Cyclic Nucleotide Phosphodiesterase Activity in Barley Seeds\textsuperscript{1,2}

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Received for publication August 1, 1972

ABSTRACT

Barley seeds (Hordeum vulgare L. cv Himalaya) contain an enzymatic activity which catalyzes the hydrolysis of adenosine cyclic 3':5'-monophosphate and adenosine cyclic 2':3'-monophosphate. A large portion of the enzymatic activity is present in the dry seed, existing in both soluble and particulate form. Secretion of the soluble phosphodiesterase from embryoless seeds is enhanced by gibberellin acid and inhibited by abscisic acid, diniotrophenol, and cycloheximide. Attempts to isolate or detect a phosphodiesterase which specifically hydrolyzes adenosine cyclic 3':5'-monophosphate were unsuccessful. Inhibition experiments indicate that probably one enzyme is involved in the hydrolysis of both of these substrates.

During barley seed germination, GA is synthesized by tissues of the embryo and translocated to the aleurone cells, thus acting as a hormonal signal (16, 21, 31). The aleurone cells surround the storage tissue component of the endosperm. They are highly differentiated and respond to the GA stimulus by synthesizing or secreting or both a number of hydrolytic enzymes which degrade the starchy endosperm and provide the developing embryo with essential metabolites. Much information is available concerning the biochemistry of the effect of GA on aleurone cells (6, 7, 9, 28, 29); however, a precise mechanism of action has not yet been elucidated. In contrast, studies on the mechanism of action of animal hormones indicate that in many cases adenosine cyclic 3':5'-monophosphate (cyclic 3':5'-AMP)\textsuperscript{3} acts as an intracellular intermediate (10, 22, 23, 25). Hormones circulating in the bloodstream activate the membrane-bound adenyl cyclase in their target tissues which catalyzes the synthesis of cyclic 3':5'-AMP from ATP. The increased cyclic 3':5'-AMP then brings about the physiological responses of the hormones. The physiological action of cyclic 3':5'-AMP is terminated by its hydrolysis to 5'-AMP via a specific cyclic 3':5'-nucleotide phosphodiesterase (10, 22, 23).

Several investigators have examined the possible role of cyclic 3':5'-AMP in barley seed metabolism (4, 5, 8, 18, 19, 20). Galisky and his colleagues (5, 8, 18) have reported that cyclic 3':5'-AMP promotes the secretion of \( \alpha \)-amylase and other hydrolytic enzymes from barley seeds in a manner similar to that evoked by GA under the same conditions. Pollard (19) has described a procedure which results in the production of a \(^{3}C\)-labeled compound which cochromatographs with cyclic 3':5'-AMP in barley aleurone cells incubated with \(^{3}C\)-adenine. Similar findings with other plant tissues have been reported using the same procedure (17, 24). Preliminary reports on the degradation of cyclic 3':5'-AMP in barley (12, 27) and other plant tissues (11, 14, 30) have also been cited.

The findings on a possible role for cyclic 3':5'-AMP in barley seed metabolism have shown some negative results. Lack of success with attempts to detect adenyl cyclase in barley and pea tissues led Lin and Varner (13) to examine the enzymatic system for the degradation of cyclic 3':5'-AMP in peas. They have characterized and partially purified a cNPDE from pea seedlings which hydrolyzed either cyclic 3':5'-AMP or cyclic 2':3'-AMP. The hydrolysis of both cyclic nucleotides is similar with respect to pH, sulphydrl reagents, temperature, urea, and metal ions. These observations suggest that a single enzyme is involved in the hydrolysis of either substrate. It was concluded that the cNPDE present in pea seedlings may function in the hydrolysis of cyclic 2':3'-AMP during the degradation of RNA (13).

A demonstration of a specific phosphodiesterase for cyclic 3':5'-AMP would constitute supporting evidence for involvement of this nucleotide in plant biochemistry. Because barley seeds have been reported to exhibit a response to exogenous applications of cyclic 3':5'-AMP, it was of interest to determine whether such an enzyme is present in these seeds. Furthermore, information on the development of cNPDE activity and the effect of GA on this development would be of general interest in documenting the biochemical sequence of events in aleurone cells during germination.

