Purification and Characterization of Wheat Germ 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase*

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An activity hydrolyzing 2',3'-cyclic nucleotides to nucleoside 2'-phosphates (2',3'-cyclic nucleoside nucleoside 3'-phosphodiesterase, CNPase; EC 3.1.4.97) was identified over 30 years ago in vertebrate and plant tissues (1, 2). A subsequent finding (3) that the central nervous system is very rich in CNPase raised considerable interest in this enzyme. The brain CNPase has been characterized in detail, found to be of glial origin, and localized predominantly in myelin (for review see Refs. 4 and 5). A similar or identical enzyme is also present, although at much lower levels, in membrane preparations isolated from other mammalian tissues or cell types (4, 5). The physiological function of animal CNPase is unknown.

The first demonstration of the functional role for CNPase activity came with the discovery of an RNA ligase which catalyzes formation of the 2'-phosphomonoester, 3',5'-phosphodiester bond (6, 7). This enzyme, functioning in tRNA splicing and viroid RNA circularization in vitro, has been identified in plants and lower eukaryotes (6-17). It ligates RNA termini bearing the 2',3'-cyclic phosphate and 5'-hydroxyl groups; conversion of 2',3'-cyclic phosphate to 2'-phosphate at the end of RNA, catalyzed by 2',3'-cyclic nucleotide 3'-phosphodiesterase, is one of the initial steps of the ligation reaction (7-11, 17). Studies with purified wheat germ and yeast RNA ligases demonstrated that activities of 2',3'- cyclic phosphodiesterase, RNA ligase, and 5'-hydroxyl polynucleotide kinase (yet another function participating in the RNA ligation process) reside in a single polypeptide (8, 11, 12, 16, 17).

In this paper, we describe the purification and characterization of another protein with CNPase activity from wheat germ. This enzyme exclusively acts on 2',3'-cyclic mononucleotides converting them to nucleoside 2'-phosphates. This is in contrast to the RNA ligase-associated phosphodiesterase which, as demonstrated below, preferentially hydrolyzes 2',3'-cyclic phosphate-terminated oligoribonucleotides.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Raw wheat germ was obtained from General Mills, Vallejo, CA. Prepackaged FPLC columns and protein molecular weight standards were from Pharmacia Biotechnology Inc. and DE52 cellulose and DE81 paper from Whatman. Cellulose plates were obtained from Merck. Radiolabeled oligonucleotides were from Amersham. Tobacco mosaic virus RNA was prepared as described (18). Aprotinin, leupeptin, benzamidine, and phenylmethylsulfonyl fluoride were from Sigma. Polyethyleneglycol 20,000 was obtained from Fluka.

All nucleoside 2',3'-cyclic phosphates (Np), nucleoside 3',5'-cyclic phosphates (NpCp), nucleoside 2'-monophosphates (Np), 3'-monophosphates (NpCp), 2'-monophosphates (NpCp), and ApGp, ApAcp, and ApAcp were obtained from Sigma. GpGp, GpGp, and poly(A,G) were products of Pharmacia Biotechnology Inc.

Enzymes—RNase N1, RNase T1, and T4 RNA ligase were obtained from Sigma, Calbiochem, and Pharmacia, respectively. Calf intestine phosphatase, RNase A, nuclelease P1, and SP8 RNA polymerase were from Boehringer. Wheat germ RNA ligase (ATP-agarose step, Ref. 16) was kindly provided by Dr. L. Pick, H. Furneaux, and J. Hurwitz, Memorial Sloan-Kettering Cancer Center, New York.

Preparation of Radioactive Substrates: (Np)GpGp, GpGp, and GpGp—A mixture of (Np),GpGp oligonucleotides (n = 3-73) was prepared as follows. (a) Unlabeled tobacco mosaic virus RNA was digested extensively with RNase T1 and the 3' terminal phosphates were removed from the resulting oligonucleotides by treatment with CIP (18) followed by phenol extraction and ethanol precipitation. (b) Two μg of the oligonucleotide mixture was incubated with [5'-32P]pCp and T4 RNA ligase to produce (Np),GpGp. Reaction mixture (20 μl) contained 0.01 M Hepes/KOH, pH 8.3, 10 mM MgCl2, 3 mM DTT, 10% dimethyl sulfoxide, 10% glycerol, 40 μM ATP, 8 μM [5'-32P]pCp (specific activity 300 Ci/mmol), and 5 units of T4 RNA ligase (18). After 12 h at 4°C, the reaction was stopped by heating for 5 min at 90°C. (c) In order to generate (Np),GpGp, the reaction volume

