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Metabolism of Cytokinin[1]

DEPHOSPHORYLATION OF CYTOKININ RIBONUCLEOTIDE BY 5′-NUCLEOTIDASES FROM WHEAT GERM CYTOSOL

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ABSTRACT

Two forms (F-I and F-II) of 5′-nucleotidases (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) which catalyze the dephosphorylation of N^6-(Δ^2-isopentenyl)adenosine 5′-monophosphate and AMP to form the corresponding nucleosides were partially purified from the cytosol of wheat (*Triticum aestivum*) germ. Both the F-I (molecular weight, 57,000) and F-II (molecular weight, 110,000) 5′-nucleotidases dephosphorylate the ribonucleotides at an optimum pH of 7. The K_m values for the cytokinin nucleotide are 3.5 micromolar (F-I enzyme) and 12.8 micromolar (F-II enzyme) in 100 millimolar Tris-maleate buffer (pH 7) at 37 C. The F-I enzyme is less rapidly inactivated by heating than is the F-II enzyme. Both nucleotidases hydrolyze purine ribonucleoside 5′-phosphates, AMP being the preferred substrate. N^6-(Δ^2-isopentenyl)Adenosine 5′-monophosphate is hydrolyzed at a rate 72 and 86% that of AMP by the F-I and F-II nucleotides, respectively. Phenylphosphate and 3′-AMP are not substrates for the enzymes. It is proposed that dephosphorylation of cytokinin nucleotide by cytosol 5′-nucleotidases may play an important role in regulating levels of "active cytokinin" in plant cells.

The occurrence of cytokinin ribonucleotide and cytokinin ribonucleoside in plant cells is well documented (2, 4, 5, 10, 11). The cytokinin ribonucleotide can be formed from cytokinin base (3, 11, 18, 20), the ribonucleoside (3, 7, 17, 18) or turnover of cytokinin-containing tRNA (13, 22). Alternatively, the ribonucleotide i^{6}Ado-5′-P[2] can be synthesized by a *de novo* pathway from the simple metabolites AMP and Δ^2-isopentenylpyrophosphate (6, 24). In the *de novo* biosynthetic pathway using a crude enzyme preparation, cytokinin ribonucleoside was also formed. Thus, 5′-nucleotidases which are capable of dephosphorylating the ribonucleotide may be contained in the crude enzyme preparation. The 5′-nucleotidase has been isolated from animal cell membrane (8, 9, 14) and cytosol[3] (19). Although the precise physiological function of this enzyme system in plant and animal cells is still unclear, the 5′-nucleotidase may play a role in the regulation of cytokinin metabolism.

We describe here the partial purification of 5′-nucleotidase from wheat germ cytosol, the properties of the enzyme, and kinetics of the dephosphorylation of cytokinin ribonucleotide by this enzyme system.

MATERIALS AND METHODS

CHEMICALS AND ENZYMES

Ado, AMP, i^{6}Ado, concanavalin A (grade IV), carbowax (mol wt, 6,000), 5′-nucleotidase (*Crotalus adamteus* venom), and wheat (*Triticum aestivum*) germ were from Sigma; i^{6}Ado-5′-P was from P-L Biochemical Co., and [8-^{14}C]Ado (59 mCi/mmol) was from Amersham-Searle Corp.

ANALYTICAL TECHNIQUES

Protein concentration was determined according to the method of Bradford (1). A Cary model 14 spectrophotometer was used to quantify purine nucleosides and nucleotides. Dephosphorylated nucleosides were separated from the corresponding nucleotides by paper electrophoresis (Camag TLE Cell, Whatman No. 3MM paper) with 0.5 M Tris-citrate buffer (pH 3.5) at 20 C and by paper chromatography (Whatman No. 3MM) in a descending fashion using the following solvent systems (v/v): A, 95% ethanol: 100 mM $(NH_4)_3BO_3$ (pH 9.0) (1:9); B, ethylacetate:1-propanol:H_2O (4:1:2); C, 1-propanol:concentrated NH_4OH:H_2O (60:20:20). Radioactivity was measured in a Nuclear-Chicago Unilux II scintillation system.