MATERIALS AND METHODS

Enzyme Extraction Procedure. Embryoless barley seeds were surface-sterilized in 1% sodium hypochlorite for 20 min, washed with sterile distilled water, and allowed to imbibe for 12 to 18 hr in a sterile petri dish containing 8 ml of water. Ten half seeds were transferred to vials containing 0.1 \( \mu \)M GA, 10 mM calcium chloride, and 10 \( \mu \)g of chloramphenicol. All glass-
ware and solutions were sterilized prior to addition of the embryoless seeds. The vials were placed in a metabolic shaker operating at 40 rpm at 25 C for 48 hr. Aliquots of the incubation medium were withdrawn and assayed directly or frozen at −20 C for later use. cNPDE activity was stable at this temperature for several months. Crude enzyme from embryoless seeds was obtained by homogenization in 4 ml of 10% (w/v) sucrose in a ground-glass homogenizer or with a mortar and pestle. Barley aleurone layers were isolated from embryoless seeds in accordance with previously described procedures (3).

**Assay for Cyclic 2':3'-AMP Phosphodiesterase Activity.** This assay is based on the enzymatic hydrolysis of cyclic 2':3'-AMP to 3'-AMP, which is then converted to adenosine by excess snake venom nucleotidase. The incubation medium consisted of 50 mm MES buffer, pH 6.0, 1 mm cyclic 2':3'-AMP, 1 mm magnesium chloride, 4 mm mercaptoethanol, 50 µg/ml snake venom (Crotalus adamanteus) nucleotidase, and crude barley seed enzyme in a total volume of 0.3 ml. The snake venom nucleotidase (Sigma Chemical Co.) contained no activators or inhibitors for barley cNPDE activity. In all experiments, corrections were made for the small cNPDE activity associated with this commercial preparation. The reaction mixtures were incubated for 120 min at 37 C, and the reactions were terminated by adding 0.03 ml of 50% (w/v) trichloroacetic acid. After centrifugation at 1800g for 10 min, the supernatant fractions were assayed for Pi by a procedure described by Chen et al. (2).

**Assay for Cyclic 3':5'-AMP Phosphodiesterase Activity.** This assay is based on enzymatic hydrolysis of 3'-H-cyclic 3':5'-AMP to a mixture of 3'-AMP and 5'-AMP. The procedure is in accordance with a method described by Thompson and Applemann (26). The nucleotides are subsequently converted to 3'-H-adenosine by excess snake venom nucleotidase. The incubation medium was identical to that used for cyclic 2':3'-AMP phosphodiesterase activity (above), except that 1 mm 3'-H-cyclic 3':5'-AMP (3.3 × 10⁶ cpm/µ mole) was used instead of cyclic 2':3'-AMP. After 120 min at 30 C, the reactions were terminated by adding 1.0 ml of a 1:3 (w/v) slurry of anion-exchange resin (Bio-Rad AG 1–8), and the tube contents were immediately centrifuged at 1800g for 10 min. Charged nucleotides bind to the resin, whereas 3'-H-adenosine remains in the supernatant fluid. Radioactivity in the supernatant fluid was determined in a liquid scintillation spectrometer. When 3'-C-cyclic 3':5'-AMP was used as a substrate the reaction mixtures were chromatographed on paper. After development, the distribution of radioactivity was determined with a radio-chromatogram scanner or liquid scintillation spectrometer.

**Protein Determinations.** Protein content was estimated by the method of Lowry et al. (15).

**Ammonium Sulfate Fractionation and Bio-Gel Chromatography.** Solid ammonium sulfate was added to a crude enzyme preparation (obtained from the incubation of embryoless seeds) until 30% saturation was achieved. After centrifugation, the supernatant was brought to 70% saturation with ammonium sulfate. Suspended material was precipitated by centrifugation at 10,000g for 10 min and redissolved in 0.5 ml of 0.01 M tris-acetate buffer, pH 7.0, and applied to a Bio-Gel P-150 column (0.7 × 30 cm) which had been equilibrated with 0.01 M tris-acetate buffer, pH 7.0. The enzyme was eluted with this buffer and collected in 0.5-ml fractions at a flow rate of 6 ml/hr.