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The abbreviations used are: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; CIP, calf intestine phosphatase; p*, 32P-labeled phosphate group; N, any of four (A, C, G, U) nucleosides; Np, Np, Np, Np, and Np, nucleoside 5', 3', 2', and 2', 3'-cyclic phosphate, respectively; TLC, thin layer chromatography; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; BSA, bovine serum albumin; Hepes, 4(2-hydroxyethyl)-1-piperazinene sulfonic acid (Mes, 4-morpholino-3-propane-sulfonic acid; Bis-tris, 2-[bis[2-hydroxyethyl]amino]-2-(hydroxypropyl)-1-propane-1,3-diol; Mops, 3-(N-morpholinopropansulfonic acid; I50, concentration of inhibitor at half-maximum inhibition; FPLC, fast protein liquid chromatography.
of RNase N1 was added. After 30 min at 37°C, SDS was added to a final concentration of 0.1% (w/v), and the reaction was incubated with CIP (5 units, 30 min at 37°C) in order to dephosphorylate any (Np),G>p* generated by RNase N1. The sample was extracted twice with phenol/chloroform/isomyl alcohol (49:49:1) mixture, and the aqeous phase was applied to a 10-ml Sephadex G-25 column equilibrated and eluted with 20 mM ammonium acetate, pH 5.5. Fractions corresponding to P1-labeled oligonucleotides were collected, lyophilized, and resuspended in H₂O. Nuclease P1 digestion and TLC analysis of the resulting product indicated that >98% of label was present in 3' terminal G>p*.

DNA was purified by digestion of one-fourth (~ 1 × 10⁶ cpm) of the resulting (Np),G>p* with nuclease P1 (0.2 μg, incubation for 1 h at 37°C in 50 mM ammonium acetate, pH 5.5). In order to obtain G>p*, the other aliquot of (Np),G>p* was incubated with nuclease P1 as above and then treated with 0.1 unit of CIP (1 h at 37°C) after addition of Tris-HCl, pH 7.5, to 50 mM concentration. Digests were applied to a cellulose plate which was developed in solvent B. Spots corresponding to G>p* and G2',p* were localized by autoradiography and excised, and radioactive substrate and product, respectively, were quantitated by scintillation counting.

One unit of CNPase is taken as the amount of enzyme hydrolyzing, under standard conditions, 1 μmol of A>p in 15 min at 30°C. Kₐ and Vₐₕₐₜ values were determined from Lineweaver-Burk plots. All velocities were calculated by a computer using the initial linear rates.

Assay of RNA Ligase-associated 2',3'-Cyclic Phosphodiesterase Activity—Ionic conditions were essentially as described by Pick and Hurwitz (16). Samples (10 μl) contained 20 mM Tris- HCl, pH 7.9, 150 mM NaCl, 6 mM Mg²⁺ acetate, 2 mM DTT, indicated amounts of RNA ligase, and either 3 mM (~15,000 cpm) radioactive substrate ((Np),G>p*, pG>p*, or G>p*) or 0.25 mM AAAG>p, AG>p, or G>p. After incubation for 1 h at 30°C, the extent of hydrolysis of the latter substrates was estimated by the microassay of P₁ (Ref. 23) as indicated above. Hydrolysis of radioactive substrates was measured by the TLC method as described for CNPase assays.

SDS-Polyacrylamide Gel Electrophoresis—12.5 or 15% polyacrylamide gels were run as described by Laemmli (24). To the protein samples, after boiling in the presence of 2% SDS and 10 mM DTT, iodoacetamide was added to 40 mM concentration and incubation continued for 30 min at room temperature. This was done in order to avoid DTT-caused artifacts appearing after silver staining (25). Silver staining procedure was according to Burk et al. (26).

Molecular Weights—Molecular weight value of the native protein was estimated by gel filtration on a FPLC Superose 12 HR 10/30 column (10 × 30 mm) equilibrated and eluted with buffer C containing 0.01% Triton X-100 and 0.2 mM K⁺ acetate. The following markers were used: BSA (67,000), ovalbumin (45,000), soybean trypsin inhibitor (20,100), and cytochrome c (12,400). Protein markers used for M, estimation from mobility in SDS-polyacrylamide gel electrophoresis are described in the legend to Fig. 3.

