Apparent Role of Adenosine Diphosphoribosyl Transferase in the Development of *Mytilus edulis* and the Inhibition of Differentiation by Ligands of the Enzyme Protein (43205)

PAL I. BAUER,* KURT KLINE,† AND ERNEST KUN*,[1]

Laboratory for Environmental Toxicology and Chemistry, Romberg Tiburon Center, San Francisco State University, and MEC Analytical Systems, Inc.,† Tiburon, California 94920*

Abstract. **The poly(ADP-ribose) polymerase or transferase (ADPRT) activity of developing embryos of *Mytilus edulis* increases with the progression of larval growth. ADPRT protein was partially purified from 2-hr-old embryos and identified by gel electrophoresis and immunotransblot, demonstrating cross-reactivity with anti-ADPRT IgG produced against the calf thymus enzyme. Two inhibitors of ADPRT, benzamide, competing with NAD at the nicotinamide binding site, and 6-amino-1,2-benzopyrone, which competes with DNA at the DNA binding site(s), both selectively arrest differentiation at the prodissoconch stage. The DNA site-oriented inhibitor, 6-amino-1,2-benzopyrone, has a much larger differentiation arresting effect than benzamide. The arrest of differentiation by 6-amino-1,2-benzopyrone is reversible. A probable ecotoxicity of ADPRT ligands on mussel differentiation is proposed.** [P.S.E.B.M. 1991, Vol 196]

Adenosine diphosphoribosyl transferase (ADPRT, EC 2.4.2.30) is a highly abundant DNA-associated nuclear protein of eukaryotes (see (1) for review). Recent evidence indicates that the enzymatic activity of this protein, consisting of the polymerization of the ADP-ribose moiety of NAD to a homopolymer that is covalently protein bound (2), represents only 1% of its molecular activity (3, 4) in cells that were not exposed to toxic DNA damage, and thus functions under near-physiologic conditions. Although this relatively small polymerase activity of ADPRT in intact cells may have distinct self-regulatory cellular importance (5), the preponderant biologic role of ADPRT is probably related to its binding to special DNA structures (6–8) and to other proteins.

One of the biologic domains where ADPRT has been implicated is differentiation (1). An advantage of lower eukaryotes as experimental models is the convenient time frame of the differentiation process that facilitates experimentation. The ADPRT activity has been identified in nuclei of sea urchin embryos (9), in *Tetrahymena pyriformis* (10), and the biologic effects, consisting of inhibition of specific stages of differentiation by certain ligands of ADPRT, were identified in *Trypanosoma cruzi* (11, 12) and *Leishmania mexicana amazonensis* (13).

In the present report we identify ADPRT in sperm, eggs, and developing embryos of the mollusk *Mytilus edulis* (14), which can complete differentiation 48 hr after *in vitro* fertilization. It is also demonstrated that two inhibitors of ADPRT, benzamide (15) acting primarily at the nicotinamide binding site of ADPRT, and, more powerfully, 6-amino-1,2-benzopyrone (6-ABP), which is selectively binding to the DNA site of the enzyme (16), reversibly inhibit differentiation at the prodissoconch stage without exerting cellular toxicity.

Materials and Methods

More than 98% homogeneous ADPRT was isolated from calf thymus as reported (17), and the preparation of rabbit anti-ADPRT IgG as well as gel elec-

[1] To whom requests for reprints should be addressed at Laboratory for Environmental Toxicology and Chemistry, Romberg Tiburon Center, San Francisco State University, P.O. Box 855, 3150 Paradise Drive, Tiburon, CA 94920.

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trophoretic enzyme assays were the same as described earlier (17, 18). Coenzymic DNA (2- to 4-kb double-stranded DNA) was the byproduct of enzyme isolation (17) and was further purified by phenol extraction. Benzamide was purchased from Sigma (St. Louis, MO) and 6-amino-1,2-benzopyrone was synthesized as described previously (16).

