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Purification and characterization of a molluscan egg-specific NADase, a second-messenger enzyme

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An egg-specific NADase has been purified to homogeneity from the ovotestis of the opisthobranch mollusk *Aplysia californica*. Unlike other NADases, the *Aplysia* enzyme generates primarily cyclic-ADP-ribose (cADPR) rather than ADP-ribose from NAD. cADPR has been shown to stimulate the release of Ca^{2+} from microsomes prepared from sea urchin egg and, when injected into intact eggs, to activate the cortical reaction, multiple nuclear cycles, and DNA synthesis. The *Aplysia* enzyme was initially identified as an inhibitor of cholera and pertussis toxin-catalyzed ADP-ribosylation. By the use of an NADase assay, it was purified from the aqueous-soluble fraction of ovotestis by sequential column chromatography. The enzyme has an apparent molecular mass of 29 kDa, a K_m for NAD of 0.7 mM, and a turnover rate of ~27 000 mol NAD · min^{-1} · mol $enzyme^{-1}$ at 30°C. Monoclonal antibodies were generated to the NADase. Immunoblots of two-dimensional gels revealed multiple isoforms of the enzyme, with pIs ranging from 8.1 to 9.8. The multiple isoforms were resolved with a cation exchange high-pressure liquid chromatography column and shown to generate cADPR. Immunohistochemical analysis of cryostat sections of *Aplysia* ovotestis shows that the enzyme is specific to the eggs and restricted to large 5- to 10-μm granules or vesicles. To date the cADPR-generating enzyme activity has been identified in various organisms, including mammals. The *Aplysia* enzyme is the first example in which the enzyme that generates cADPR has been purified. All of the available evidence indicates that this NADase is a second-messenger enzyme, implying that other NADases may serve a similar function.

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Introduction

The NADases are a diverse group of enzymes that, perhaps artificially, have been grouped together because of their common activity: they cleave the nicotinamide-ribose bond of NAD to produce nicotinamide and adenosine diphosphoribose (ADPR). They have been identified in many organisms from microbes to mammals. In mammals, many NADases have been shown to be membrane-associated ectoenzymes, i.e., their activity is directed into the extracellular environment (Muller *et al.*, 1983; Kim *et al.*, 1988). However, it is apparent that all NADases do not generate ADPR. Recently a metabolite of NAD has been shown to release Ca^{2+} from internal stores and has been identified as cyclic-ADP-ribose (cADPR) (Clapper *et al.*, 1987; Lee *et al.*, 1989). cADPR has been shown to be more potent than IP_3 in releasing Ca^{2+} from internal stores in sea urchin eggs (Dargie *et al.*, 1990). The enzyme that generates cADPR has never been purified to homogeneity, and nothing is known about its primary structure.

This paper is the first part of a two-part study. It describes the purification and characterization of an aqueous-soluble egg-specific NADase from the ovotestis of *Aplysia californica*. The second paper, which follows, describes the cloning and sequencing of the cDNA for the enzyme. We initially identified and purified the enzyme as an inhibitor of bacterial toxin-catalyzed ADP-ribosylation. Further investigation of the purified protein revealed it to be a potent NADase. However, analysis of the products of the purified egg enzyme by several criteria (Lee and Aarhus, 1991 and in this paper) indicates that the *Aplysia* NADase, unlike other NADases, generates primarily the compound cADPR.

Results

Identification and purification of the NADase

The NADase was discovered when attempts to ADP-ribosylate G-proteins in the ovotestis of

Aplysia, with bacterial exotoxins, specifically failed, whereas in other *Aplysia* tissues ADP-ribosylation was observed. When ovotestis was mixed with any other tissue, ADP-ribosylation could not be induced (Hellmich and Strumwasser, 1989). The inhibitory factor was shown subsequently to be an aqueous-soluble protein capable of splitting NAD.

Purification of the NADase was achieved using a three-step chromatographic procedure, illustrated by a sodium dodecyl sulfate (SDS)-polyacrylamide gel of each step (Figure 1). Inhibition of the toxin-catalyzed reaction was used initially as an assay to monitor the progress of the purification. When it was clear that the protein was an NADase, further purifications were monitored by an NADase assay, as described by Moss *et al.* (1976) and under Methods.

Table 1 shows the quantitation of a typical purification in which the NADase activity is followed. The starting material consisted of 604 mg of protein obtained from 5.3 g (wet weight) ovotestis. The high speed aqueous-soluble supernatant (Figure 1, lane 2) was enriched by a factor of four for NADase-specific activity compared with the crude homogenate. The highest enrichment of enzyme activity in the entire purification procedure occurred with the cation exchange column, where the NADase activity increased 23-fold. The peak of NADase activity from this column contains predominantly two proteins of 48–50 and 29 kDa when the resin was CM-Sepharose CL-6B (Figure 1, lane 3). The 48- to 50-kDa protein was less evident with the Accell-CM resin. The elution profiles of the protein content (measured at 280 nm) and the NADase activity, for the Accell-CM cation exchange column, are shown in Figure 2A along with the NaCl gradient. There are two peaks of NADase activity and at least two to three peaks of protein absorbance.

A single peak of NADase activity comigrates with the 29-kDa protein through a gel-filtration column with K_{av} of 0.73 (Figure 1, lane 5). Carbonic anhydrase (29 kDa) had an identical partition coefficient (K_{av}) during an independent run. After the cation exchange and gel-filtration columns, the recovery of NADase activity was 46% of the crude homogenate, and the enrichment of specific activity was ~200-fold.

The last step in the purification involved applying the pooled NADase activity to a hydroxylapatite column equilibrated with 0.01 M sodium phosphate, pH 7.8. Bound proteins were eluted with a linear gradient from 0.01 to 0.3 M sodium phosphate. Figure 2B illustrates the coelution of protein and NADase activity between 0.2 and 0.3 M sodium phosphate. The pooled NADase activity from this column ran as a single band on an SDS-polyacrylamide gel, whether stained by Coomassie blue (Figure 1, lane 7) or silver (not shown). After this three-column procedure, the protein was subjected to amino-acid sequencing, as described in the accompanying paper, where only a single *N*-terminal amino acid was observed (Glick *et al.,* 1991). Typically, this three-column procedure yields ~1 mg of purified NADase when starting with 600 mg of total aqueous-soluble ovotestis protein, with 27% recovery of enzyme activity.