EXTRACTION AND FRACTIONATION OF ENZYMES

Wheat germ (45 g) frozen with liquid N_2 was homogenized in a Waring Blendor in 7 volumes/weight of buffer A [50 mM Tris-HCl buffer (pH 7), 10 mM 2-mercaptoethanol, and 12 mM $MgCl_2$] containing polyvinylpolypyrrolidine (4.5 g). The homogenate was filtered through double layers of cheesecloth. The filtrate was centrifuged for 15 min at 15,000*g* and the resulting supernatant was centrifuged again for 20 min at 20,000*g*. The supernatant is referred to as extract. The following steps were employed to further purify the extract:

Step 1. Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the extract to 20% saturation. After 30 min, the precipitate was removed by centrifugation at 20,000*g* for 20 min and the supernatant was brought to 60% by ammonium sulfate. The precipitate, collected by centrifugation at 25,000*g* for 20 min, was redissolved in 20 ml buffer A. The protein solution (136 mg protein) was dialyzed against 2 liters buffer A for 16 h and reduced to 10 ml by carbowax.

Step 2: DEAE-cellulose Chromatography. The concentrated protein solution was applied onto a DEAE-cellulose (Whatman DE-23) column (2.5 × 27 cm) equilibrated with buffer A. The column was eluted with 1.4 bed volumes buffer A followed by linear gradient of NaCl (0 to 0.5 M; total volume, 180 ml) in the

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[2] Abbreviations: i^{6}Ado-5′-P, N^6-(Δ^2-isopentenyl)adenosine 5′-monophosphate; Ade, adenine; Ado, adenosine; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine.

[3] The term "cytosol" is used only in distinction to "membrane bound." The extraction procedures do not rigorously preclude the possibility of contamination by nucleus enzymes.

same buffer. The 5'-nucleotidase fractions were pooled and again concentrated by carbowax.

Step 3: Sephadex G-100 Chromatography. The enzyme solution was filtered through a Sephadex G-100 column (2.5 × 34 cm) equilibrated with buffer A. The column was eluted with the same buffer. The fractions containing 5'-nucleotidase activity were pooled and stored at −20 C.

ENZYME ASSAYS

The 5'-nucleotidase was assayed in the following incubation mixture (total volume, 1 ml): 100 mM Tris-maleate buffer (pH 7), 12 mM $MgCl_2$, 3 mM AMP or other purine ribonucleotide, 1 mg BSA, and the enzyme preparation. After 15 min incubation at 37 C, the reaction was terminated by the addition of 1 ml 10% (w/v) cold trichloroacetic acid. The precipitated protein removed by centrifugation and Pi released was determined by the method of King (16).

Alkaline phosphatase activity was assayed by measuring the release of Pi from AMP. The assay mixture contained 50 mM Tris-HCl buffer (pH 10), 20 mM $MgCl_2$, 1 mg BSA, and 5 mM AMP in a total volume of 1 ml. The reaction was initiated by the addition of the enzyme. Incubation was at 37 C for 10 min and 1 ml 10% (w/v) cold trichloroacetic acid was added to terminate the reaction. The Pi released in the soluble fraction was measured by King's method (16).

Acid phosphatase was assayed according to Joyce and Grisolia (15). The activity of adenosine kinase was assayed by the method described previously (3).

One unit of the enzyme is defined as the amount of enzyme which produces 1 μmol Pi/min under the assay conditions.

RESULTS

PURIFICATION OF 5'-NUCLEOTIDASE

DEAE-cellulose chromatography resolved 5'-nucleotidase into two major peaks of activity (Fig. 1, peaks I and II). The peak I enzyme solution contained alkaline phosphatase, and its activity amounted to about 15 to 20% of that of 5'-nucleotidase activity. Acid phosphatase activity was not detected in these two enzyme peaks (data not shown). Each of the pooled peak I and peak II enzyme fractions was further purified by Sephadex G-100 chromatography. The major enzyme fractions devoid of alkaline and acid phosphatases after the Sephadex G-100 columns were pooled