Larvae of *Mytilus edulis* were isolated and the effects of ADPRT ligands were tested as follows. Blue mussels were obtained from Seafarms West, San Diego, CA, and spawning was induced in adult animals by heat shock at 20–22°C. Suspensions of separated gametes were placed in crystallizing dishes and examined for viability through microscopic inspection. Fertilization (200 eggs/ml) in sea water was performed by mixing the gametes (10^6 sperms/ml) and allowing them to stand for a minimum of 2 hr with gentle aeration. Fertilized eggs were separated by filtration through 20-μm pore Nitex filters that allow passage of sperm and debris. The fertilized eggs were resuspended to form a stock suspension and were counted in a Sedgewick-Rafter cell. Initial density of the fertilized eggs was adjusted to 15/ml, as determined by counting of three aliquots. Exposure to ADPRT ligands was conducted in sea water at 20 ± 2°C with 14-hr light and 10-hr dark periods. Constancy of temperature, pH, salinity, and dissolved O_2 were monitored at 24-hr intervals. At the end of 48 hr, the embryos were fixed in Formalin (5% final concentration). The average number of normal and abnormal larvae were determined by direct counting and morphologic analysis by microscope in five 1-ml aliquots. The D-hinge or prodissoconch I larvae and abnormal development forms were readily identified and quantitated at low magnification (×40). Less than 10% abnormal forms were present in controls, containing no ADPRT ligands. Criteria of identifications recommended by the American Society of Testing of Materials (E-724-80) were closely followed.

Results

The activity of ADPRT in Mytilus sperms, eggs, and fertilized eggs 2 hr after fertilization is illustrated in Figure 1. Rates of ADPRT transfer from NAD to protein-bound acid-precipitable products were followed for 5 min in extracts of fertilized eggs by techniques described in the legend of Figure 1. It is striking that a rapid increase in ADPRT activity occurred in fertilized eggs as soon as 2 hr after fertilization. Since extensive sonication (see legend, Fig. 1) results in a large number of DNA breaks in broken embryo cells, close to V_{max} of ADPRT has been actually determined that correlates with enzyme content, suggesting that the increase in enzyme activity parallels the development of embryos and probably reflects the biosynthesis of ADPRT that occurs simultaneously with cell division cycles of the embryo. Since apparent ADPRT activity in frozen and

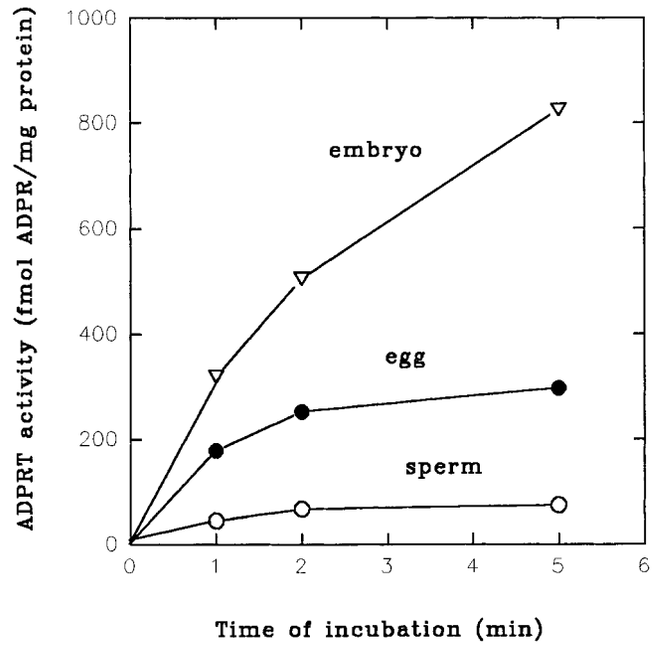

Figure 1. The ADPRT activity of *M. edulis* sperms, eggs, and embryos. Collection of eggs and sperm of *M. edulis*, fertilization and incubations were carried out as described in Materials and Methods. At the end of the incubation (2 hr after fertilization) the embryos were collected by centrifugation (10 min at 400*g* at 4°C) and 0.3 ml of packed pelleted cells from each sample were suspended in a 4-fold volume of extraction buffer (100 m*M* Tris-HCl, pH 8.0; 5 m*M* $MgCl_2$; 10 m*M* 2-mercaptoethanol; 0.4 *M* NaCl). The samples were frozen and thawed three times (by submerging in solid CO_2/acetone mixture) and were sonicated with 6 × 10 bursts using a Branson sonifier (50% output) at 4°C, with 1 min cooling between each sonication cycle. Finally, the samples were centrifuged at 10,000*g* for 5 min and the supernatants assayed for ADPRT activity as described earlier, with 100 n*M* NAD as substrate (5, 16).

thawed, then sonicated cell types (Fig. 1) critically depends on the availability of DNA termini, required for maximal enzymatic activity, we tested the adequacy of DNA fragmentation by adding DNase I (10 μg/ml) and found no further augmentation of ADPRT activities.

