location of the Ap_4A hydrolase in 4-day-old cells of a tomato cell suspension culture (138). Microscopic analysis of the distribution of the Ap_4A hydrolase at different stages of the cell cycle visualized by parallel 4,6-diamidino-2-phenylindole staining, revealed that the protein accumulates within nuclei of cells in the interphase but is absent in the nucleus as well as cytoplasm during all stages of mitosis.

Interconversions between adenine mononucleotides and diadenosine polyphosphates that can occur in plant cells are shown in Figure 3.

5. CONCLUDING REMARKS AND PERSPECTIVES

As has been shown in this review, mononucleoside polyphosphates and dinucleoside polyphosphates are ubiquitous compounds and although their existence in plant cells has yet to be demonstrated, plants possess both the anabolic enzymes responsible for p_nN and Np_nN' synthesis, and the catabolic enzymes, some of which are highly specific, that degrade these minor dinucleotides to NTP, NDP and/or NMP that are found in other organisms. Therefore results obtained in studies of the biochemistry of these compounds in plant systems may be of wider significance. A good example is the study of (*asymmetrical*) Ap_4A hydrolases whose catalytically active nudix motif proved to be conserved in various related enzymes.

It is plausible that plants are even more active with respect to the synthesis of p_nNs and Np_nN's than other organisms since, in addition to aminoacyl-tRNA synthetases, they possess coumarate:CoA ligase. This typically plant enzyme, essential for phenylpropanoid metabolism, has to be particularly active for the efficient synthesis of such important compounds as lignins and flavonoids. I anticipate the demonstration of other p_nNs and/or Np_nN' "synthases" in plants. These could be transferases involved in the metabolism of carbohydrates that use NDP-sugars as intermediates. At certain stages of plant development and in certain organs, the reactions leading, for example, to starch accumulation have to proceed very intensively. However, in the event of an inadequate supply of either acyl- or sugar acceptors, these transferases may transfer the nucleotide moiety (AMP- or UMP-) from the accumulating acyl~pN intermediates on to polyphosphates or nucleoside polyphosphates. This suggestion will require experimental verification since, as has been shown earlier (21), not all enzymes (ligases) that catalyze the reversible transfer of the nucleotidyl- moiety from NTP on to the cognate organic acid, such as an amino acid, fatty acid, phenolic acid or sugar-phosphate, with concomitant release of PP_i, are able to transfer the NMP-moiety onto polyphosphates and/or nucleoside polyphosphates.

So far, there has not been a single report describing the biological effects exerted by p_nNs or Np_nN's on plant cells. A variety of intriguing effects exerted by these compounds have been shown in animal and microbial systems, however (8), and these should inspire plant physiologists to test p_nNs or Np_nN's in experimental plant models. I hope that this review will encourage somebody to do so.

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