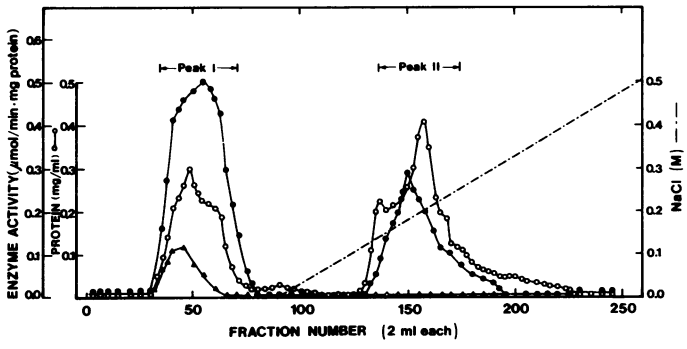

FIG. 1. DEAE-cellulose column chromatography of 5'-nucleotidase from wheat germ cells. Protein solution (9.5 ml, 125 mg protein fractionated by 20 and 60% ammonium sulfate) was applied to a column (2.5 × 27 cm) equilibrated with buffer A [50 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 12 mM 2-mercaptoethanol]. The column was eluted with 175 ml buffer A followed by a linear gradient of 0 to 0.5 M NaCl. One hundred μl of each fraction were used to measure enzyme activities. Conditions for enzyme activity assays are described in the text. (●——●), 5'-nucleotidase activity; (△——△), alkaline phosphatase activity. 5'-AMP was used as the substrate.

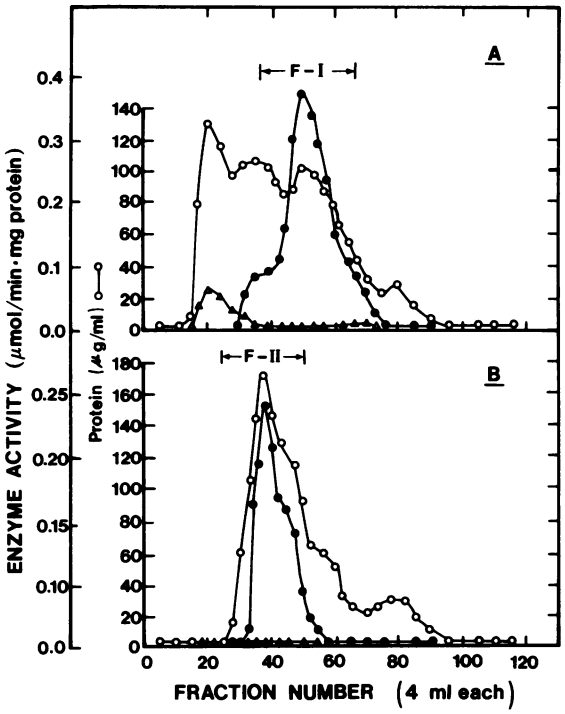

FIG. 2. Purification of wheat germ cytosol 5'-nucleotidases by Sephadex G-100 column filtration. The active fractions of peak I and peak II 5'-nucleotidase obtained from DEAE-cellulose chromatography were applied on Sephadex G-100 columns (2.5 × 34 cm) equilibrated with buffer A. The columns were eluted with the same buffer. The 5'-nucleotidase activity (●——●), alkaline phosphatase activity (△——△), and acid phosphatase activity (results not shown) were determined with aliquots from the indicated fractions as described in the text. 5'-AMP was used as the substrate.

and these repurified peak I and peak II enzymes are referred to as F-I and F-II 5'-nucleotidase, respectively (Fig. 2, A and B).

Rechromatography of the F-I and F-II 5'-nucleotidases on Sephadex G-100 columns showed similar elution profiles, and the enzymes were not purified further. The degree of purification after Sephadex G-100 chromatography was approximately 79- and 63-fold for the F-I and F-II, respectively. No significant adenosine kinase activity was detected in these enzyme preparations; thus, phosphorylation of nucleoside to form nucleotide seems negligible.

From Sephadex G-200 chromatography (2.5 × 30 cm), the relative mobility of the F-I nucleotidase was equivalent to a mol wt of 57,000 and that of the F-II nucleotidase corresponded to a mol wt of 110,000. The molecular weights were calculated from standard molecular weight marker proteins: myoglobin, 17,000; bovine plasma albumin, 66,000; ovalbumin, 43,500; and catalase, 240,000.

The activity of the crude enzyme before DEAE-cellulose chromatography decreased about 10% a day at 4 C, whereas the F-I or F-II enzyme preparation lost less than 5% of the original activity when stored over a period of 5 days at 4 C in buffer A. This enzyme preparation is stable for several months in buffer A containing 20% glycerol at −20 C.