Identification of ADPRT in Developing Embryos of *M. edulis*. This was accomplished by partial purification of ADPRT from embryos 2 hr after fertilization. Ten milliliters of packed embryos were obtained by sedimentation at 400*g* for 10 min followed by dilution to 100 ml in extraction buffer and five successive sonications, each for 1 min, with a Branson sonifier, keeping the suspension at 4°C in an ice bath. Microscopic examination showed that more than 95% of the cells were disrupted by this treatment. After further extraction for 20 min with mild stirring, the cell debris were removed by centrifugation at 5000*g* at 4°C, and the supernatant was loaded onto a hydroxylapatite column of 20-ml bed volume and developed as described (17). The eluted protein solution was desalted and fractionated on a benzamide-Sepharose affinity column as described for the thymus enzyme (17). The resulting

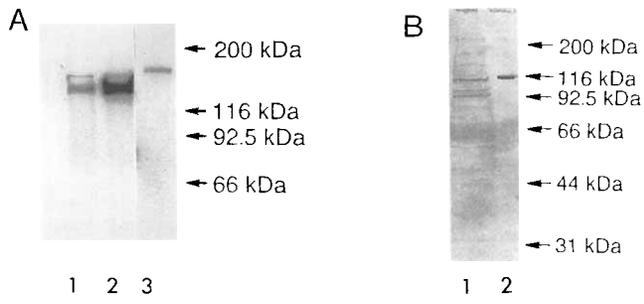

Figure 2. Electrophoretic and immunochemical characterization of ADPRT, partially purified from *M. edulis* embryos. (A) The partially purified ADPRT from *M. edulis* embryos was incubated either with 100 n*M* or 200 μM NAD under the conditions described in the legend of Table I. Aliquots were mixed with an equal volume of 2× sample buffer on the acid-urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (5) and loaded onto 10% acrylamide urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. After electrophoresis, the gels were soaked in 20% trichloroacetic acid to wash away unbound radioactive materials, and after a final wash in 10% acetic acid the gels were dried and exposed to autoradiography. Lane 1, mono-ADP-ribosylated ADPRT prepared without added octamer C. Lane 2, mono-ADR-ribosylated ADPRT prepared in the presence of octamer C; Lane 3, poly(ADP-ribosylated) ADPRT prepared in the presence of octamer C (400 n*M*). (B) The partially purified enzyme (25 μg of protein) was exposed to electrophoresis on an 8% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel according to the method of Laemmli (5). As a marker protein, ADPRT purified from calf thymus by the method of Buki *et al.* (17) (0.2 μg) was placed into Lane 2 (Fig. 2B). Following electrophoresis, the proteins were transblotted onto nitrocellulose sheets and the ADPRT immunostained as described earlier (17). Lane 1, *M. edulis* ADPRT; Lane 2, calf thymus ADPRT.

Table I. Effects of 6-ABP and Benzamide on the *M. edulis* ADPRT Enzyme[a]

NAD concentration	Inhibitor	ADPRT specific activity
		pmol ADPR/mg protein × min
100 n*M*	None	14.7
100 n*M*	0.1 m*M* benzamide	1.30
100 n*M*	1.0 m*M* 6-ABP	2.28
		nmol ADPR/mg protein × min
200 μM	None	4.9
200 μM	0.1 m*M* benzamide	0.38
200 μM	1.0 m*M* 6-ABP	0.51

[a] ADPRT (30 μg of protein) was preincubated with the drugs for 5 min. The reaction mixture contained 100 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, and 400 n*M* octamer C. The reaction was started by adding [^{32}P]NAD, and 2-min rates were measured.