**Paper Chromatography.** Whatman No. 1 filter strips were used, and chromatography was performed at room temperature. For descending chromatography, the solvent systems were ethanol-1 M ammonium acetate, pH 7.4 (7:3) (solvent system I); isopropanol-ammonium hydroxide-0.1 M boric acid (6:1:3) (solvent system II); and saturated ammonium sulfate-0.1 M phosphate buffer, pH 6.0-isopropanol (70:19:2) (solvent system III). Authentic nucleotides and their derivatives were used as markers and located under shortwave ultraviolet light.

**Source of Barley Seeds, Hormones, and Nucleotides.** Barley seeds (Hordeum vulgare L. cv. Himalaya), 1969 harvest, were obtained from the Department of Agronomy, Washington State University, Pullman. Synthetic (RS)-abscisic acid was a gift of the R. J. Reynolds Tobacco Co., Research Department. Gibberellic acid and nucleotides were purchased from Sigma Chemical Co. or Calbiochem. The 3'-H-cyclic 3':5'-AMP and 3'-C-cyclic 3':5'-AMP were purchased from Schwarz/Mann. Paper chromatography of these labeled compounds in solvent system I showed that there were impurities located in the area of authentic adenosine, adenine, and 3'-AMP or 5'-AMP. To remove these impurities the labeled cyclic 3'-AMP, stock solutions were streaked on paper and chromatographed in solvent system I overnight. The cyclic 3'-5'-AMP band was eluted free from bases, nucleosides, and other nucleotides with deionized water (recovery rate of at least 80%) and was sufficiently dilute to use directly in the enzyme assays.

**RESULTS AND DISCUSSION**

**Products of Enzymatic Hydrolysis of Cyclic 2':3'-AMP and Cyclic 3':5'-AMP.** Lin and Varner (13) have established that cyclic 3':5'-AMP is hydrolyzed by a partially purified cNDPE from pea seedlings to a mixture of 5'-AMP and 3'-AMP. This is in contrast to the 3':5'-cNPDE isolated from animal tissues which hydrolyzes cyclic 3':5'-AMP exclusively to 5'-AMP (10). A cNPDE activity is present in embryoless barley seed homogenates and in incubation medium, which similarly catalyzes the hydrolysis of 3'-cyclic 3':5'-AMP to a mixture of 5'-AMP and 3'-AMP and converts cyclic 2':3'-AMP to 3'-AMP. The identity of these metabolites was ascertained by paper chromatography with solvent systems I, II, and III. To enhance the production of 3'-cyclic 3':5'-AMP and 3'-cyclic 5'-AMP, their respective unlabeled nucleotides were added to the reaction mixture. Since nucleotidase activity against 3':AMP and 5'-AMP is present in these homogenates, adenosine is obtained as a major metabolite when either cyclic 2':3'-AMP or cyclic 3':5'-AMP is degraded by crude barley seed homogenates. The rate of 3'-AMP hydrolysis is considerably greater than 5'-AMP nucleotidase activity. Because of the presence of these nucleotidases, it was impossible to determine the exact ratio of 5'-AMP to 3'-AMP in the hydrolysis of 3'-cyclic 3':5'-AMP by cNPDE.

The possibility that a transferase (1) may have catalyzed the formation of 3'-AMP from 5'-AMP (or vice versa) was explored by separately incubating 3'-cyclic 3':5'-AMP and 3'-cyclic 5'-AMP with barley seed enzyme at 30 C for 2 hr and chromatographing the reaction mixtures in solvent system II. No evidence of transferase activity was observed.

**Characteristics of Barley Seed cNPDE Activity.** cNPDE activity against either cyclic 2':3'-AMP or cyclic 3':5'-AMP is present in both the supernatant and pellet fractions after centrifugation of barley seed homogenate at 100,000g for 60 min. The soluble and particulate enzymes exhibit similar pH optima, the same phosphodiesterase hydrolysis products and are similarly affected by EDTA and metal ions. A soluble cNPDE is secreted from embryoless seeds into their surrounding medium. This source of crude enzyme was used to examine some of the characteristics of cNPDE activity or alternatively it was fractionated with ammonium sulfate and passed over a Bio-Gel P-150 column and then used for studies.
Barley cNPDE activity was reduced in the presence of EDTA. The EDTA had no effect on nucleotidase activity. Addition of magnesium chloride or zinc sulfate partially restores activity (Table I). A significant inhibition of cyclic 3':5'-AMP hydrolysis by sodium fluoride is obtained with little or no effect on the hydrolysis of cyclic 2':3'-AMP. A similar observation has been reported for cNPDE from pea seedlings (13).