Characterization of the purified NADase and its isoforms

The NADase assay, used for kinetic analysis of the purified ovotestis NADase, measured the amount of [^{14}C]nicotinamide produced from the hydrolysis of [^{14}C]NAD after a 1-min incubation at 30°C in the presence of 5×10^{-9} M purified NADase. The analysis of the enzyme activity indicated that the NADase had a K_m of 0.7 mM and a V_{max} of ~32 nmol NAD · min^{-1} · 35 ng^{-1} NADase (Figure 3). The V_{max} of the NADase corresponds to a turnover rate of 26 516 mol NAD · min^{-1} · mol enzyme^{-1}.

Further characterization of the purified NADase indicated that multiple isoforms existed. Both HPLC analysis on a cation-exchange column and two-dimensional gels indicated that the purified enzyme consisted of multiple protein peaks and spots, respectively. A Coomassie blue-stained two-dimensional (2-D) gel revealed several protein spots (Figure 4A); the lightest staining spot was the most acidic (pI of ~8.1) and the darker staining clusters of spots were more basic (pI of ~9.8). The apparent molecular mass of all the spots were about the same, 29 kDa.

To determine whether all the spots identified by the 2-D gel separation of purified NADase were the same protein, we used a monoclonal antibody (MAb) raised against the purified NADase to probe a Western blot of the 2-D gel. The specificity of the MAb (1AT2E9) was illustrated by the fact that it stained a single band (29 kDa) of a Western blot of the high speed supernatant of crude homogenate from ovotestis (Figure 5). When used to stain a blot of the 2-D gel, this MAb labels all the Coomassie-stained spots seen on the 2-D gel, indicating that the different spots were isoforms of the same protein (Figure 4B).

The protein isoforms were separated by cation-exchange HPLC to determine whether they

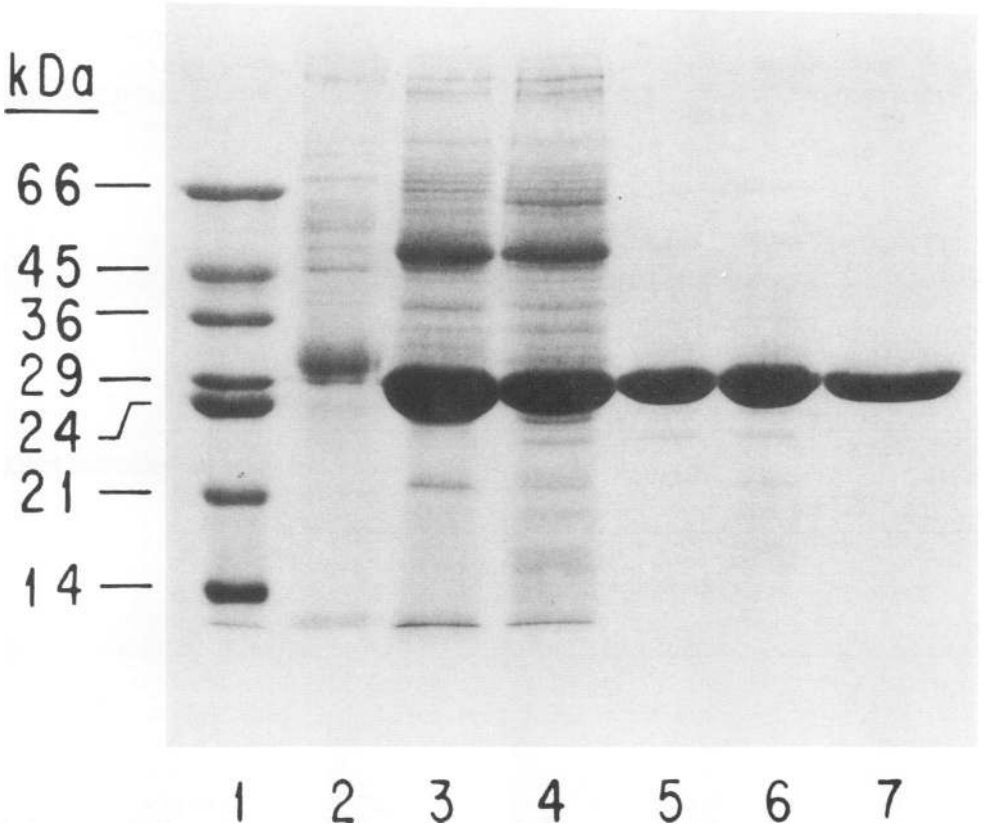

Figure 1. **Purification of the NADase from the soluble fraction of *Aplysia* ovotestis.** Coomassie blue-stained SDS-polyacrylamide gel (12% acrylamide) of the starting material and the peak NADase activity from each column in a three-step purification. Lane 1, molecular weight markers; lane 2, *Aplysia* ovotestis high-speed supernatant; lane 3, peak from cation exchange column (CM-Sepharose CL-6B); lane 4, starting material (after concentration) applied to the gel-filtration column; lane 5, peak from the gel-filtration column; lane 6, starting material (after concentration) applied to hydroxylapatite column; lane 7, peak from the hydroxylapatite column.

could generate cADPR (procedure described below). The column resolved four major peaks (Figure 6), which were collected for characterization of their activities after incubation with NAD. The protein in peak 1 (Figure 6) neither had NADase activity nor could it generate cADPR, whereas the remaining three protein peaks could generate cADPR proportionally to the amount of protein. Peaks 2, 3, and 4 generated 92, 74, and 49 pmol cADPR/15 min at 0°C, respectively. With the use of the area of the absorbance at 280 nm as a measure of pro-

Table 1. Purification of NADase (*Aplysia californica*)

