ADP-ribosyltransferase in isolated nuclei from sea-urchin embryos

Atsushi ISOAI and Ikuo YASUMASU

Department of Biology, School of Education, Waseda University, 1-6-1, Nishi-waseda, Shinjuku-ku, Tokyo 160, Japan

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The activity of ADP-ribosyltransferase in nuclei isolated from sea-urchin embryos was estimated by the incorporation of [adenosine- ^{14}C]NAD $^{+}$ into the acid-insoluble fraction. Hydrolysis of this acid-insoluble product by snake venom phosphodiesterase yielded radioactive 5'-AMP and phosphoribosyl-AMP. The incorporation of [^{14}C]NAD $^{+}$ was inhibited by 3-aminobenzamide and nicotinamide, potent inhibitors of ADP-ribosyltransferase. [^{14}C]NAD $^{+}$ incorporation into the acid-insoluble fraction results from the reaction of ADP-ribosyltransferase. The optimum pH for the enzyme in isolated nuclei was 7.5. The enzyme, in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-NAD $^{+}$ and 0.5 mM-dithiothreitol, exhibited the highest activity at 18°C in the presence of 14 mM-MgCl $_2$. The apparent K_m value for NAD $^{+}$ was 25 μM . The activity of the enzyme was measured in nuclei isolated from the embryos at several stages during early development. The activity was maximum at the 16–32-cell stage and then decreased to a minimum at the mesenchyme blastula stage. Thereafter its activity slightly increased at the onset of gastrulation and decreased again at the prism stage.

It is well known that poly(ADP-ribosyl)ation is one of the modification reactions of nuclear proteins, such as histones (Nishizuka *et al.*, 1968; Ueda *et al.*, 1975; Burzio *et al.*, 1979; Ogata *et al.*, 1980), high-mobility-group proteins (Tanuma & Johnson, 1983) and Mg $^{2+}$ - and Ca $^{2+}$ -dependent endonuclease (Yoshihara *et al.*, 1975). Poly(ADP-ribosyl)ation is catalysed by nuclear ADP-ribosyltransferase, which transfers the ADP moiety of NAD $^{+}$ to acceptor proteins to form a poly(ADP-ribose) (Hayaishi & Ueda, 1977). This enzyme has been detected in various organisms (Ueda *et al.*, 1982). The biological function of poly(ADP-ribose) is not completely understood at present, but many works suggest that this modification of nuclear proteins is involved in the regulation of DNA replication (Burzio & Koide, 1970), DNA repair (Durkacz *et al.*, 1980) and cell differentiation (Caplan *et al.*, 1979; Farzaneh & Pearson, 1979).

For the first step to find out the role of poly(ADP-ribose) in sea-urchin development, we investigated some properties of ADP-ribosyltransferase in isolated nuclei from sea-urchin embryos and observed the changes in the enzyme activity during early development.

Materials and methods

Gametes were obtained from the sea-urchin *Hemicentrotus pulcherrimus* by an injection of 0.5 M-

KCl into the body cavity. After insemination, the eggs were allowed to develop at 20°C.

Preparation of nuclei

Nuclei were isolated by the method described by Albanese *et al.* (1980). Embryos were washed twice with a solution (0.1 M-sodium phosphate, pH 6.0, 0.1 M-LiCl, 0.5 mM-phenylmethanesulphonyl fluoride) supplemented with 24 mM-EDTA. Then the embryos were washed once with the solution without EDTA, and homogenized in a Dounce homogenizer with a Teflon pestle. Triton X-100 was added (final concn. 0.2%) to the homogenate, which was then incubated for 5 min at 0–2°C. Thereafter the nuclei were pelleted by centrifugation at 2600 *g* for 10 min and washed twice with the solution.

Assay of ADP-ribosyltransferase activity

Nuclei isolated from the 16–32-cell-stage embryos, in which the maximum activity of the enzyme was observed, were used unless indicated. The basal reaction mixture contained, in 150 μl , 50 mM-Tris/HCl, pH 7.5, 0.5 mM-dithiothreitol, 14 mM-MgCl $_2$, 0.5 mM-[adenosine- ^{14}C]NAD $^{+}$ (2×10^5 c.p.m./tube), and an appropriate amount of nuclei in 0.25 M-sucrose. Incubation was performed at 18°C for 20 min. The reaction was started by adding nuclei and, after 20 min

incubation, a 50 μ l portion of the reaction mixture was applied to a filter paper (Whatman 3MM). The filter paper was washed three times with 5% (w/v) trichloroacetic acid, and analysed for ^{14}C radioactivity by using toluene-based scintillation fluid in a liquid-scintillation spectrometer (Aloka LSC-700; Aloka Co., Tokyo, Japan). To obtain the total activity, deoxyribonuclease I (50 μg /tube) was added in some cases to the reaction mixture. The activity of ADP-ribosyltransferase was expressed as ^{14}C radioactivity incorporated into acid-insoluble fraction/20 min per mg of protein or per μg of DNA in the nuclei. The other reaction conditions are indicated in the Figure legends.

