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IMMUNOCHEMICAL PROPERTIES OF UNMODIFIED AND AUTOMODIFIED POLY(ADP-RIBOSE) SYNTHETASES*

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SUMMARY A specific antibody against poly(ADP-ribose) synthetase of calf thymus was produced in the rabbit. The antibody formed a single immunoprecipitin line with purified as well as crude poly-(ADP-ribose) synthetase preparations, and quantitatively precipitated the enzyme activity. The antibody was cross-reactive, to much lesser extents, with the enzymes of rat, mouse and chicken livers and of HeLa cells. The antibody was also reactive with auto-poly(ADP-ribosyl)ated calf thymus enzyme, and used for characterization of the poly(ADP-ribosyl) enzyme linkage.

Mammalian cell nuclei contain a chromatin-bound enzyme, poly-(ADP-ribose) synthetase, which catalyzes the formation of a homopolymer of repeating ADP-ribose units from NAD (1-3). Although the biological function of the polymer is not yet fully understood, a close correlation between the polymer synthesis and DNA replication (4), DNA repair (5, 6), cell differentiation (7, 8), or neoplastic transformation (8, 9) has been suggested. Various nuclear proteins, both histones and nonhistone proteins, serve as the acceptors of this modification reaction (1-3). Recently, Yoshihara et al. (10) reported that poly(ADP-ribose) synthetase itself might accept ADP-ribose. In order to confirm this finding and examine the nature of the auto-poly(ADP-ribosyl)ation reac-

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Abbreviation: SDS, sodium dodecyl sulfate.

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tion, we recently produced a specific antibody against the enzyme in the rabbit. In this paper, we report, using this antibody, the confirmation of automodification of poly(ADP-ribose) synthetase and also some immunochemical properties of unmodified and automodified enzymes of various species.

MATERIALS AND METHODS

Materials: [Ade-^{14}C]NAD was obtained from Radiochemical Centre, Amersham. NAD, calf thymus DNA (type I), calf thymus histone (type II-A) and calf liver RNA (type IV) were the products of Sigma. Soluene 350 was purchased from Packard. Poly(ADP-ribose) synthetase was purified from calf thymus to apparent homogeneity by the method of Ito et al. (11).

Assays: Poly(ADP-ribose) synthetase activity was assayed as described previously (11). One unit of enzyme activity was defined as the amount to incorporate 1 nmol of ADP-ribose into acid-insoluble material per min under the above condition. Protein was assayed by the method of Lowry et al. (12). The average chain length of poly(ADP-ribose) was determined by digestion with snake venom phosphodiesterase (13).

Immunological procedures: Antibody against calf thymus poly(ADP-ribose) synthetase was prepared by an initial subcutaneous injection of 0.5 mg of purified enzyme mixed with complete Freund's adjuvant to a rabbit, followed by a booster (0.25 mg) one month later. Blood was collected two weeks after the booster, and the IgG fraction was purified (14). Antibody (IgG) was concentrated to 20 mg protein/ml with the aid of a Centriflo membrane cone (Amicon).

Ouchterlony double immunodiffusion was performed on a microslide glass mounted with 1% agar in phosphate-buffered saline (PBS) (0.15 M NaCl and 10 mM sodium phosphate, pH 7.2) (15).

Precipitation of poly(ADP-ribose) synthetase with antibody was performed by the following procedure. Twofold serial dilutions of antibody with PBS were mixed with 2 units of calf thymus poly(ADP-ribose) synthetase (hydroxyapatite fraction) in 50 µl of PBS. The mixtures were incubated for 30 min at 37°C, followed by another 30-min incubation at 0°C. The mixtures were centrifuged at 12,000 X g for 20 min, and a 20-µl aliquot of each supernatant was assayed for the enzyme activity.

Cross-reactivity of the antibody with poly(ADP-ribose) synthetases of various species was examined by the same procedure except that varying amounts of enzyme were used. Immunoreactivity index was defined by the amount (in terms of mg protein) of antibody required to precipitate 1 unit of enzyme.