Purification step	Total activity (μmol/min)	total protein (mg)	Specific activity ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	Recovery
Crude	362	604	0.6	100
Supernatant (150 000 g)	319	133	2.4	88
Cation exchange (Waters, Accell-Cm)	178	3.2	55	49
Gel-filtration (Sephacryl-S200)	166	1.3	126	46
Hydroxylapatite	97	0.8	120	27

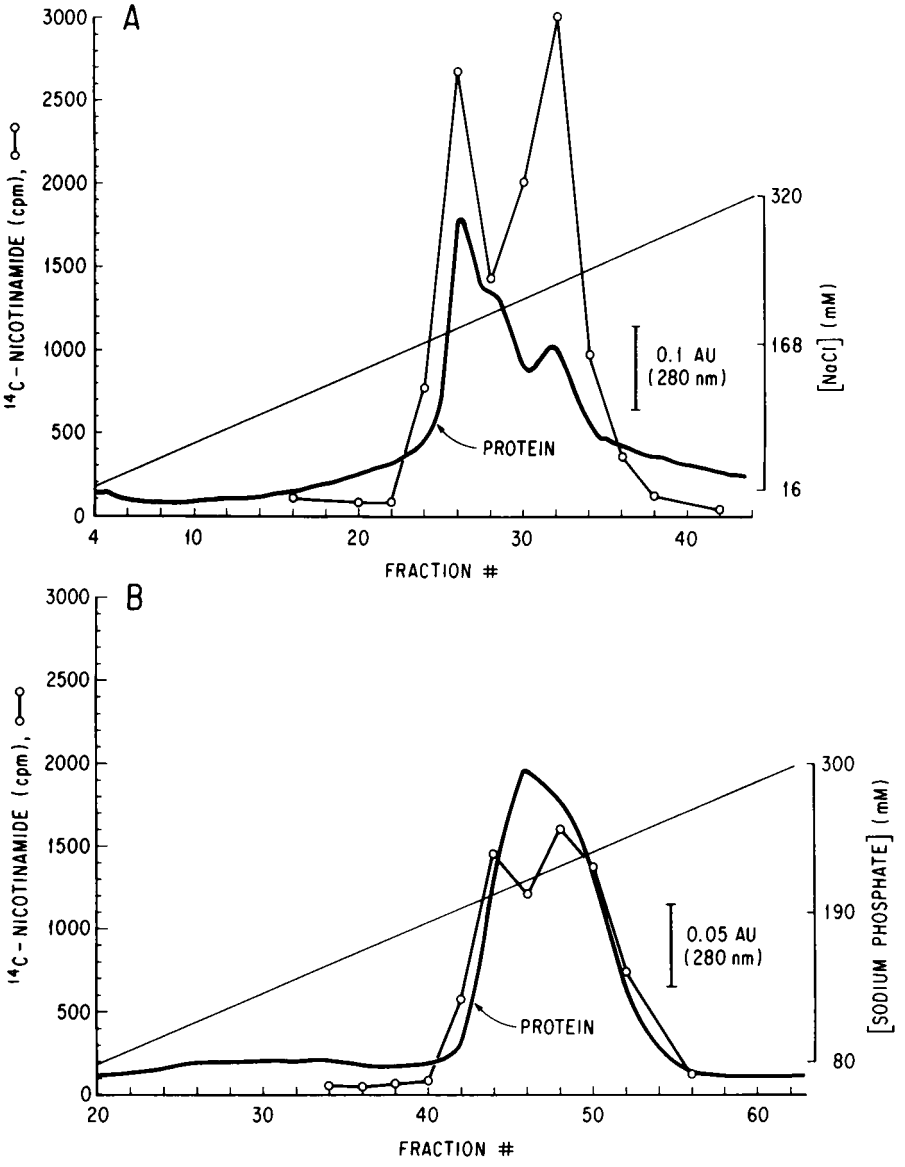

***Figure 2.* Coelution of NADase activity with protein from the cation exchange and hydroxylapatite columns.** The resin was Accell-CM in A and hydroxylapatite in B. The thick line shows the elution of protein from both columns, monitored at 280 nm. O, the elution of NADase activity. The NADase activity in each fraction was assayed as described under Methods. The gradients (thin line) are shown for each column.

tein, the cADPR/protein ratios of peaks 2, 3, and 4 were 0.64, 0.63, and 0.59, respectively.

Characterization of the NADase products

To determine whether cADPR was generated as a product, we sent 30 μg purified *Aplysia* NADase to Dr. Hon Cheung Lee (University of Minnesota). From HPLC and nuclear magnetic resonance analysis, he established that the product of the enzyme action on NAD was not ADPR but cADPR (Lee and Aarhus, 1991). He also found that, in an in vitro sea urchin preparation, the product of the *Aplysia* enzyme released Ca^{2+}. Our paper confirms, by mass spectrometry, that the predominant product of the *Aplysia* NADase is cADPR.

The products of the NADase were characterized after incubating the purified enzyme with NAD for 1, 5, or 15 min (0°C) and separating the products on either of two anion exchange columns (Vydac 303NT405, Bio-Rad AG MP-1) by HPLC, as described under Methods. Figure 7 illustrates three HPLC traces obtained with the two different columns. The top trace (Figure 7A) shows the elution profile of the standards (nicotinamide, NAD, cADPR, and ADPR, in that order) from the Bio-Rad column, and the bottom