Identification of poly(ADP-ribose)

To 150 μ l of the reaction mixture obtained as mentioned above, an equal volume of 20% trichloroacetic acid was added after incubation for 20 min at 18°C, and acid-insoluble material was collected by centrifugation at 8000 *g* for 5 min. The pellet was washed twice with 10% trichloroacetic acid and twice with ethanol. The washed pellet was suspended in 200 μ l of 50 mM-Tris/HCl, pH 6.8, containing 20 mM-MgCl₂ and 50 μg of snake venom phosphodiesterase, and was incubated at 37°C for 60 min. The reaction was stopped by adding 200 μ l of 10% trichloroacetic acid. After centrifugation at 10000 *g* for 5 min, the supernatant was analysed by paper chromatography as described by Shima *et al.* (1969).

The acid-insoluble material, obtained by incubation of nuclei with [^{14}C]NAD⁺, was also suspended in 100 μ l of 0.1 M-HCl or 0.1 M-Tris/glycine buffer, pH 10.5, and incubated at 37°C for 60 min. The acid-insoluble material thus treated was applied to a filter paper, then washed three times with 5% trichloroacetic acid and analysed for ^{14}C radioactivity.

Determination of protein and DNA

Protein contents were measured by the method of Lowry *et al.* (1951), with bovine serum albumin

as the standard. DNA contents were measured by the method of Burton (1956), with calf thymus DNA as the standard.

Chemicals

[adenosine- ^{14}C]NAD⁺ (sp. radioactivity 288 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. NAD⁺ and deoxyribonuclease I were purchased from Boehringer, Mannheim, West Germany. 3-Aminobenzamide and nicotinamide were the products of Tokyo Kasei Co., Tokyo, Japan. Phenylmethanesulphonyl fluoride and snake venom phosphodiesterase were from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of analytical grade.

Results

Identification of poly(ADP-ribose)

Radioactive product was obtained in acid-insoluble fraction after the incubation of isolated nuclei with [^{14}C]NAD⁺. This acid-insoluble radioactive material was resistant to hydrolysis in 0.1 M-HCl at 37°C for 60 min, and was hydrolysed, to some extent, by heating at 37°C in 0.1 M-Tris/glycine buffer, pH 10.5, for 60 min (Table 1). The acid-insoluble material became partially acid-soluble after treatment with snake venom phosphodiesterase (Table 1). The acid-soluble products thus obtained were identified as 5'-AMP and phosphoribosyl-AMP by paper chromatography (result not shown). Thus the radioactive product obtained after the incubation of isolated nuclei with [^{14}C]NAD⁺ was identified as poly(ADP-ribose), which was produced by the reaction catalysed by ADP-ribosyltransferase.

Optimum conditions for ADP-ribosyltransferase activity

Fig. 1 shows the relationship between reaction time and poly(ADP-ribose) synthesis in isolated nuclei from sea-urchin embryos at the 16–32-cell

Table 1. Digestion of acid-insoluble material in the nuclei incubated with [^{14}C]NAD⁺

For this experiment, nuclei were obtained from the 16–32-cell-stage embryos. The nuclei, containing ^{14}C -labelled material after an incubation with [^{14}C]NAD⁺ for 20 min at 18°C, were kept at 37°C for 60 min in 0.1 M-HCl or 0.1 M-Tris/glycine buffer, pH 10.5, or were incubated with snake venom phosphodiesterase (250 μg /ml) at 37°C for 60 min.

Treatment	^{14}C radioactivity in acid-insoluble fraction after treatment (c.p.m.)	Percentage of acid-insoluble radioactivity after treatment (%)
Control	6018	—
0.1 M-HCl	6777	0
0.1 M-Tris/glycine buffer, pH 10.5	2025	34
Snake venom phosphodiesterase	3180	52

stage. The incorporation of [^{14}C]NAD into the acid-insoluble fraction was linear for 20 min and then attained a plateau after about 40 min of reaction.

Fig. 1. Time course of poly(ADP-ribose) synthesis in isolated nuclei from sea-urchin embryos

The reaction mixture is described in the Materials and methods section. The reaction at 18°C was carried out for various times as indicated. Poly(ADP-ribose) synthesized during the reaction is expressed as c.p.m. ($\times 10^{-3}$) of [^{14}C]adenosine incorporated into acid-insoluble material. The nuclei were obtained from 16–32-cell-stage embryos.