Tests were also performed to examine the effect of the methylxanthines, caffeine and theophylline, on cNPDE. The crude enzyme preparations of both the soluble and particulate cNPDE activities showed inhibition with both compounds but this property was lost with the purification of the enzyme indicating that their effect may be other than an interaction with cNPDE itself.

**Effect of Imbibition and Plant Hormones on Secretion of cNPDE.** If cyclic 3':5'-AMP hydrolysis is due to the action of an enzyme which is different from that involved in the degradation of cyclic 2':3'-AMP, then there may be distinct patterns in their respective activities during imbibition or in response to hormones which influence enzyme synthesis and secretion in barley seeds. To test this possibility, dry embryoless seeds were incubated in the presence or absence of GA and cNPDE activity was measured after various periods of incubation. The results indicate that cNPDE is present in the dry seed and that its activity increases gradually throughout the 50-hr incubation period (Fig. 3). After 24 hr, cNPDE activity can be detected in the incubation medium and GA increases the amount released (Fig. 3). In this system, the secretion of cNPDE is coincident with RNase release. GA-enhanced release is inhibited by abscisic acid, cycloheximide, or dinitorphenol (Table II). The amount of enzyme secreted into the medium from isolated aleurone layers increases as the concentration of GA is increased to 0.1 μM (Fig. 4), at the same time no secretion of cNPDE activity was detected from the accompanying endosperm tissue. Abscisic acid or GA caused neither direct inhibition nor activation of cNPDE. In each case, patterns were similar for the hydrolysis of either cyclic 3':5'-AMP or cyclic 2':3'-AMP (Table II, and Figs. 3 and 4).

### Table I. Effect of Inorganic Ions on cNPDE Activity

Crude enzyme from the incubation medium surrounding embryoless barley seeds was treated with EDTA and dialyzed prior to the addition of MgCl₂ or ZnSO₄. cNPDE assays were performed as described under “Materials and Methods.” The results are the averages of duplicate determinations.

<table>
<thead>
<tr>
<th>Inorganic Ions</th>
<th>Cyclic 2':3'-AMP</th>
<th>Cyclic 3':5'-AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated¹</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1.5 mM EDTA</td>
<td>34</td>
<td>60</td>
</tr>
<tr>
<td>3 mM MgCl₂</td>
<td>52</td>
<td>96</td>
</tr>
<tr>
<td>0.5 mM ZnSO₄</td>
<td>79</td>
<td>93</td>
</tr>
<tr>
<td>0.001 mM NaF</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.01 mM NaF</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>0.1 mM NaF</td>
<td>97</td>
<td>74</td>
</tr>
<tr>
<td>0.3 mM NaF</td>
<td>91</td>
<td>59</td>
</tr>
<tr>
<td>0.6 mM NaF</td>
<td>89</td>
<td>42</td>
</tr>
<tr>
<td>1 mM NaF</td>
<td>86</td>
<td>36</td>
</tr>
</tbody>
</table>

¹ Enzymatic activities of the untreated enzyme were respectively 27.0 and 3.7 nmol of product/hr.0.01 ml of medium for cyclic 2':3'-AMP and cyclic 3':5'-AMP.
were also employed. Plant Physiol., 52: 281.

zymes Dowex and some centrifugation, gel filtration, has RNase. here.

activity, ATPase. and conditions. Total phosphodiesterase activity per 10 half-seeds or per 1.7 ml of medium surrounding these seeds is shown below. The assays were performed under standard conditions. The following concentrations were used: GA, 0.1 μM; ABA, 25 μM; dinitrophenol (DNP), 0.1 mM, and cycloheximide (CHI) 10 μg/ml.

Table II. Effect of GA, ABA, Dinitrophenol, and Cycloheximide on cNPDE Activity in Embryless Barley Seeds and Their Surrounding Media

Surface-sterilized dry embryoless seeds were transferred directly to the test solutions. cNPDE activities were determined after 48 hr of incubation. Total phosphodiesterase activity per 10 half-seeds or per 1.7 ml of medium surrounding these seeds is shown below. The assays were performed under standard conditions. The following concentrations were used: GA, 0.1 μM; ABA, 25 μM; dinitrophenol (DNP), 0.1 mM, and cycloheximide (CHI) 10 μg/ml.