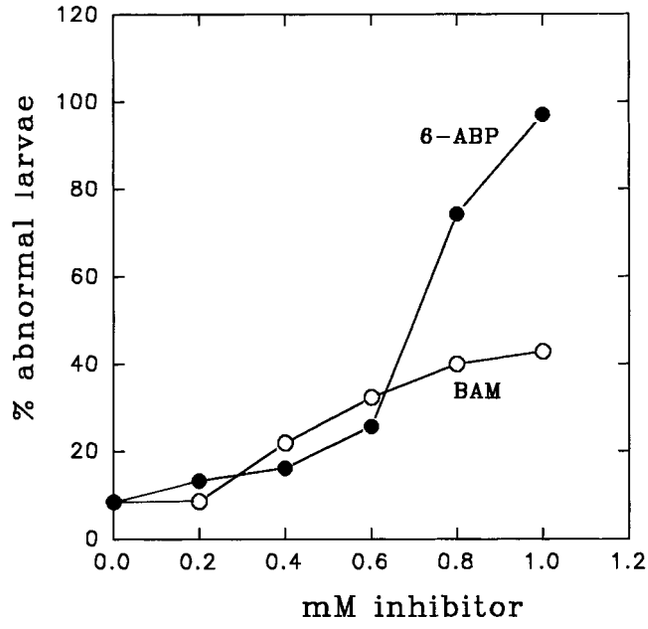

Figure 3. Effect of 6-ABP and benzamide on the development of *M. edulis* larvae. The *in vitro* toxicity tests were carried out as described in Materials and Methods. The experiments were done in duplicates that deviated by ±15% from the mean. Control samples contained less than 10% of abnormally developed larvae.

Table II. The Reversible Effect of 6-ABP on the Development of *M. edulis* Larvae: Reversibility of the 6-ABP Action in the *In Vitro* Toxicity Tests[a]

	Survival rate (%)[b]	% of development arrested larvae[c]
Control (untreated)	100	10.3 ± 5.0
6-ABP-treated control (inhibitor washed out)	100	24.8 ± 3.2
6-ABP remaining	94.8	97.7 ± 2.3

[a] *M. edulis* embryos were produced as described in Materials and Methods (2-hr embryos at the 32–64 cell stage) and were further incubated in artificial sea water for 4 hr in the presence of 1 m*M* 6-ABP. At the end of this incubation, the embryos were washed exhaustively with sea water on a 10-μm pore size filter to remove the drug. Finally, the embryos were resuspended in sea water and aliquots transferred into chambers containing either 6-ABP or equal amounts of buffer only. The samples were further incubated for 48 hr and evaluated for normal and abnormal embryo content by microscopic examination.

[b] Number of counted larvae as percentage of the number of inoculated embryos.

[c] Percentage of larvae that did not reach the D-hinge stage.

preparation represented a 50-fold purified ADPRT, based on specific activity (17), and this preparation still contained bound DNA since it exhibited enzymatic activity without added DNA, but activity was augmented by the synthetic DNA analog octamer C, indicating incomplete saturation of ADPRT by the co-isolated bound DNA. In Figure 2A, the electrophoretic separation of mono-ADP-ribosylated ADPRT of *Mytilus* is shown when 100 n*M* NAD was the substrate concentration (5, 17, 18) and the activation of mono-ADP-ribosylation by octamer C is illustrated in Figure 2, Lane 2. In the presence of 200 μM NAD and octamer C, the enzyme was poly(ADP-ribosylated) and its migration retarded due to oligomeric (ADPR)n, as apparent from Figure 2, Lane 3.

Immunochemical identification of ADPRT extracted from Mytilus is illustrated in Figure 2B, which is an immunotransblot. Figure 2, Lane 1 contains two major immunoreactive bands exhibiting M_r of 116 and 92 kDa, the former being the native enzyme and the latter a proteolytic degradation product containing epitopes of ADPRT. Figure 2, Lane 2 shows the immunologic identification of authentic thymus ADPRT (17). Since the purpose of this study was to identify the ADPRT protein, no detailed characterization of the polymer itself was undertaken, as described earlier (2).

Inhibition of the Mytilus ADPRT by Benzamide and by 6-ABP. As shown in Table I, both inhibitors were effective as assayed at one inhibitory concentration. The k_i of benzamide was 1.5 μM, competitive with respect to NAD and that of 6-ABP, 47 μM, competitive with respect to octamer C (16), illustrating that the two inhibitors act on ADPRT by different mechanisms.