Figs. 2 and 3 respectively show the effects of temperature and pH on ADP-ribosyltransferase activity. The activity was maximum at 18°C and was close to zero at temperatures higher than 30°C . The activity was low at pH values below 7 and above 8.5. The maximum activity was obtained at pH 7.5. Fig. 3 shows a typical example from four experiments. In all cases, the highest activity was obtained at pH 7.5. As shown in Fig. 3, the activity is slightly lower at pH 7.8 than at pH 7.5 and at pH 8.0. Similar results were obtained in all four experiments. In the present study, however, significantly low activity at pH 7.8 was not reproducibly obtained. At present, optimum pH

Fig. 3. Effect of pH on ADP-ribosyltransferase activity

The reaction mixture was as described in the Materials and methods section, except for the pH of the 50 mM-Tris/HCl buffer. The reaction at 18°C was carried out for 20 min at the pH values indicated in the Figure. The enzyme activity is expressed as in Fig. 2. The nuclei were obtained from embryos at the 16–32-cell stage.

Fig. 2. Effect of temperature on ADP-ribosyltransferase activity in isolated nuclei from sea-urchin embryos

The procedures are given in the Materials and methods section. The reaction was performed for 20 min at various temperatures. The enzyme activity is expressed as c.p.m. ($\times 10^{-4}$) of [^{14}C]adenosine incorporated into acid-insoluble material/20 min per mg of protein of nuclei isolated from the 16–32-cell-stage embryos.

Fig. 4. Effect of Mg^{2+} ions on ADP-ribosyltransferase activity

The reaction mixture was as described in the Materials and methods section, except for the concentration of $MgCl_2$. The reaction was performed at 18°C for 20 min in the presence of $MgCl_2$ at various concentrations. The enzyme activity is expressed as shown in Fig. 2. The isolated nuclei obtained from embryos at the 16–32-cell stage were used as the enzyme source.

was tentatively designated as 7.5. As shown in Fig. 4, the enzyme in the isolated nuclei from sea-urchin embryos exhibited maximum activity in the presence of 14 mM- $MgCl_2$. The requirement of Mg^{2+} for the ADP-ribosyltransferase reaction is known in other cell types (Nishizuka *et al.*, 1967; Brightwell *et al.*, 1975).

Fig. 5 shows double-reciprocal relationships between NAD^+ concentration and ADP-ribosyltransferase activity. The apparent $K_m(NAD^+)$ was 25 μM . At NAD^+ concentrations higher than 0.125 mM, substrate activation occurred.

On the basis of these observations, the reaction was carried out in the present study at 18°C for 20 min in 50 mM-Tris/HCl, pH 7.5, containing 14 mM- $MgCl_2$, 0.5 mM-dithiothreitol and 0.5 mM- NAD^+ .

As shown in Fig. 6, the incorporation of [^{14}C] NAD^+ into nuclei was inhibited by 3-aminobenzamide and nicotinamide, which are powerful inhibitors of ADP-ribosyltransferase (Purnell & Whish, 1980).

Fig. 5. Double-reciprocal plot of ADP-ribosyltransferase activity with substrate concentration

The reaction mixture was as described in the Materials and methods section. The reaction at 18°C for 20 min was carried out in the presence of NAD^+ at various concentrations. Activity (v) is expressed as c.p.m. ($\times 10^{-4}$) of [^{14}C]adenosine incorporated into acid-insoluble material/20 min per mg of protein.

Fig. 6. Effect of inhibitors on ADP-ribosyltransferase activity

The reaction was carried out as described in the Materials and methods section, in the presence of 3-aminobenzamide (●) or nicotinamide (○). The enzyme activities in the presence of inhibitors are expressed as percentages of that in their absence.

ADP-ribosyltransferase activity during early development

Fig. 7 shows the changes of the physiological and the total ADP-ribosyltransferase activity during early development. The total enzyme

Fig. 7. Changes in the physiological and the total ADP-ribosyltransferase activity during early development

The physiological (●) and the total (○) activities were measured as indicated in the Materials and methods section. Each value in the Figure is the mean of three experiments. Vertical bar shows S.E.M. To obtain the total enzyme activity, deoxyribonuclease I (50 µg/tube) was added in the reaction mixture. The enzyme activity is expressed as c.p.m. ($\times 10^{-3}$) of [^{14}C]adenosine incorporated into acid-insoluble material from [^{14}C]NAD⁺/20 min per µg of DNA. The stages of development (times after fertilization at 20°C) were the 16–32-cell stage (4h), the blastula stage before hatching (8h), the mesenchyme blastula stage (16h), the mesenchyme blastula just before gastrulation (18h), the middle-gastrula stage (24h), the prism stage (36h) and the pluteus stage (45h).

activity was measured in the nuclei treated with deoxyribonuclease I, in accordance with the report that DNA breakage causes a marked stimulation of ADP-ribosyltransferase in chromatin (Berger *et al.*, 1978). The maximum physiological and total activity was obtained at the 16–32-cell stage (4h after fertilization at 20°C) and decreased during development up to the mesenchyme blastula stage (16h after fertilization). Thereafter the activity became slightly higher at the onset of gastrulation (18h after fertilization) and decreased again at the prism stage (36h after fertilization). During development after the gastrula stage (24h after fertilization), the total activity became almost the same as the physiological one. This may be due to the high nuclease activity in the nuclei during their isolation.