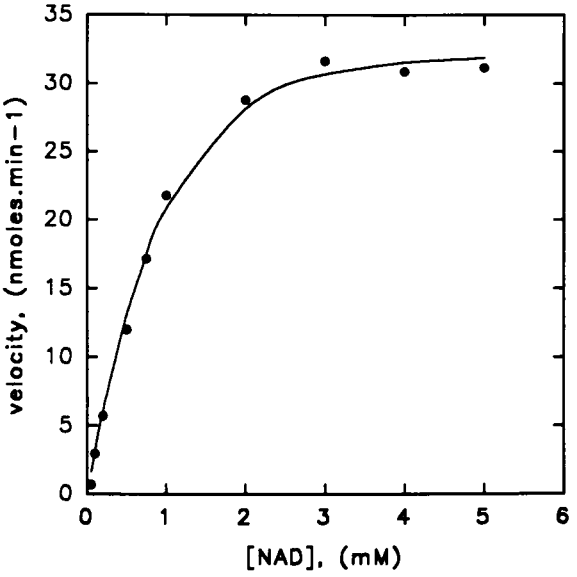

Figure 3. **Velocity of NADase reaction versus NAD concentration.** Various concentrations (0.05–5.0 mM) of [carbonyl-^{14}C]NAD (specific activity = 1.6 nCi/nmol) and 5×10^{-9} M ovotestis NADase were incubated in 300 mM potassium phosphate buffer (pH 7.0) at 30°C. The amount of NAD cleaved during the incubation period was determined by measuring the amount of [^{14}C]nicotinamide produced. The data were fitted to the equation f = a + b∗exp(−c∗x) using a nonlinear curve fitting program (SigmaPlot, version 4.0). The estimated K_m of the NADase is 0.7 mM and the V_{max} is ~32 nmol · min^{-1} · 35 ng enzyme^{-1}. This corresponded to a turnover rate of ~27 000 mol NAD · min^{-1} · mol NADase^{-1} at 30°C.

trace shows the products of the enzyme after 15 min at 0°C. The major product, after incubation with NADase, elutes between NAD and ADPR at ~15 min. This product was identified as having a mass (M-H, m/z) of 540 by negative ion fast atom bombardment mass spectrometry, which is consistent with the determination by Lee *et al.* (1989) on cADPR as generated by the sea urchin enzyme activity.

Figure 7B shows the elution profile of the products on the Vydac column. cADPR elutes from this column between nicotinamide and NAD at 1 min. After incubations at 1 and 15 min, at 0°C, cADPR accounts for 97% and 87%, respectively, of the sum of the two products (cADPR and ADPR), as measured from the area of the peaks on the Vydac column.

Immunolocalization

The MAb (1AT2E9) was used to probe cryostat sections of the *Aplysia* ovotestis. Immunohistochemical staining of cryostat sections of the ovotestis show that the enzyme is contained

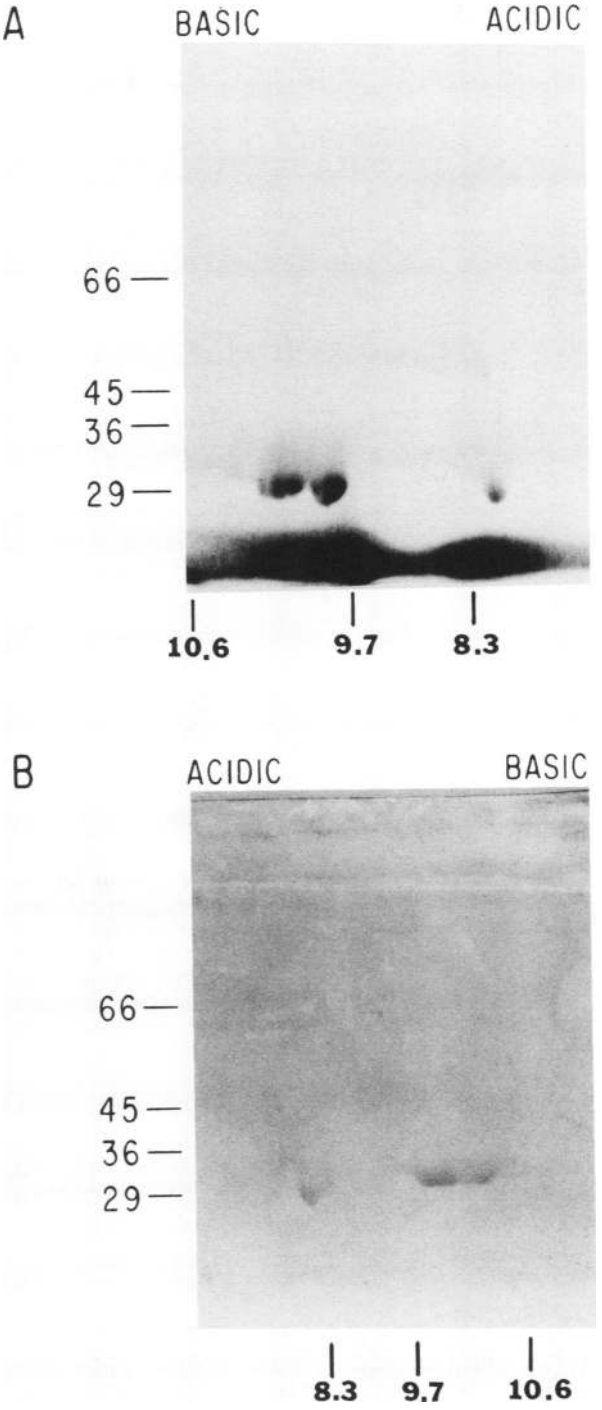

Figure 4. **2-D gel and Western blot of NADase.** (A) 10 μg of purified ovotestis NADase was separated on an IEF/SDS-polyacrylamide gel (12% acrylamide) and stained with Coomassie blue. Molecular mass markers (kDa) are shown by lines on the left. The position of the pI markers is shown at the bottom. (B) Western blot of a 2-D gel separation of the purified NADase, probed with the anti-NADase MAb.