Molecular Weights—Molecular weight value of the native protein was estimated by gel filtration on a FPLC Superose 12 HR 10/30 column (10 × 30 mm) equilibrated and eluted with buffer C containing 0.01% Triton X-100 and 0.2 mM K⁺ acetate. The following markers were used: BSA (67,000), ovalbumin (45,000), soybean trypsin inhibitor (20,100), and cytochrome c (12,400). Protein markers used for M, estimation from mobility in SDS-polyacrylamide gel electrophoresis are described in the legend to Fig. 3.

Chromatography—Products of enzymatic reactions were analyzed by cellulose TLC in solvent A (isosweric acid/NH₄OH/H₂O, pH 4.3 (57/38/3)).

Protein Determination—Protein concentration was measured by the method of Bradford (27) using the reagent obtained from Bio-Rad and BSA as a standard.

Protein Inhibitors—Protein inhibitors were added to all buffers utilized during protein purification (16). The following mixture is referred to in the text as protease inhibitor mix: 0.1 mM EDTA, 0.1 mM benzamidine, 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride. Values correspond to final concentrations in the buffers.

Buffer—Buffer A contained 20 mM Tris/acetate, pH 8.0, 0.5 mM DTT, 10% glycerol, 0.01% Triton X-100, and protease inhibitor mix. Buffer B contained 20 mM Bis-tris, pH 6.0, 0.5 mM DTT, 10% glycerol, 0.01% Triton X-100, and protease inhibitor mix.

RESULTS

Purification of 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase

All operations except loading and running of the FPLC columns (steps 4-7, performed at room temperature) were carried out at 0-4°C.

Step 1: 180,000 × g Supernatant—Dry wheat germ (40 g) was ground in a mortar until a fine powder was obtained (10-15 min) and suspended in 150 ml of extraction buffer (50 mM Tris/acetate, pH 7.6, 50 mM K⁺ acetate, 3 mM Mg²⁺ acetate, 0.5 mM DTT) containing the protease inhibitor mix at 10 times the concentration described under "Experimental Procedures." After an additional 5 min of grinding, the suspension was centrifuged at 25,000 × g for 20 min. The resulting supernatant was then centrifuged for 2.5 h at 50,000 rpm in
the Ti-60 Beckman rotor. 140 ml of the 180,000 x g supernatant was collected.

Re-extraction of the 25,000 x g pellet with extraction buffer containing either 1% Triton X-100 or Triton X-100 and 1 M ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity. It appears, therefore, that, contrary to the mammalian CNPase (4, 5), ammonium acetate did not yield substantial additional amounts of CNPase activity.

Step 2: Ammonium Sulfate Fractionation—Solid ammonium sulfate was added to the 180,000 x g supernatant fraction to 35% of saturation. Precipitated protein was removed by centrifugation (15,000 x g, 20 min), and ammonium sulfate was added to the resulting supernatant to 65% of saturation. Precipitated protein was collected by centrifugation, resuspended in 40 ml of buffer A containing 40 mM K+ acetate, and dialyzed against the same buffer (2 x 2 liters).

Inclusion of Triton X-100 in buffers used during this and subsequent steps increased CNPase recovery. In addition, after the protein has been subjected to high concentrations of (NH₄)₂SO₄ (precipitation steps and hydrophobic chromatography; see below), the activity of CNPase was greatly stimulated by the addition of Triton X-100.

Step 3: DEAE-cellulose—Ammonium sulfate fraction (45 ml) was applied to a DE52 column (2.5 x 30 cm) equilibrated with buffer A containing 40 mM K+ acetate. After washing the column with 150 ml of the same buffer, proteins were eluted with 800 ml of linear 40–300 mM K+ acetate gradient in buffer A. Six-ml fractions were collected every 10 min. CNPase activity eluted from 10 ml of Buffer A containing 40 mM K+ acetate and dialyzed against the same buffer (2 x 2 liters).

Separation of CNPase activity into two peaks on DEAE-cellulose was observed during four independent purifications, using different batches of wheat germ. The basis of this separation is not known. It does not appear to be due to an inhibitor eluting between the two peaks since fractions from this region did not inhibit CNPase activity. The separation observed could be due to the existence of different enzyme isotypes, chemical modification, or limited proteolysis which did not destroy the enzymatic activity. CNPase eluting in peaks A and B showed similar substrate specificity (similar Kᵅ-values for A₃-p, similar relative activities with different N₃-p substrates; see below). Neither of the fractions hydrolyzed pA₃-p, pG₃-p, or 3',5'-cAMP (see below).