CHARACTERISTICS OF ENZYME ACTIVITY

Optimum pH. Both F-I and F-II 5'-nucleotidases had a pH optimum around 7 with either AMP or i^6Ado-5'-P as a substrate; these data were obtained with 100 mM Tris-maleate buffer at pH values from 5 to 7, and with 100 mM Tris-HCl buffer at pH 7 to 9. The results are illustrated in Figure 3 (only the data of the F-I enzyme activity are shown).

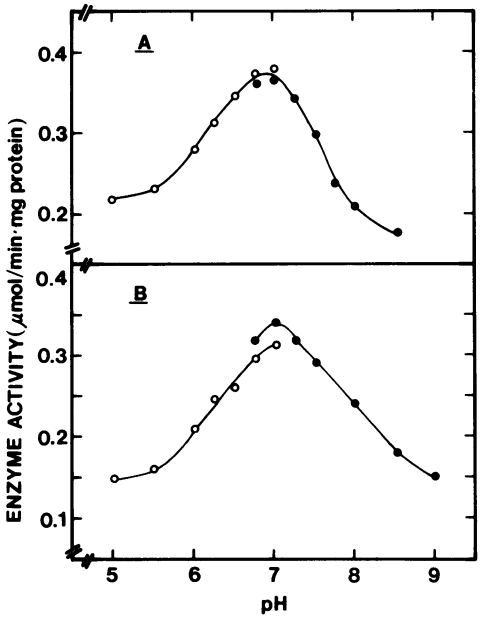

FIG. 3. Effect of pH on AMP (A) and i^{6}Ado-5'-P (B) dephosphorylation by 5'-nucleotidase. Standard assay conditions were used, except that the pH of the buffer was varied. The results for the F-I enzyme activity are shown. The following buffers at indicated pH were used: (○——○), 100 mM Tris-maleate; (●——●), 100 mM Tris-HCl.

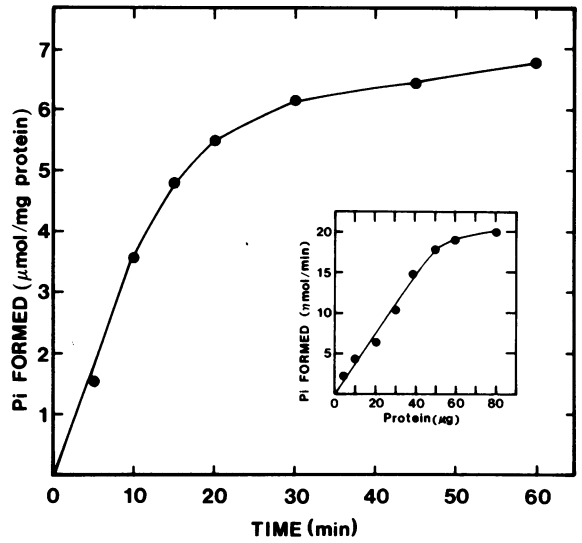

FIG. 4. Time course of i^{6}Ado-5'-P dephosphorylation with the F-I enzyme. Reactions were carried out under standard assay conditions except incubation times were varied. Only the data of the F-I enzyme activity is shown. The effect of protein concentration on i^{6}Ado-5'-P dephosphorylation is shown in the inset.

Dephosphorylation Time Course. The time course studies indicated that the rate of i^{6}Ado-5'-P dephosphorylation by either F-I or F-II 5'-nucleotidase reached a maximum in 10 to 15 min and leveled off (Fig. 4). The leveling off of enzyme activity after 15 min incubation may be due to the limited presence of the substrate, end product inhibition, and/or the deterioration of the enzyme in aqueous solution at 37 C. The linearity of i^{6}Ado-5'-P dephosphorylation, with respect to enzyme concentration, is shown in Figure 4 (inset).

Reaction Products. The liberation of Pi as one of the reaction products is implicit in the assay. Ado or i^{6}Ado was identified as the other product by paper chromatography in three solvent systems. Approximate R_F values for Ado and i^{6}Ado, respectively, were: solvent system A, 0.68 and 0.79; solvent system B, 0.18 and 0.87; solvent system C, 0.57 and 0.88. Paper electrophoretic analysis of the reaction products after 15-min incubation also showed that only i^{6}Ado or Ado was formed from i^{6}Ado-5'-P or from AMP, respectively. To substantiate further the product identification, the putative i^{6}Ado or Ado spot separated by paper electrophoresis was cut and eluted with water. The UV analysis of the eluted products

Table I. *Substrate Specificity of 5'-Nucleotidases*

One-ml reaction mixtures contained 100 μmol Tris/maleate buffer (pH 7), 12 μmol $MgCl_2$, 3 μmol substrate, 1 mg BSA, and 40 μg enzyme. The reaction was carried out at 37 C for 15 min. All rates are relative to the hydrolysis of 5'-AMP. Each value represents the mean of three experiments with two replicates each. The maximum standard error was ±7%.