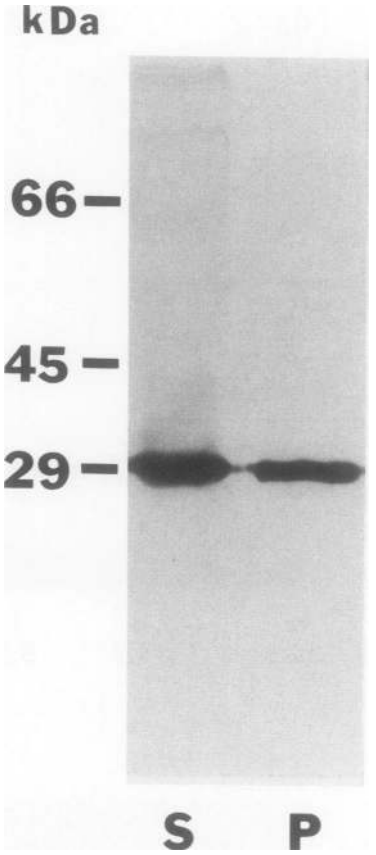

Figure 5. **Western blot of the high-speed aqueous soluble fraction of *Aplysia* ovotestis and the purified NADase probed with an anti-NADase MAb.** Twenty micrograms of protein from the high-speed supernatant (S) and 0.3 μg of purified ovotestis NADase (P) were blotted onto nitrocellulose and probed with the anti-NADase MAb as described under Methods. The position of various molecular mass markers is indicated by the lines on the left.

within 5- to 10-μm granules or vesicles within the eggs (E in Figure 8). The negative control, consisting of sections of ovotestis in which the MAb was replaced by SP2 myeloma cell culture supernatant, showed only the yellow autofluorescence of the yolk granules (Y).

Discussion

This paper describes the purification and characterization of an *Aplysia* NADase that generates, as its primary product, the Ca^{2+}-mobilizing compound cADPR from NAD. Clapper *et al.* (1987) were the first to demonstrate that a sea urchin egg extract contained an enzymatic activity that converted NAD into a metabolite capable of stimulating Ca^{2+} release in a cell free system. Additional studies have shown that the active metabolite of NAD is cADPR (Lee *et al.*, 1989) and that the enzyme activity can be found in a variety of mammalian tissues (Rusinko and Lee, 1989). The cADPR-generating enzyme, however, has not been purified from sea urchin or any other source, with the exception of the *Aplysia* enzyme described in this paper.

The mechanism by which the activity of the NADase is regulated in *Aplysia* is currently unknown. The presence of multiple isoforms of the *Aplysia* enzyme, as revealed by 2-D gel electrophoresis and by cation exchange HPLC, indicates that the isoforms vary from each other by charge, which may be due to different phosphorylation states. Glick *et al.* (1991) in the following paper have identified potential phosphorylation sites within the sequence of the protein. However, data from the individual isoforms indicate that the enzyme activities (peaks 2, 3, and 4 of Figure 6) are essentially the same at converting NAD to cADPR (ratios of cADPR generated/protein are 0.59–0.64). It is possible that a kinetic analysis of these isoforms might reveal subtle differences not apparent from a single time of incubation.

Peak 1 could not cleave NAD or generate cADPR and may represent an inactive form of the enzyme. This conclusion is based on the fact that the MAb reacted with all the Coomassie blue-stained spots that were separated by 2-D gel electrophoresis. It is possible, however, that our method of purification produces some inactive enzyme as a result of some proteolysis not detectable by SDS-gel electrophoresis.

One potential method of regulating the enzyme is suggested by the immunohistochemical results. Of the various cell types in the ovotestis, only the eggs contain the NADase, and its distribution within the ova is restricted to large (5- to 10-μm) granules or vesicles. Compartmentalization of the enzyme in these granules or vesicles would allow the cell to regulate the activity of the NADase by physically separating it from its substrate, so that cADPR is synthesized only under specific conditions. The following paper (Glick *et al.*, 1991) presents evidence that the proenzyme contains a hydrophobic signal sequence, implying that the NADase is likely to be a secretory protein. It is not known from our present work whether the NADase is active within these granules, but we believe that it is more likely that it is active only after being released into the cytoplasm or secreted from the egg.

NADase activity has been identified throughout the animal kingdom, from microbes such as *Neurospora* (Kaplan *et al.*, 1951) to mammals (Muller *et al.*, 1983; Kim *et al.*, 1988). In mammals some of the highest NADase activity has

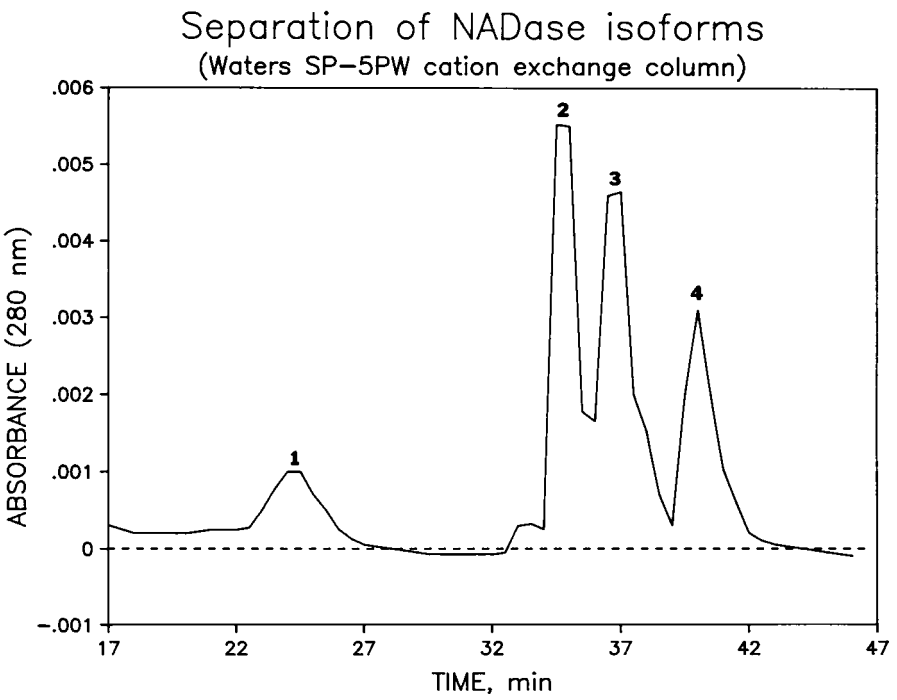

Figure 6. **HPLC chromatogram showing the multiple isoforms of the purified ovotestis NADase eluted from a Waters SP-5PW cation exchange column.** Eighteen micrograms of purified NADase was injected onto the SP-5PW column equilibrated with 10 mM HEPES, pH 7.0. The column was washed for 5 min at 1 ml/min and eluted over 120 min (0.5 ml/min) with a linear salt gradient (0–0.5 M NaCl, 10 mM HEPES, pH 7.0). The purified NADase was resolved into four major peaks. Each peak was collected, concentrated three-fold (centricon 3, Amicon), and assayed for the production of cADPR by reacting with 2 mM NAD for 15 min, at 0°C, in 20 mM potassium phosphate, pH 7.0.