Step 4: Mono Q at pH 8.0—The concentrated DEAE-cel- lulose fraction (peak B, 15 ml) was divided into four aliquots, and each was subjected to ion exchange chromatography on Mono Q HR 5/5 column (5 x 50 mm) equilibrated with buffer A containing 40 mM K+ acetate. The column was washed with 6 ml of the same buffer, and proteins eluted with 15 ml of a linear 40–520 mM K+ acetate gradient in buffer A. One-ml fractions were collected every 1 min. CNPase activity eluted at 0.3 mM K+ acetate. Active fractions from all four chromatographies were combined and dialyzed for 3 h against 1 liter of buffer B containing 40 mM K+ acetate.

Step 5: Mono Q at pH 6.0—Dialyzed protein from the previous step (10 ml) was applied to Mono Q HR 5/5 column equilibrated with buffer B containing 40 mM K+ acetate. The column was washed with 5 ml of the same buffer, and proteins eluted with 20 ml of a linear 40–424 mM K+ acetate gradient in buffer B. One-ml fractions were collected every 1 min. Fractions containing CNPase activity, eluting at 240 mM K+ acetate, were pooled and dialyzed for 3 h against 1 liter of buffer C containing 1.5 mM (NH₄)₂SO₄.

Step 6: Phenyl-Superose—Dialyzed protein (3 ml) was applied to a phenyl-Superose HR 5/5 (5 x 50 mm) column equilibrated with buffer C containing 1.5 mM (NH₄)₂SO₄. The column was washed with 5 ml of the same buffer, and proteins eluted with 15 ml of a linear decreasing 1.5 to 0 mM gradient of (NH₄)₂SO₄ in buffer C. 0.4-ml fractions were collected every 1 min. Peak fractions eluting between 0.9 and 0.7 mM (NH₄)₂SO₄ were pooled (2.8 ml) and dialyzed against 1 liter of buffer C containing 0.01% Triton X-100 and 0.2 mM K+ acetate. Sample was then concentrated about 5-fold by keeping the dialysis bag in dry polyethylene glycol 20,000 for 2 h and finally dialyzed for 2 h against 0.5 liter of buffer C containing 0.01% Triton X-100 and 0.2 mM K+ acetate.

Step 7: Superose 12—Dialyzed protein (0.5 ml) was subjected to gel filtration on Superose 12 HR 10/30 column (10 x 300 mm) equilibrated with buffer C containing 0.01% Triton X-100 and 0.2 mM K+ acetate. Fractions of 0.4 ml were collected every 1 min. Fractions 39-42 (see Fig. 2) were pooled and stored in aliquots at −70°C. Fractions prior to fraction 39 were not combined with others since they contained trace amounts of a contaminating Mᵦ = 48,000 protein as judged by gel electrophoresis.

The procedure described above resulted in a 16,000-fold purification of CNPase. A summary of the purification procedure is given in Table I.

Properties of the Purified Enzyme

Purified enzyme appeared homogenous on SDS-polyacrylamide gel (Fig. 3). In addition, analysis of the Superose 12 fractions (last step of purification) by SDS-15% polyacrylamide gel electrophoresis indicated that elution of CNPase activity exactly paralleled elution of a polypeptide with an apparent Mᵦ = 24,000 (Fig. 2). A similar molecular weight value was obtained from 12.5% polyacrylamide gel electrophoresis (Fig. 3, calculations not shown). The Mᵦ of native CNPase estimated by gel filtration on Superose 12 columns (see "Experimental Procedures") corresponded to 23,000 (data not shown). It is concluded that wheat germ CNPase is a single polypeptide chain enzyme of approximate Mᵦ of 23,500.

Purified CNPase was stable for at least 5 months when stored at −70°C and was not affected by several cycles of

![Fig. 1. Purification of CNPase by DEAE-cellulose chromatography. Chromatography conditions are described under "Results." The arrow indicates the start of the salt gradient. Fractions containing CNPase activity were pooled as indicated by horizontal bars A and B. Inset, CNPase activity in column effluent. 4 ml of each fraction were incubated with 5 mM A₃-p, and products were analyzed by cellulose TLC as described under "Experimental Procedures." Fractions from other regions of the column did not contain CNPase activity measurable by this assay. Positions of A₃-p, A₁₃-p, and A₃-p markers are indicated.](image-url)
Results. 0.2 μl of each fraction was assayed. Inset, co-elution of the 
M, 24,000 protein band with the CNPase activity. 10 μl of each 
fraction were electrophoresed on SDS-15% polyacrylamide gel. Mo-
lecular weight markers were those indicated in Fig. 3. Only the 
relevant portion of a silver-stained gel is shown. Positions of trypsin 
inhibitor (20,100) and carbonic anhydrase (30,000) markers are in-
dicated.