The biologic effectivity of the two inhibitors is depicted in Figure 3, and it is evident that the DNA binding site-oriented inhibitor 6-ABP (16) is considerably more effective in arresting the development of Mytilus embryos, as seen from the appearance of abnormal larvae (Fig. 3, ordinate).

The inhibitory effect of 6-ABP on the development of normal Mytilus embryos was reversible. This was demonstrated as follows. Fertilized eggs were allowed to develop for 2 hr, then 1 mM 6-ABP was added to one series and none to a parallel series that represented controls that have not been exposed to the drug. After 4 hr of exposure to the drug, half of the embryo suspension was washed free of 6-ABP by repeated (three times) centrifugal washings, and the population of abnormal (D-hinge) embryos determined in both drug-exposed and washed and continuously drug-exposed embryo suspensions at the end of 48 hr. Results are shown in Table II. In controls, which have never been in contact with 6-ABP, about 10% abnormal larvae were counted. In embryos that were exposed to 6-ABP for 46 hr, about 98% developmentally arrested embryos accumulated, whereas removal of the drug after 4-hr exposure largely restored normal development, and only 25% of arrested embryos were detectable. Survival rate was not affected, thus the effect of 6-ABP appeared to be unrelated to nonspecific toxicity, but more probably to apparently selective inhibition of some critical step in differentiation.

Discussion

The results described here demonstrate the critical role of ADPRT in the development of Mytilus embryos. Apparently this role is universal since similar involvement of ADPRT in the development of a variety of organisms is known (9–13). The exact role of ADPRT in differentiation is not known, and this role has been projected for the most part from the development-arresting effects of benzamide-type (15) nicotinamide-binding site-oriented inhibitors (1) of ADPRT. The isolation and immunochemical identification of ADPRT in Mytilus leaves little doubt about the ubiquitous and highly conserved nature of this protein in mussel cells, and points to the possible ecologic importance of naturally occurring or industrial pollution-introduced substances that are ligands of ADPRT, since they are likely to interfere with the development of this widely distributed organism (14).

The newly formulated physiologic cellular biochemical role of ADPRT as being primarily involved in macromolecular binding processes in chromatin that are secondarily modified by the "metabolic," i.e., poly(ADP-ribose) synthesizing activity of this protein (3–8), does provide a working hypothesis for the explanation of the inhibitor effects of certain ADPRT ligands on differentiation. The distinction between the mode of action of various ADPRT ligands as inhibitors can provide certain directives. The most commonly used inhibitors, benzamide and some of its analogs, are known competitors of NAD at the nicotinamide binding site(s) of ADPRT (15), and it is commonly thought that their biologic action should be related to this enzyme-binding site. The pharmacologic action of benzamide on cell function is complicated by its limited rate of penetration into cells in culture, resulting in 10–15 μM steady-state intracellar concentration when applied at about 1 mM extracellular level (19). Nonspecific toxic effects of benzamide were observed at 5 mM or higher extracellular concentration (20), therefore we never exceeded maximal 1 mM extracellular doses. However, benzamide also binds to a second, DNA-recognizing, site of ADPRT (21), a site that more selectively binds 6-ABP (16), which is not a competitive inhibitor of NAD. The fact that the strong binding of benzamides to the nicotinamide site(s) does not correlate with its development-arresting potency (Fig. 3), whereas the DNA binding site-specific 6-ABP (16) is more effective, tends to suggest that enzyme sites that regulate DNA binding could play a more important function in arresting differentiation, possibly through an ADPRT ligand-induced and ADPRT-mediated topologic effect on DNA (6). Recent results show that about 10–15% of extracellularly applied 6-ABP exists intracellularly, and this concentration is only 2- to 3-fold higher than the k_i calculated for the pure enzyme. However, this largely unexplored hypothetical mechanism of drug-induced secondary effects of ADPRT on DNA function requires a great deal of experimental testing. The Mytilus model, besides indicating a new environmental toxicologic mechanism, has experimental advantages for the exploration of the biologic role of ADPRT in differentiation.

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