been shown to be associated with the macrophages of various tissues and directed toward the extracellular environment (Artman and Seeley, 1978, 1979; Amar-Costesec *et al.*, 1985). The NADases also vary greatly in size (molecular mass ranges from 25 to 120 kDa), location (membrane associated and aqueous soluble), and enzymatic activity. The assumption that the function of this varied group of enzymes is simply the regulation of intracellular NAD is most likely incorrect. In fact it should be realized that the most commonly used methods of examining NADase activity in the literature—the KCN assay, which measures the amount of NAD remaining uncleaved (Colowick *et al.*, 1951), and the radiolabeled NAD method, which detects radioactive nicotinamide (Moss *et al.*, 1976)—would not identify cADPR. Reevaluation of the activity of other NADases might reveal, as in the *Aplysia* enzyme, products other than nicotinamide and ADPR such as cADPR. The Ca^{2+}-mobilizing activity of cADPR makes the reevaluation of other NADases of obvious importance. A similar conclusion has been reached by Lee and Aarhus (1991).

Methods

Purification of the ovotestis NADase

Aplysia californica (250–300 g) were obtained from Alacrity Marine Biological Services (Redondo Beach, CA) and Sea Life Supply (Sand City, CA). Ovotestes (5–12 g) were isolated and either placed directly into liquid nitrogen for storage and later use or placed into ice-cold homogenization buffer (250 mM sucrose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 8.0, 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride [PMSF]) and homogenized (5:1 buffer volume to wet weight of tissue) in a glass-on-glass Dounce homogenizer. The homogenized tissues were fractionated in a two-step centrifugation procedure. The first step was a low-speed centrifugation (20 000 × *g* for 20 min at 4°C). The pellet from the low-speed spin was discarded and the supernatant subjected to a second high speed spin (150 000 × *g*, 90–120 min at 4°C).

Chromatography was performed on the Waters (Milford, MA) 650E Advanced Protein Purification System at room temperature (20–22°C). The first column consisted of a cation exchange resin (Waters, Accell-CM, 1 × 30 cm), except in our earliest purifications, which used CM-Sepharose CL-6B (Pharmacia, Piscataway, NJ) in a low-pressure mode. The high-speed supernatant was diluted 15-fold with 20 mM HEPES (pH 8.0) and loaded on to the Accell-CM column, equilibrated with 20 mM HEPES (pH 8.0) at 8 ml/min. Typical amounts of total protein loaded onto the first column ranged from 100 to 200 mg.

The bound protein was eluted from the column over 120 min, with a linear salt gradient (0–500 mM NaCl, 20 mM HEPES, pH 8.0) at a rate of 1 ml/min, and collected in 2.0-ml fractions. The peak of NADase activity eluted from the cation exchange column with 180–250 mM NaCl. These fractions were pooled (18–20 ml), concentrated (centricon 3, Amicon, Danvers, MA) to 3–5 ml, and injected onto a 1 × 60 cm gel-filtration column (Sephacryl-S200, Pharmacia) equilibrated with 20 mM HEPES, pH 8.0. The peak of NADase activity was eluted (1 ml/min) from the gel-filtration column and collected in 1-ml fractions.

This peak of NADase activity was loaded onto a 1 × 10 cm hydroxylapatite column (Bio-Gel HTP, Bio-Rad, Rich-

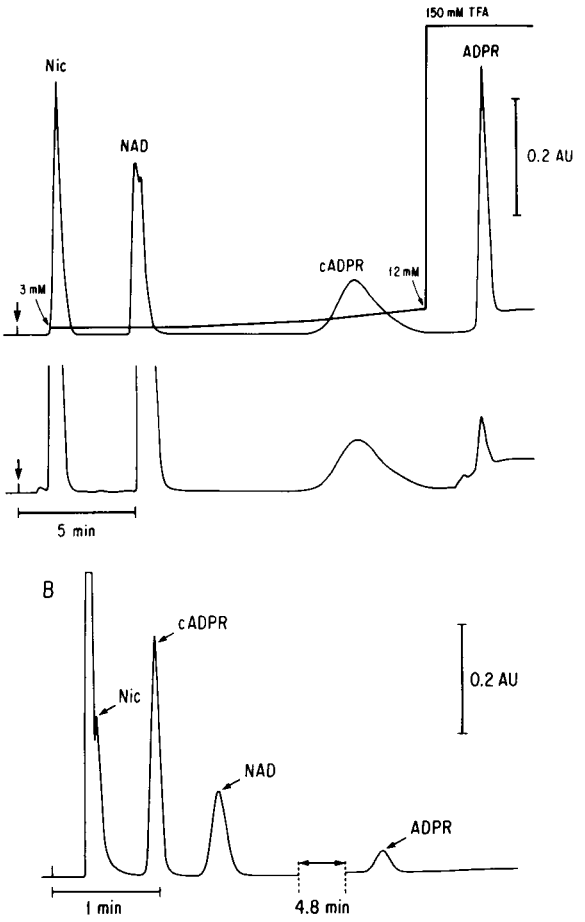

Figure 7. **HPLC chromatograms of the products generated by the purified ovotestis NADase.** (A, top) the elution profile of the standards (nicotinamide [Nic], NAD, cADPR, and ADPR) separated by HPLC on a Bio-Rad AG MP-1 column, as described under Methods. The gradient for trifluoroacetic acid (TFA) is superimposed on the elution profile. Absorbance (AU) was measured at 254 nm. (Bottom) the elution profile of the products of the purified NADase, after incubation with 2 mM NAD, for 15 min at 0°C. cADPR eluted at ~15 min. (B) the elution profile of the products of the purified NADase, after a 5-min incubation with 2 mM NAD, at 0°C, separated on a Vydac 303NT405 ion exchange column as described under Methods. The retention times were nicotinamide, 0.4 min; cADPR, 1.0 min; NAD, 1.5 min; ADPR, 7.4 min.

mond, CA) equilibrated with 10 mM sodium phosphate (pH 7.8) and eluted, over 120 min, with a linear sodium phosphate gradient (10–300 mM sodium phosphate, pH 7.8) at 0.5 ml/min.