Mono


do not hydrolyze internucleotide 3',5'- and 
A2'p6'A as substrates). 
Addition of NaCl to 0.2 or 0.4 M concentration inhibited 
the reaction by 34 and 57%, respectively. Addition of DTT or 
N-ethylmaleimide (each 1 mM) was without effect; preincu-
bation of CNPase with 1 mM N-ethylmaleimide for 20 min at 
25 °C was also not inhibitory. EDTA at 5 or 10 mM concentra-
tion did not affect CNPase activity.

The effect of different divalent cations (chloride salts) on 
CNPase activity was tested. At 10 or 500 μM concentrations, 
Co2+, Ni2+, Mg2+, or Ca2+ had no significant effect. Ca2+, Zn2+, 
Fe2+, and Fe3+ had no observable effect at 10 μM concentra-
tion, but were inhibitory at 500 μM (74, 82, 33, and 66% 
inhibition, respectively). Cu2+ was most inhibitory (70 and 
95% inhibition at 10 and 500 μM, respectively). Mn2+ slightly 
stimulated CNPase activity (19, 37, 31, and 23% stimula-
tion

Freezing and thawing. The enzyme was stable for at least 3 
days when stored at 4 °C in buffer C containing 0.2 M K+ 
acetate and 0.01% Triton X-100. Heating of the purified 
enzyme for 10 min at 55 °C did not decrease the activity. 
Heating for 10 min at 65 or 80 °C decreased activity by 20.2 
and 82.2%, respectively. Purified CNPase did not contain 
detectable nonspecific phosphatase activity (assayed with [γ-
32P]ATP and [α-32P]GTP as substrates) and 2',3'- or 
3',5'-nucleotidase activity (assayed with A3'pG, A2'p6'A, and pA, respec-
tively). It also did not hydrolyze internucleotide 3',5'- and 
2',5'-phosphodiesterases (assayed with A3'pG, A2'p6'A, and A2'p6'A as substrates).

Table 1
Steps of purification of wheat germ CNPase

<table>
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<tr>
<th>Purification step</th>
<th>Total protein mg</th>
<th>Specific activity units/mg</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 180,000 × g supernatant</td>
<td>5,455</td>
<td>0.44</td>
<td>100</td>
<td>1</td>
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<td>2. Ammonium sulfate</td>
<td>1,710</td>
<td>1.37</td>
<td>97.5</td>
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<td>3. DEAE-cellulose Peak A</td>
<td>52</td>
<td>17.1</td>
<td>37.1</td>
<td>39</td>
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<td>4. Mono Q, pH 6.0</td>
<td>1.32</td>
<td>818</td>
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<td>5. Mono Q, pH 8.0</td>
<td>0.54</td>
<td>1,553</td>
<td>34.5</td>
<td>4,380</td>
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<tr>
<td>6. Phenyl-Sepharose</td>
<td>0.031</td>
<td>5,466</td>
<td>7.0</td>
<td>12,400</td>
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<td>7. Superose 12</td>
<td>0.018</td>
<td>7,100</td>
<td>5.3</td>
<td>16,100</td>
</tr>
</tbody>
</table>

* One unit corresponds to the amount of enzyme hydrolyzing 1 μmol of A3',pG in 15 min at 30 °C under standard conditions.

The following assays were performed with 5 mM A3',pG as substrate. In 50 mM Tris-HCl, pH 7.0 hydrolysis was linear for up to 5 min, but the inclusion of 0.01% Triton X-100 or 0.01% BSA allowed the reaction to proceed almost linearly for 30 min (Fig. 4B and data not shown). Kinetic parameters (Km, Vmax, effect of temperature; see below) for A3',pG hydrolysis were similar in the presence of Triton X-100 and BSA.

![Figure 2](image)

**Figure 2. Purification of CNPase by gel filtration on FPLC Superose 12 column.** Filtration was performed as described under "Results." 0.2 μl of each fraction was assayed. Inset, co-elution of the M, 24,000 protein band with the CNPase activity. 10 μl of each fraction were electrophoresed on SDS-15% polyacrylamide gel. Molecular weight markers were those indicated in Fig. 3. Only the relevant portion of a silver-stained gel is shown. Positions of trypsin inhibitor (20,100) and carbonic anhydrase (30,000) markers are indicated.