Substrate (3 mM)	Relative Activity	
	F-I 5'-nucleotidase	F-II 5'-nucleotidase
	%	
5'-AMP	100	100
i^{6}Ado-5'-P	72	86
IMP	79	85
GMP	98	77
ADP	31	40
ATP	34	17
cAMP	25	27
3'-AMP	2	3
Phenylphosphate	0	4

Table II. *Kinetic Constants for 5'-Nucleotidases*

Experimental conditions are as described in Table I, except that the data of K_m and V_{max} were obtained from eight different substrate concentrations.

Enzyme Fraction	K_m		V_{max}		$V_{max}:K_m$	
	AMP	i^{6}Ado-5'-P	AMP	i^{6}Ado-5'-P	AMP	i^{6}Ado-5'-P
	μM		μmol/min·mg protein		ratio	
F-I	3.2	3.5	0.45	0.34	0.14	0.097
F-II	11.5	12.8	0.23	0.17	0.02	0.013

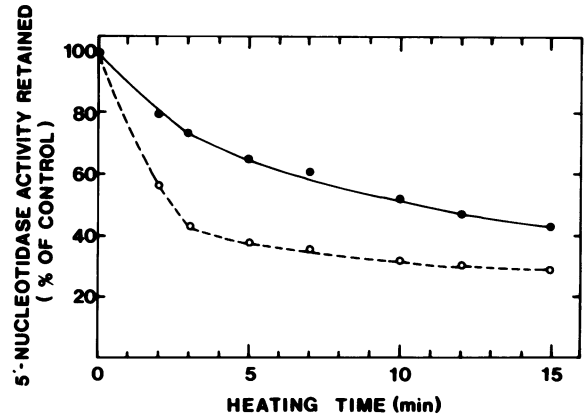

FIG. 5. Heat inactivation of F-I and F-II 5'-nucleotidases. The enzymes were heated at 60 C for the times indicated and then added to the reaction mixtures for 15-min incubation at 37 C. In control experiments, enzymes were not heated to 60 C. AMP was used as the substrate. (●——●), F-I enzyme; (○- - -○), F-II enzyme.

showed λ_{max} was 268 nm for i^{6}Ado and 260 nm for Ado at neutral pH, values identical to those of authentic samples. Thus, the reaction products of i^{6}Ado-5'-P or AMP were indeed i^{6}Ado or Ado in addition to the Pi.

Effect of Divalent Cations. Both F-I and F-II enzymes functioned optimally at a Mg^{2+} concentration of 12 mM under standard assay conditions. $MnCl_2$ at 12 mM was 74% as effective as the same concentration of $MgCl_2$. When 10 mM of $CaCl_2$ was added to an assay mixture containing 12 mM $MgCl_2$, the enzyme activity of the F-I or F-II 5'-nucleotidase decreased to about 70% level of its original activity. It was considered by Gibson and Drummond (12) that Ca^{2+} interacts with Mg^{2+} binding site as a competitive inhibitor of 5'-nucleotidase obtained from certain sources.

Effect of Concanavalin A. Purified membrane 5'-nucleotidase from various cell types has been shown to be inhibited by concanavalin A (*Canavalia ensiformis* lectin) (8). The reported inhibition resulted from a direct binding of concanavalin A with membrane glycoproteins, such as membrane 5'-nucleotidase, having sugars with D-arabinose configuration. To test whether wheat germ cytosol 5'-nucleotidases contains such sugars, various concentrations of concanavalin A (0.1 μM to 5 mM) were incubated with 25-μg of either F-I or F-II protein in the standard assays. Inasmuch as no inhibition of the cytosol 5'-nucleotidase was observed, wheat germ cytosol 5'-nucleotidase is different from enzymes isolated from plasma membrane.