NADase assay

The NADase activity of the fractions from each of the three columns was assayed with Dowex anion exchange columns, prepared following the procedure of Moss *et al.* (1976). The NADase reaction was initiated by adding 10 μl of each fraction to a tube containing 390 μl of NAD (7 mM NAD, 20 mM potassium phosphate, pH 7.0) with a trace amount of [carbonyl-^{14}C]NAD (specific activity = 1.6 nCi/nmol), obtained from Amersham (Arlington Heights, IL). After incubating the reaction mixture on ice for 5 min, we applied duplicate samples (100 μl) to the Dowex column, equilibrated with 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5). The [^{14}C]nicotinamide was eluted with four 1-ml washes of 20 mM Tris-HCl, pH 7.5. After elution, 1 ml of each 4-ml sample was added to 7 ml of scintillation fluid (Aquasol-2, NEN Research Products, Boston, MA) and counted in a LKB liquid scintillation counter (LKB 1218 RackBeta, Piscataway, NJ) for 5 min.

For kinetic studies of the purified NADase, 5×10^{-9} M enzyme was incubated for 1 min in 300 mM potassium phosphate buffer, pH 7.0, at 30°C with various concentrations of NAD (0.05–5.0 mM, specific activity = 1.6 nCi/nmol), in a total volume of 0.3 ml. At t = 0 and t = 1 min, 0.1-ml samples were applied to the Dowex column and eluted as described above.

2-D gel electrophoresis

The procedure for the separation of the NADase by 2-D gel electrophoresis is as described by Ausubel *et al.* (1987) with the following exception: the isoelectric gels (0.1 × 10 cm) were run at 800 V for only 2–3 h. pI was estimated by running a parallel tube gel using cytochrome C, modified to produce isoforms ranging from pI 4.1 to 10.6 (pI Marker protein kit, United States Biochemical, Cleveland, OH).

HPLC

The products generated by the purified NADase were separated on two different anion exchange columns (Vydac 303NT405 [0.46 × 5 cm] and Bio-Rad AG MP-1 [1 × 10 cm]), as described by Lee *et al.* (1989). Samples were prepared for both columns as follows: at t = 0, 1×10^{-7} M purified NADase was added to 2 mM NAD in 20 mM potassium phosphate buffer, pH 7.0. After incubation at 0°C for 1–15 min, the reaction was stopped by the addition of an equal volume of acetone (100 μl) and incubated on ice an additional 10–15 min. The precipitated protein was removed by centrifugation (14 000 × *g* at 4°C for 10 min) and the acetone evaporated from the supernatant under a stream of nitrogen gas.

To separate the enzyme products on the Vydac column, we used a protocol established by Lee *et al.* (1989), except that we substituted ammonium acetate for ammonium formate in solvent A and acetic acid for formic acid in solvent B. Twenty microliters of the sample was injected onto the Vydac column; equilibrated with 0.045 M CH_3COONH_4 (solvent A), pH 4.6, with H_3PO_4; and eluted with a linear gradient from 0 to 100% 0.5 M NaH_2PO_4 (solvent B), pH 2.7, with CH_3COOH in 10 min at a flow rate of 2 ml/min. Sample elution from the column was monitored at 254 nm. Control samples (minus NADase) containing known standards (nicotinamide, NAD, ADPR, and ADP) were treated as described above.

The protocol for the Bio-Rad AG MP-1 column was as described by Lee *et al.* (1989), except that the column was equilibrated in water rather than 2% trifluoroacetic acid in water. cADPR eluted as a highly symmetrical peak (retention time of 15 min, between 4 [6 mM] and 8% [12 mM] trifluoroacetic acid).

Sample preparation of cADPR for mass spectrometry

We reacted 2 mM NAD with 1×10^{-7} M purified NADase (3 ml 20 mM potassium phosphate, pH 7.0) at 0°C for 30 min. After acetone precipitation as described above, 2.5 ml of NADase was injected onto the Bio-Rad column (AG MP-