![Figure 3](image)

**Figure 3. SDS-12.5% polyacrylamide gel electrophoresis of CNPase at different purification stages.** The following fractions were analyzed: DEAE-cellulose, 15 μg of protein (lane 1); Mono Q/ 
Phe 8, 1.6 μg (lane 2); Mono Q/pH 6, 2 μg (lane 3); phenyl-Superose, 0.5 μg (lane 4), and Superose 12, 0.2 μg (lane 5). Protein standards in lane M: phosphorylase b (94,000), BSA (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

![Figure 4](image)

**Figure 4. Hydrolysis of A3',pG by the purified wheat germ CNPase. A.** Dependence on enzyme concentration. Assays were in-
cubated for 30 min. B. Kinetics of hydrolysis at 0 (A), 9 (C), 20 (X), 
and 30 °C (O). Assays (15 μl) contained 2.25 ng of CNPase. C. pH 
optimum. Assays (20 μl) containing 3 ng of CNPase were incubated 
for 30 min. The following buffers were used: Mes/NaOH (O), Mops/
NaOH (C), and Tris-HCl (X).

Thereafter, Triton X-100 was included in all reaction mix-
tures.

The rates of A3',pG hydrolysis were similar at 20, 30, and 
37 °C; at 0 and 9 °C, the rates were 45 and 69% of the value 
found at 30 °C (Fig. 4B and data not shown). The amount of 
substrate hydrolyzed was linearly dependent on enzyme con-
etration up to about 2 ng of enzyme per 20 μl, and the 
optimal activity was found at pH 7.0 (Fig. 4).

Addition of NaCl to 0.2 or 0.4 M concentration inhibited 
the reaction by 34 and 57%, respectively. Addition of DTT or 
N-ethylmaleimide (each 1 mM) was without effect; preincu-
bation of CNPase with 1 mM N-ethylmaleimide for 20 min at 
25 °C was also not inhibitory. EDTA at 5 or 10 mM concentra-
tion did not affect CNPase activity.

Reaction Requirements of Purified CNPase

The following assays were performed with 5 mM A3',pG as 
substrate. In 50 mM Tris-HCl, pH 7.0 hydrolysis was linear 
for up to 5 min, but the inclusion of 0.01% Triton X-100 or 
0.01% BSA allowed the reaction to proceed almost linearly 
for 30 min (Fig. 4B and data not shown). Kinetic parameters 
(Km, Vmax, effect of temperature; see below) for A3',pG hydrolysis were similar in the presence of Triton X-100 and BSA.
at 1, 10, 100, and 500 μM concentrations, respectively). It was verified that the observed effects of metal ions were not due to their influence on phosphatase digestion performed during the CNPase assay.

The effect of nucleotides and of P, on enzyme activity was tested with 2 mM G>p* as substrate (see "Experimental Procedures"). At 10 mM concentrations, G2'p and G3'p showed only a small inhibition (12 and 19%, respectively). However, pG and P, were strongly inhibitory, with apparent values of 0.8 and 0.9 mM, respectively. Similarly, P, gave an apparent I0.5 of 0.8 mM. Kinetic analysis of the inhibitory effects of pG and P, showed them to be competitive inhibitors. It is likely that the strong inhibition by nucleoside 5'-phosphates is due to their phosphate group being more accessible than that in nucleoside 2'- or 3'-phosphates.

Substrate Specificity

Wheat germ CNPase hydrolyzed all four nucleoside 2',3'-cyclic phosphates to the corresponding 2'-phosphomonoesters (Fig. 5). Identity of N2'p products was further confirmed by cellulose TLC in solvent B (data not shown). In this solvent, nucleoside 2'-phosphates separate from nucleoside 5'-phosphates (not separable in solvent A) although they migrate together with the respective 2',3'-cyclic phosphates. The production of nucleoside 3'-phosphates was also not observed when labeled G>p* substrate and high concentrations of CNPase were used (Fig. 7A and results not shown). Under these conditions, generation of G2'p* at a level corresponding to about 0.5% of the G3'p* produced would have been detected. Nucleoside 3',5'-cyclic phosphates (3',5'-cAMP and 3',5'-cGMP) were not hydrolyzed as analyzed by cellulose TLC (Fig. 5) or phosphatase assay (data not shown).