Substrate Specificity. The relative activities of the F-I and F-II enzymes toward a number of nucleotides and other phosphate esters are shown in Table I. Among the nucleotides tested, 5'-AMP was the best substrate. The activities of F-I and F-II enzymes toward i^{6}Ado-5'-P were about 72 and 86%, respectively, that of 5'-AMP. These enzymes specifically hydrolyzed purine ribonucleoside 5'-phosphates, but not 3'-AMP or phenylphosphate. Dephosphorylation of ADP, ATP, and AMP also occurred, but at a lower rate.

The K_m and V_{max} values were calculated from Lineweaver-Burk plots with data from at least five different substrate concentrations (4.2×10^{-7} to 2.1×10^{-4} M) (Table II). At pH 7 and 37 C, using F-I enzyme, the K_m values were calculated to be 3.2 and 3.5 μM for AMP and i^{6}Ado-5'-P, respectively, whereas, using F-II enzyme, the K_m values were 11.5 and 12.8 μM for AMP and i^{6}Ado-5'-P, respectively. The V_{max}:K_m ratio indicate that AMP is about 44 and 54% more efficient as a substrate than i^{6}Ado-5'-P for the F-I and F-II nucleotidase, respectively.

Heat Inactivation. The results above suggest that there are at least two forms of cytosol 5'-nucleotidase present in wheat germ cells. If this is true, then these two forms of enzyme may respond differently to heat inactivation. Figure 5 shows the patterns of heat inactivation of the F-I and F-II 5'-nucleotidase. The F-II enzyme was inactivated in a biphasic manner, with a rapid initial decline in activity followed by a slower rate of inactivation. In contrast, the F-I enzyme was less rapidly inactivated by heating and did not show the biphasic behavior. These results, together with the data of substrate specificity studies, clearly show that F-I and F-II enzymes are multiple forms of 5'-nucleotidases.

DISCUSSION

The results of the study presented here indicate that 5'-nucleotidases from wheat germ cytosol catalyze the dephosphorylation of AMP and i^{6}Ado-5'-P. Kinetics of the isolated 5'-nucleotidase indicated the presence of two enzyme forms in the wheat germ cytosol. Multiple forms of 5'-nucleotidase from the rat mammary gland (9), bovine milk fat globule membrane (14), and *Bacillus subtilis* K (21) also have been reported. These data suggest that more than one form of 5'-nucleotidase from a particular organism is involved in hydrolyzing 5'-purine nucleotides. The properties of enzymes obtained from different sources appear to differ considerably. Gibson and Drummond (12) examined the pigeon heart 5'-nucleotidase and demonstrated that this enzyme was located mainly in the supernatant of 100,000*g* centrifugation, and AMP was the preferred substrate, whereas cytosol 5'-nucleotidase from chicken liver was shown to hydrolyze IMP and GMP more rapidly than AMP (19). The dephosphorylation reaction was not inhibited by concanavalin A; thus, the 5'-nucleotidases isolated from wheat germ are different from membrane-bound 5'-nucleotidase which was shown to be inhibited by concanavalin A (8).

The cytosol 5'-nucleotidases from wheat germ cells showed a specificity for 5'-nucleotides without significant hydrolysis of 3'-nucleotide and phenylphosphate (Table I). AMP and i^{6}Ado-5'-P were dephosphorylated rapidly, whereas the hydrolysis of ADP, ATP, and cAMP proceeded at a lower rate. Although nonspecific phosphatases are generally considered not to be involved in AMP metabolism (23), the extent of dephosphorylation of cytokinin ribonucleotide in plant cells by other phosphatases, such as acid phosphatase, and membrane-bound 5'-nucleotidase remains to be investigated.

Whether cytokinin base *per se* serves as the "active form" of cytokinin has not been unequivocably resolved; however, enzymic regulation of the interconversion of cytokinin nucleotide, nucleoside, and base may play an important role in regulating an adequate level of "active cytokinin" in plant cells. For example, cytokinin base can be catabolized continuously by various enzymes to form adenine and other compounds (11, 13), resulting in a loss of cytokinin base. Such a deficiency must be replenished in order to maintain the levels of available cytokinin. Deribosylation of cytokinin ribonucleoside would provide the needed cytokinin base. The depleted ribonucleoside, in turn, could be synthesized from dephosphorylation of the corresponding ribonucleotide. Thus, dephosphorylation of cytokinin ribonucleotide may be regulated in a complex manner that includes the intracellular concentrations of the "active cytokinin" pool.

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