<table>
<thead>
<tr>
<th>Phosphodiesterase Activity</th>
<th>Pl</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Half-seeds</td>
<td>Medium</td>
</tr>
<tr>
<td>H2O</td>
<td>9.2</td>
<td>1.7</td>
</tr>
<tr>
<td>GA</td>
<td>9.3</td>
<td>5.8</td>
</tr>
<tr>
<td>GA + ABA</td>
<td>8.4</td>
<td>1.7</td>
</tr>
<tr>
<td>GA + DNP</td>
<td>7.9</td>
<td>0.9</td>
</tr>
<tr>
<td>GA + CHI</td>
<td>7.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Attempts to Separate 3':5'-cNPDE Activity. Among the enzymes which might be responsible for the observed cNPDE activity, prominent ones in barley are RNase, 3'-nucleotidase, and ATPase. Bio-Gel fractionation separates cNPDE activity from RNase. The separation of cNPDE from 3'-nucleotidase has been attempted with no success using the following techniques: gel filtration, isoelectric focusing, sucrose density gradient centrifugation, and gel electrophoresis (12). In addition to some of these techniques, heat inactivation, acetone fractionation, cellulose powder (Whatman CF 11) chromatography, and Dowex column (Bio-Rad AG 50-W-4) chromatography were also not capable of effecting a separation from nucleotidase activity nor a separation between 3':5'-cNPDE and 2':3'-cNPDE activity.

In view of the lack of success with these procedures, inhibition experiments were devised to test whether 3'-nucleotidase or ATPase might be responsible for the cNPDE activity. Neither 3'-nucleotidase nor ATPase activities are inhibited by cyclic 3':5'-AMP 1 mM, using substrate concentrations of 0.1 mM for 3'-AMP and ATP. Tests with cyclic 2':3'-AMP could not be made because its rate of hydrolysis at these concentrations is sufficient to produce Pi itself, hence preventing an accurate determination of the 3'-AMP and ATP hydrolysis rates.
via Pi determination. In addition, the results from the experiments with metal ion treatments (Table I) were also obtained concomitantly for the substrates, 3'-AMP and ATP. The rate of hydrolysis of neither of these two substrates was affected by 1.5 mM EDTA. While zinc sulfate restores cNPDE activity from EDTA inhibition, it brings about a drastic reduction of nucleotidase and ATPase activity. These experiments support the possible existence of a true cNPDE with no phosphate or nucleotidase activity, as was demonstrated for pea cNPDE by Lin and Varner (13).

Inhibition of Hydrolysis of Cyclic 2':3'-AMP and Cyclic 3':5'-AMP. The Michaelis constant for cyclic 2':3'-AMP hydrolysis (0.7 mM) differs from that of cyclic 3':5'-AMP hydrolysis (0.4 mM). If one enzyme is involved in the degradation of both nucleotides, then the hydrolysis of one substrate would be expected to be inhibited by the addition of the other substrate. Cyclic 3':5'-AMP competitively inhibits the hydrolysis of cyclic 2':3'-AMP (Fig. 5), and cyclic 2':3'-AMP inhibits the hydrolysis of cyclic 3':5'-AMP.

These results suggest that the soluble cNPDE in barley seeds is due to the action of a single enzyme and that it is nonspecific, catalyzing the hydrolysis of either cyclic nucleotide. If ribonuclease action in plants results in an accumulation of cyclic 2':3'-AMP, this phosphodiesterase may function to complete the degradation of RNA to 3'-AMP as previously suggested (13). Should unequivocal evidence be obtained for cyclic 3':5'-AMP in barley seeds, then it is possible that the soluble barley seed enzyme is involved in its hydrolysis. In a series of preliminary experiments, however, we have been unable to demonstrate the presence of cyclic 3':5'-AMP, adenyl cyclase, or a cyclic 3':5'-nucleotide-dependent protein kinase in barley seeds. The presence of a nonspecific cNPDE, therefore, does not necessarily imply the involvement of cyclic 3':5'-AMP in barley seed metabolism.

Acknowledgments—We wish to express our appreciation to Dr. R. W. Breidenbach, R. M. Sachs, and J. R. Whitaker for their helpful suggestions. We also thank Drs. P. P. C. Lin and J. E. Varner for sending us a copy of their manuscript while it was in press.

LITERATURE CITED