The values of K, and Vmax were estimated for four nucleoside 2',3'-cyclic phosphates (Table II). Based on relative values of Vmax/Km, the specificity of CNPase toward the four nucleotides is A>p>C>p>G>p.

Comparison of the Substrate Specificities of Wheat Germ CNPase and RNA Ligase-associated 2',3'-Cyclic 3' Phosphodiesterase

Wheat germ contains an RNA ligase activity which ligates 2',3'-cyclic phosphate-terminated and 5'-hydroxyl-terminated RNAs via a 2'-phosphomonoester, 3',5'-phosphodiester linkage (6-10, 16, 17). Formation of this linkage requires the concerted action of three enzymatic activities: 2',3'-cyclic nucleotide 3'-phosphodiesterase, 5'-hydroxy polynucleotide kinase, and RNA ligase (7-12, 16, 17). It appears that these activities are intrinsic to a single polypeptide species of Mr = ~100,000 (8, 11, 12, 16, 17). The 2',3'-cyclic nucleotide 3'-phosphodiesterase activity is responsible for conversion of cyclic phosphate at the 3' end of RNA into the 2'-phosphorylated form.

It was of interest to compare the substrate specificities of CNPase toward the four nucleotides: A>p>C>p>G>p.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K, (mM)</th>
<th>Vmax (μmol/min/mg)</th>
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<tbody>
<tr>
<td>A&gt;p</td>
<td>13.1 ± 1.2</td>
<td>2094 ± 75</td>
</tr>
<tr>
<td>G&gt;p</td>
<td>9.2 ± 0.7</td>
<td>276 ± 17</td>
</tr>
<tr>
<td>C&gt;p</td>
<td>25.2 ± 4.0</td>
<td>2140 ± 294</td>
</tr>
<tr>
<td>U&gt;p</td>
<td>25.3 ± 6.6</td>
<td>602 ± 130</td>
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</table>

Reactions were performed in 50-μl volumes of 0.7-12.5 mM substrate concentrations. All velocities were calculated from the initial linear rates. Values were fitted to the Lineweaver-Burk equation by the linear regression method assuming proportional errors. Parameters in the table are the weighted means, together with their standard errors, of two (for U>p) or three (A>p, G>p, and C>p) determinations.

Fig. 6. Activity of wheat germ CNPase and RNA ligase

Fig. 5. Hydrolysis of cyclic nucleotides by wheat germ CNPase. 20-μl reaction mixtures were incubated with (even-numbered lanes) or without (odd-numbered lanes) 6 ng of purified CNPase and contained the following nucleotides (each 5 mM): G>p (lanes 1 and 9); A>p (lanes 3 and 4); U>p (lanes 5 and 6); C>p (lanes 7 and 8); 3',5'-cGMP (lanes 9 and 10); 3',5'-cAMP (lanes 11 and 12). 7 μl of each sample was subjected to cellulose TLC in solvent A, and nucleotides were visualized under UV light. The positions of nucleotide markers are indicated.
that similar inhibitors effectively inhibit proteolysis of RNA
the only one active exclusively with 2',3'-cyclic mononucleo-
activity, the protein characterized in this work appears to
represent a phosphodiester domain of RNA ligase released
separated from each other during the ammonium sulfate and
both enzymes, RNA ligase (8, 10, 16) and CNPase should be
was hydrolyzed by CNPase, but the oligonucleotides AAAG>p
Ligase-associated phosphodiesterase hydrolyzed (Np) G>p*
and pG > p* with similar efficiency, whereas G>p* was
hydrolyzed approximately 25 times more slowly (Fig. 7B). G>p*
was the only substrate hydrolyzed by CNPase; under
conditions when over 70% of input G>p* was converted to
G2'p*, no hydrolysis of (Np)G>p* of pG>p* was observed
(Fig. 7A). Similarly, when 2.5 mM pG>p* was incubated with
60 ng of CNPase for 1 h at 30 °C, the formation of radioactive
pG2'p* was not detected (results not shown).

The other set of substrates used for comparison of both enzymes included AAAG>p, AG>p, and G>p. Again, G>p was hydrolyzed by CNPase, but the oligonucleotides AAAG>p and AG>p were not (Fig. 7C). AAAG>p was also not a substrate (results not shown). RNA ligase was able to open the 2',3'-cyclic phosphate in all substrates; oligonucleotides were however hydrolyzed more efficiently than G>p (Fig. 7D).