Preparation and immunoprecipitation of auto-poly(ADP-ribosyl)-ated synthetase: Two units of calf thymus poly(ADP-ribose) synthetase (hydroxyapatite fraction) were incubated at 25°C for 20 sec with a mixture (0.1 ml) containing 0.1 M Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 µM [^{14}C]NAD (500 cpm/pmol), and 0.1 mg/ml calf thymus DNA. The incubation was terminated by

the addition of 5 µl of 0.1 M 3-aminobenzamide; the latter is a potent inhibitor of poly(ADP-ribose) synthetase (16).

The enzyme preparation thus automodified was incubated at 25°C for 30 min with serial dilutions of antibody in 0.2 ml of 1 M NaCl containing 10 mM sodium phosphate (pH 7.2). After 30 min at 0°C, immunoprecipitate was collected by centrifugation (12,000 X g, 20 min), and washed three times with 1 M NaCl containing 10 mM sodium phosphate (pH 7.2). The precipitate was solubilized with 0.2 ml of 1 M NaOH, and the radioactivity determined by a liquid scintillation method (11).

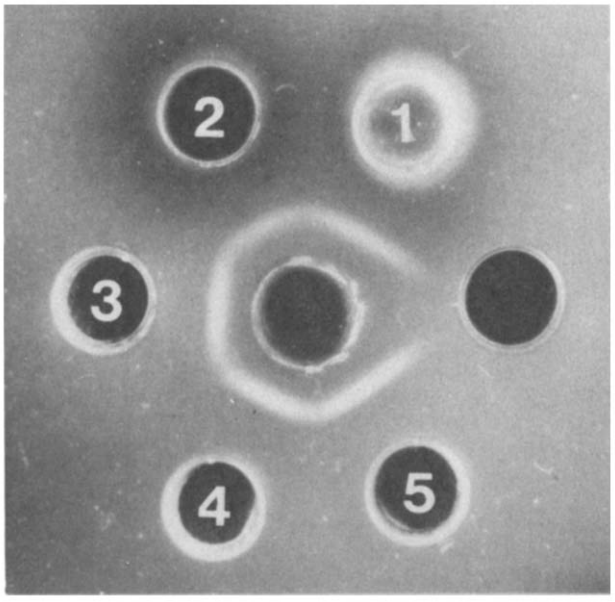
Polyacrylamide gel electrophoresis of auto-poly(ADP-ribosyl)ated enzyme: Ten units of poly(ADP-ribose) synthetase, automodified as above, were incubated with 1 mg of antibody in 0.2 ml of 1 M NaCl containing 10 mM sodium phosphate (pH 7.2). Immunoprecipitate was collected by centrifugation, washed once with 1 M NaCl containing 10 mM sodium phosphate (pH 7.2) and twice with 1 M NaCl containing 0.1 M glycine/HCl (pH 2.7), and then dissolved in a solution (50 µl) containing 2% SDS, 1% 2-mercaptoethanol, 0.01% bromphenol blue and 30% glycerol. After 60 min at 25°C, the mixture was subjected to electrophoresis in cylindrical SDS (0.1%)-polyacrylamide (5%) gels (0.5 X 7 cm) as described by Weber and Osborn (17). After electrophoresis, the column was cut longitudinally in half; one half was stained with Coomassie blue, and the other was sliced into 2-mm pieces. The pieces were treated (for 6 hr at 50°C) with 1 ml of Soluene 350, and examined for radioactivity (18).

Chemical treatment of auto-poly(ADP-ribosyl)ated synthetase: Immunoprecipitate of auto-poly(ADP-ribosyl)ated enzyme, prepared as above, was dissolved in 6 M guanidine/HCl containing 20 mM acetic acid. Twenty microliters of this solution were incubated with 2.5 µl of either 1 M HCl or 1 M NaOH, or 20 µl of 4 M NH_2OH (pH 7.0) or 0.2 M potassium phosphate (pH 7.0) at 25°C for various time lengths. Incubation was terminated by the addition of 20% CCl_3COOH. Acid-insoluble material was collected on a Millipore filter (pore size = 0.45 µm), and examined for radioactivity.