Discussion

In the nuclei of sea-urchin embryos, incubation in the presence of [^{14}C]NAD⁺ produced acid-insoluble radioactive materials, which yielded 5'-

AMP and phosphoribosyl-AMP on treatment with snake venom phosphodiesterase. It has been demonstrated that poly(ADP-ribose) is digested by snake venom phosphodiesterase to produce 5'-AMP and phosphoribosyl-AMP (Fujimura *et al.*, 1967; Hasegawa *et al.*, 1967). This radioactive material formed in the isolated nuclei was also resistant to acid hydrolysis and was split by alkaline hydrolysis. Alkaline hydrolysis has been known to split poly(ADP-ribose) from proteins (Adamietz *et al.*, 1978; Riquelme *et al.*, 1979). Thus the production of radioactive material in the isolated nuclei from sea-urchin embryos is concluded to be due to the reaction catalysed by ADP-ribosyltransferase. Additionally it was observed that 3-aminobenzamide and nicotinamide inhibited the incorporation of [^{14}C]NAD⁺ into the nuclei. This also supports the conclusion mentioned above.

The optimum temperature for the enzyme activity of sea-urchin embryos was somewhat lower than those in other cell types (Burzio & Koide, 1970; Stone & Shall, 1973; Farzaneh & Pearson, 1979; Okolie & Onyezili, 1983), and was within the physiological range of temperature for the development of the embryos of the sea-urchin *Hemicentrotus pulcherrimus*.

The apparent K_m of the enzyme for NAD⁺ was 25 µM in the sea-urchin embryos. This value is lower than the reported values of 0.27 mM for Ehrlich ascites cells (Romer *et al.*, 1968), 0.85 mM for rat liver nuclei (Clark *et al.*, 1971) and 1.5 mM for mouse fibroblast (LS-cell) nuclei (Stone & Shall, 1973). The value obtained in the nuclei of sea-urchin embryos is similar to the K_m value of 23 µM obtained for the purified enzyme from pig thymus (Tsopanakis *et al.*, 1978). The substrate activation of the enzyme in isolated nuclei from sea-urchin embryos was also observed, as for the purified enzyme (Ito *et al.*, 1979). In crude enzyme preparations for other cell types, substrate activation has not been found.

During early development of the sea-urchin, the activity of ADP-ribosyltransferase, both physiological and total, changes markedly. At the 16–32-cell stages (4h after fertilization at 20°C), the activity per nucleus was markedly higher than in the other stages. Before this stage, cell number in an embryo is quite small, and hence the amount of nuclei isolated from embryos was quite small, if the same number of embryos as those at the later stages was used as starting material. Hence the isolation of nuclei from the eggs at the earlier stages than the 16–32-cell stage was not performed, because of limitation of the materials. Hence it remains unknown whether or not the activity in the eggs at stages earlier than the 16–32-cell stage is higher than in the eggs at this stage.

The activity per nucleus at the blastula stage (8 h after fertilization) was markedly higher than at the mesenchyme blastula stage (16 h after fertilization), but was lower than that at the 16–32-cell stage. The activity seems to decrease gradually during this period of development. During the period between the 16–32-cell stage and the blastula stage, cell number increases exponentially, to about 700 cells/embryo at the blastula stage (Niikura *et al.*, 1984). Hence the activity of the enzyme per embryo was enhanced, although slightly. On the contrary, the activity per embryo as well as per nucleus probably decreased during the period of development between the blastula and the mesenchyme blastula stage, where the cell number in an embryo increases at quite a low rate. The high activity of the enzyme seems to be maintained during the developmental period where cell proliferation occurs. But it is not clear whether or not poly(ADP-ribosylation) is involved in cell proliferation in the present study.

In the embryos at the onset of gastrulation (18 h after fertilization), as well as those at the middle-gastrula stage (24 h after fertilization), the activity was considerably higher than in the embryos at the mesenchyme blastula stage. After the onset of gastrulation, differentiation of endodermal, mesodermal and ectodermal cells occurs. The modification of nuclear proteins by the ADP-ribosyltransferase seems to support cell differentiation in the sea-urchin embryos, as well as in other cell types (Caplan *et al.*, 1979; Farzaneh & Pearson, 1979). Also at the 16–32-cell stage, differentiation of mesoderm has been demonstrated to occur in the embryos (Okazaki, 1975). The high activity of this enzyme at this stage may also cause poly(ADP-ribosylation) of the nuclear proteins to contribute to cell differentiation.

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