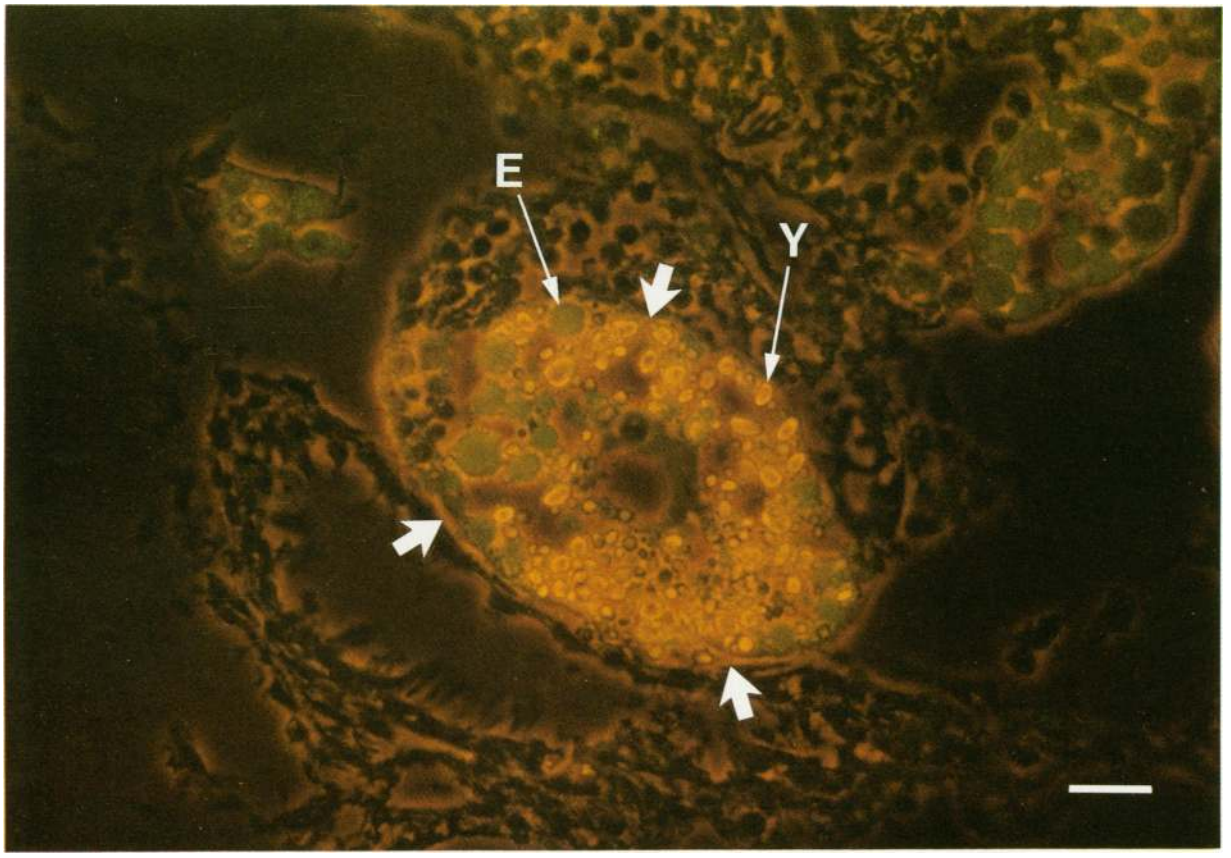

Figure 8. **Immunohistochemical localization of NADase in cryostat sections of *Aplysia* ovotestis.** Combination fluorescent/phase-contrast photomicrograph of *Aplysia* ovotestis after staining with the anti-NADase MAb. The ovoid-shaped oocyte (outlined by three thick arrows) contains yellow autofluorescing granules (Y) and additional granules (E) containing the NADase that fluoresce green after exposure to the mouse anti-NADase MAb and FITC-coupled goat anti-mouse immunoglobulin. Scale = 20 μm.

1) and the cADPR peak was collected. The 15-ml sample, containing cADPR, was aliquoted, as 1 ml samples, into microfuge (Eppendorf, Madison, WI) tubes. They were evaporated in a Speed-Vac concentrator (Savant, Farmingdale, NY) for 4 h. This resulted in a five-fold concentration of the initial sample (final volume was 3 ml). This was evaporated to dryness in a Buchler (Fort Lee, NJ) vortex evaporator for 4 h. The dried sample was resuspended in 200 μl water, frozen, and submitted to the MIT Mass Spectrometry Facility for determination of mass, using negative ion fast atom bombardment. Once the mass of *Aplysia* cADPR was shown to be identical to the mass of sea urchin cADPR (Lee *et al.*, 1989), purified *Aplysia* cADPR was used as a standard (e.g., Figure 7).

Preparation of MAb

Purified *Aplysia* ovotestis NADase was used as the immunogen to produce MAb. The immunization procedure and schedule were as follows: 25 μg of purified protein was mixed with 100 μl Freund's complete adjuvant and injected intraperitoneally into a 5-wk-old, female Balb/c mouse. The initial injection was followed by two subsequent injections of 25 μg of protein each, in Freund's incomplete adjuvant, at ~2-wk intervals. For the fusion, Sp2 myeloma cells were used and the conventional protocol (Kohler and Milstein, 1975) was followed. Polyethylene glycol (PEG 1500) was purchased from Boehringer Mannheim (Mannheim, Germany) and cell culture media were obtained from either Whittaker Bioproducts (Walkersville, MD) or GIBCO Laboratories (Chagrin Falls, OH).

The hybridomas were initially screened on nitrocellulose (Schleicher & Schuell, Keene, NH), with the purified NADase as the antigen, in a Bio-Rad dot blot apparatus. Immunostaining of the mouse monoclonal was performed with the Vectastain (Vector Laboratory, Burlingame, CA) ABC kit, which uses a biotinylated secondary anti-mouse antibody that binds avidin-HRP. The HRP was detected by using the chromogen 4-chloro-1-naphthol (Sigma, St. Louis, MO). Western blots were produced following the procedure of Towbin *et al.* (1979) and probed with the same Vectastain ABC kit described above.

Immunohistochemistry

Tissue preparation. *Aplysia* ovotestis was fixed in 4% formaldehyde, 100 mM potassium phosphate, pH 7.4, and 10% sucrose for 1–4 h at room temperature. The fixed tissue was then equilibrated in a large volume of 30% sucrose, frozen in liquid nitrogen-cooled Freon-22, embedded in OCT compound (Lab-Tek Products, Naperville, IL) and sectioned at 5 μm (−28°C). The sections were placed on gelatinized

(0.5% gelatin, 0.05% chromium potassium sulfate) glass slides and dried at room temperature.

Immunostaining. The sections of ovotestis were washed with phosphate-buffered saline (PBS), pH 7.4, blocked with 1% bovine serum albumin for 1 h, and incubated overnight at 4°C with hybridoma supernatant containing the monoclonal antibody (1:2 to 1:5 dilution of supernatant in PBS). After the incubation with primary antibody, the slides were rinsed in PBS and incubated for 1 h with an anti-mouse IgG-IgM FITC-coupled secondary antibody. The negative control consisted of substituting myeloma supernatant (from Sp2 cells) for the hybridoma supernatant.

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