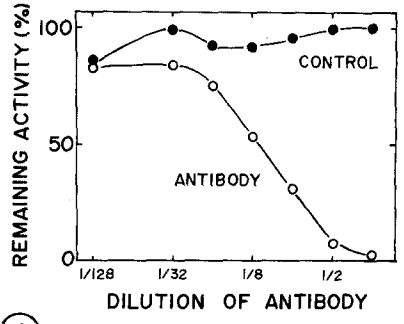
RESULTS AND DISCUSSION

Properties of antibody against poly(ADP-ribose) synthetase:

The antibody gave a single precipitin line with no spur upon double immunodiffusion with all enzyme samples from the various stages of purification (Fig. 1), indicating that there was a single and common antigen throughout the purification. The enzyme preparation used for immunization did not contain significant amounts of DNA (11), and the antibody did not react with calf thymus DNA, calf thymus histone or calf liver RNA. When the purified enzyme preparation was reacted with the antibody after mixing with calf thymus DNA, an apparently identical precipitin line



(1)



(2)

Fig. 1. Double immunodiffusion analysis with anti-synthetase antibody and enzyme preparations at various purification steps. The central well contained antibody (IgG), and the wells 1,2,3,4, 5 contained, respectively, crude extract of calf thymus (151 units/ml), protamine fraction (108 units/ml), DNA-agarose fraction (167 units/ml), hydroxyapatite fraction (170 units/ml), and Sephadex G-150 fraction (180 units/ml). Each enzyme fraction was concentrated with the aid of a Centriflo cone.

Fig. 2. Immunoprecipitation of calf thymus poly(ADP-ribose) synthetase. Partially purified enzyme (hydroxyapatite fraction, 2 units) was incubated with various quantities of anti-synthetase IgG (o) or control (preimmune) IgG (•), and the enzyme activity in the supernatant obtained by centrifugation was assayed as described in MATERIALS AND METHODS.

with that of DNA-free enzyme was observed (data not shown). These results suggested that the antibody was directed against the enzyme protein itself.

When poly(ADP-ribose) synthetase and the antibody were incubated together and the mixture centrifuged, the enzyme activity was lost quantitatively from the supernatant (Fig. 2). From the linear portion of the titration curve, 0.1 mg of the antibody was estimated to precipitate 1 unit of the calf thymus enzyme.

Table I.

Cross-reactivity of antibody against poly(ADP-ribose) synthetase

Enzyme source	Immunoreactivity index[b)]
Calf thymus (hydroxyapatite fraction)	0.1
Rat liver (nuclear extract)[a)]	3.4
Mouse liver (nuclear extract)[a)]	3.1
Chicken liver (nuclear extract)[a)]	5.6
HeLa cell (nuclear extract)[a)]	2.2

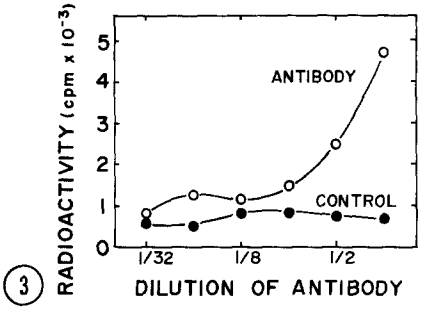
a) Nuclei were prepared from rat, mouse or chicken liver by the method of Chauveau et al. (19) or from HeLa cells by the method of Sporn et al. (20). Isolated nuclei were homogenized with 3 volumes of 50 mM Tris/HCl (pH 8.0) containing 0.3 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol and 50 mM sodium bisulfite, using a Polytron homogenizer. Homogenate was centrifuged at 12,000 X g for 15 min, and the separated supernatant was used as the enzyme source.

b) See "MATERIALS AND METHODS".