DISCUSSION

The 2',3'-cyclic nucleotide 3'-phosphodiesterase characterized in this work appears to be distinct from RNA ligase, another protein isolated from wheat germ and possessing the 2',3'-cyclic 3'-phosphodiesterase activity. These two enzymes differ in chromatographic properties, molecular weights, and substrate specificities. Based on purification protocols for both enzymes, RNA ligase (8, 10, 16) and CNPase should be separated from each other during the ammonium sulfate and DEAE-cellulose steps employed in this work. It is also unlikely that the CNPase polypeptide of Mr = 23,000 might represent a phosphodiesterase domain of RNA ligase released by proteolysis of the Mr = ~100,000 ligase protein. A mixture of protease inhibitors was used throughout purification of CNPase, and Pick and Hurwitz (16) have shown previously that similar inhibitors effectively inhibit proteolysis of RNA ligase during its purification from wheat germ.

Of the three known enzymes with 2',3'-phosphodiesterase activity, the protein characterized in this work appears to be the only one active exclusively with 2',3'-cyclic mononucleo-
tide substrates. Vertebrate CNPase is known to hydrolyze cyclic mononucleotides and cyclic phosphate-terminated oligonucleotides (3, 28; see also Refs. 17 and 29 for activity of calf brain CNPase with oligonucleotide substrates). Similarly, as demonstrated above, wheat germ RNA ligase is able to hydrolyze 2',3'-cyclic nucleotide and oligonucleotide substrates, although the latter are used much more efficiently. This property of RNA ligase is not surprising since RNAs with 2',3'-cyclic termini are the physiological substrates of this enzyme, and their conversion to the 2'-phosphorylated molecules is an initial step of the ligation reaction (7-10, 17).

Interestingly, pG>p and the oligonucleotide substrate (Np), G>p* are hydrolyzed by RNA ligase with similar efficiencies. It appears therefore that the phosphate group esterified at the 5' position of the terminal cyclic nucleotide is an important structural feature in substrate recognition by RNA ligase. It would be of interest to find out whether pN>p (or pN2') may also function as an acceptor of a 5'-phosphorylated RNA terminus in subsequent steps of the ligation reaction which results in formation of the 2'-phosphomonoester, 3',5'-phosphodiester linkage (7-10, 17). It has been demonstrated previously (30) that pN2'p can act as a donor in this reaction where 2',3'-cyclic phosphate-terminated oligonucleotide is used as an acceptor.

The function of plant 2',3'-cyclic 3'-phosphodiesterase and of the membrane-associated enzyme from animals is unknown. Although both enzymes have quite similar kinetic parameters (high $K_v$ values for cyclic nucleotides and similar catalytic efficiencies in the range of 10,000–100,000 min$^{-1}$; Refs. 31–34 and this work), it is rather unlikely that they might be related structurally or functionally. The plant CNPase, contrary to the mammalian enzyme (4, 5, 31–34), appears to be a soluble protein. In addition, the two enzymes have distinct substrate specificities, pH optima, and molecular weights (25,500 versus 48,000–50,000 for the mammalian CNPase; Refs. 31–34).

As to the metabolic function of plant CNPase, two possibilities may be considered. (i) CNPase functions in catabolism of 2',3'-cyclic nucleotides produced by degradation of cellular RNAs by cyclizing endoribonucleases. Indeed, ribonucleases generating 2',3'-cyclic nucleotides rather than nucleoside 3'-phosphates as degradation products have been identified in plant extracts (35, 36). It should be noted, however, that another cyclic nucleotide phosphodiesterase able to hydrolyze 2',3'-cyclic nucleotides to nucleoside 3'-phosphates is present in plant cells (37 and references therein). This enzyme has broader substrate specificity; it also hydrolyzes the 3',5'-cyclic nucleotides producing nucleoside 3'- and 5'-phosphates and cleaves the pyrophosphate linkages in nucleoside diphosphates and triphosphates. (ii) Nucleoside 2',3'-cyclic phosphates or 2'-phosphates could function as regulatory molecules in plants and CNPase might be one of the enzymes involved in control of their level. Indeed, there are indications that G>p and A>p may function as turgor regulating leaf movement factors in plants (38).

Acknowledgments—We are indebted to Drs. L. Pick, H. Furneaux, and J. Hurwitz (Memorial Sloan-Kettering Cancer Center, New York) for providing us with wheat germ RNA ligase and General Mills Inc. (Vallejo, CA) for multiple samples of wheat germ. We thank Jan Hofsteenge, Thomas Hohn, and Brian Hemmings of the Friedrich Miescher-Institut for helpful comments and criticism.

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