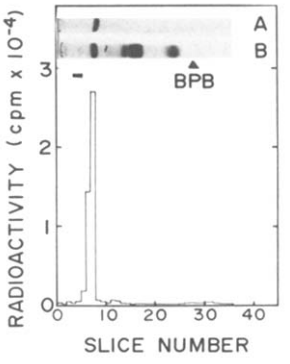
Although the antibody produced no visible precipitin line in the Ouchterlony test with crude enzyme preparations of rat, mouse, and chicken livers and HeLa cells, cross-reaction was detected with the immunoprecipitation test (Table I). The apparent immunoreactivity indexes for these enzymes were >20-fold higher than that for calf thymus enzyme. However, these values were considered tentative, since poly(ADP-ribose) synthetase activity was assayed in crude enzyme preparations containing partially fragmented DNA, histones, nonhistone proteins, and poly(ADP-ribose)-degrading enzymes, all of which have been known to affect apparent poly(ADP-ribose) synthesis to varying degrees (1-3).

Immunoprecipitation and characterization of auto-poly(ADP-ribosyl)ated synthetase:

When poly(ADP-ribose) synthetase preincubated with [^{14}C]NAD was mixed with the antibody, radioactive immunoprecipitate was



(3)



(4)

Fig. 3. Immunoprecipitation of auto-poly(ADP-ribosyl)ated synthetase. Partially purified enzyme (hydroxyapatite fraction, 2 units), was incubated with [^{14}C]NAD and then with various quantities of anti-synthetase IgG (o) or control (preimmune) IgG (●) as described in MATERIALS AND METHODS. Immunoprecipitates were collected by centrifugation, and their radioactivities determined.

Fig. 4. SDS-polyacrylamide gel electrophoresis of auto-poly(ADP-ribosyl)ated synthetase. Electrophoresed gel column was cut in half; one half was used for radioactivity determination and the other was stained. Insets A, unmodified enzyme; B, immunoprecipitates of auto-poly(ADP-ribosyl)ated enzyme. BPB, bromphenol blue.

produced (Fig. 3). Since the antibody did not cross-react with ADP-ribose nor poly(ADP-ribose), as judged by membrane binding assays (21) (data not shown), this result indicated that poly(ADP-ribose) synthetase itself was poly(ADP-ribosyl)ated (10) and that the antibody reacted with the auto-modified enzyme.

These ideas were further supported by analysis of the immunoprecipitate with SDS-polyacrylamide gel electrophoresis; only one distinct peak of radioactivity migrated to the position close to poly(ADP-ribose) synthetase (Fig. 4). Judging from the facts that the average chain length of poly(ADP-ribose) in this experiment was 4.1 ADP-ribose units, and that poly(ADP-ribose) of this size hardly affected the electrophoretic mobility of an acceptor protein (18), this result appeared to show the immunoprecipitation of auto-modified enzyme.

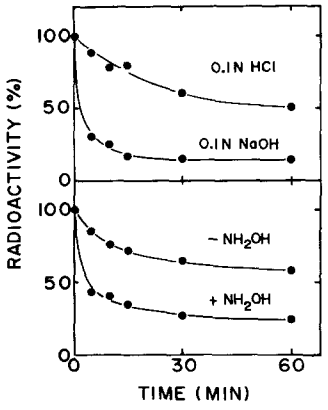


Fig. 5. Chemical stabilities of poly(ADP-ribosyl) enzyme linkage in various pH values (upper) or neutral NH_2OH (lower). Experimental conditions were described in MATERIALS AND METHODS.

The linkage between poly(ADP-ribose) and the enzyme was labile in neutral NH_2OH and mild alkali (Fig. 5). The decreases in acid-insoluble radioactivity at 25°C in 1 hr in 0.1 M HCl, 0.1 M NaOH, 0.1 M potassium phosphate (pH 7.0) and 2 M NH_2OH (pH 7.0) were 51, 84, 42 and 73%, respectively. These stabilities were similar to those of ADP-ribosyl histone ester linkages(22, 23), although the poly(ADP-ribosyl) enzyme linkage appeared to be slightly less stable at neutral pH.

One of the most distinguished feature of poly(ADP-ribose) synthetase is poly(ADP-ribosyl)ation of the enzyme itself (6,10, 18,24). Recently, we found that this automodification may play an important role in DNA repair (6). Further investigation of this reaction both *in vitro* and *in vivo* using the specific antibody will help unveiling the biological significance of this modification reaction in chromatin.

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