Poly(ADP-ribosylation) of Nuclear Proteins

ENZYMATIC ELONGATION OF CHEMICALLY SYNTHESIZED ADP-RIbose-HISTONE ADDUCTS*

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Poly(ADP-ribosylation) is a unique post-translational modification of nuclear proteins (1–3). The modification reaction is supposed to proceed by an initial attachment of an ADP-ribose monomer, followed by successive elongation using NAD as the donor of ADP-ribose. Both the modifying enzyme (poly(ADP-ribosyl)ation) and modified proteins have been demonstrated in various eukaryotic organisms and tissues in vitro (1–7) as well as in vivo (8–12). Although there are some indications suggesting a relationship between this modification and the regulation of gene expression (13–15), its precise biological function has not yet been elucidated (16, 17). Among many proteins reported to be modified, histone was the first example (1–3), and the subgroups H1 and H2B have been documented to be the major acceptors (4–11, 18). Poly(ADP-ribosyl)ation has been partially purified from rat liver (19–22), calf thymus (23, 24), pig thymus (25) or ascites tumor cells (26). Common unique features of these enzyme preparations are the absolute dependence of the activity on DNA and further stimulation by histones added along with DNA (16–19). The latter effect of histones is not due to a supply of acceptors but rather to unmodified H1. The transferred ADP-ribose forms a linear polymer, poly(ADP-ribose), having the adenosine terminus. In the presence of ADP-ribose histone H1 adduct, a majority (50 to 90%) of new labeled ADP-ribose–H1 adduct preincubated with the synthetase and unlabeled NAD. Comparative studies using variously labeled adducts and NADs reveal that the elongation proceeds exclusively by a terminal addition mechanism, i.e. ADP-ribose being transferred to the adenosine terminus. In the presence of ADP-ribose–histone H1 adduct, a majority (50 to 90%) of new chains originates from the preattatched ADP-ribose and the remainder from similar structures on an endogenous acceptor contained in the synthetase preparation.

These results taken together suggest that poly(ADP-ribosyl)ation is primarily engaged in chain elongation, but not in direct ADP-ribosylation of histones (chain initiation); the latter reaction is probably catalyzed by an as yet unidentified enzyme or requires other factor(s) or conditions.

Materials and Methods

Materials and Enzymes—P-NAD, ADP-ribose, calf thymus DNA (highly polymerized), and ovomucoid trypsin inhibitor were purchased from Sigma; Neurospora crassa NADase, Crotalus adamanteus venom phosphodiesterase, and Escherichia coli alkaline phosphatase were from Worthington; bovine trypsin and proteinase K were from Calbiochem. Calf thymus histone H1 was prepared by the method of Johns (29).

Labeled NADs and ADP-riboses—[AMP-32P]NAD, [ribose(in NMN)-14C]NAD and [ribose(in NMN)-3H]NAD were prepared from [α-32P]ATP, D-ribose1,5-glucose, and D-ribose1,4-glucose, respectively, as described previously (30). The latter precursors, D-ribose1,5-glucose, and [Ado-U-14C]NAD were purchased from the Radiochemical Centre, Amersham; [Ado-2,8-3H]NAD was from New England Nuclear. [Ado-3H]ADP-ribose, [α-32P]ADP-ribose, ADP-[14C]ribose, and ADP-[13C]ribose were prepared from respective precursor NADs using N. crassa NADase.

Purification of Poly(ADP-ribose) Synthetase—Poly(ADP-ribose) synthetase was purified from rat liver nuclei up to the final (phosphocellulose) step by the method previously described (22).

Assay of Poly(ADP-ribose) Synthetase Activity—The standard

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mixture contained 20 μmol of Tris-Cl (pH 8.0), 2 μmol of MgCl₂, 250 nmol of dithiothreitol, 2 nmol of Ado-[14C]-NAD (50,000 cpm/nmol), equal amounts (10 to 30 μg) of calf thymus DNA and histone H₁, and purified enzyme in a total volume of 0.2 ml. Reaction was carried out for 10 min at 37°C and was terminated by addition of 3 ml of 20% Cl₃COOH. Acid-insoluble radioactivity was collected on a Millipore filter (HA type) and counted in a toluene-based scintillator.

Preparation of ADP-ribose-Histone Adducts—Nonenzymatic ADP-ribosylation of histone H₁ and reduction of the Schiff base were carried out by a modification of the method of Kun et al. (28). Calf thymus histone H₁ (11.1 mg/ml) was incubated in a solution (30 μl) containing 167 mM sodium carbonate (pH 10) and 1 μmol of ADP-ribose. After incubation for 3 h at 37°C, ammonium acetate (2.3 mM, pH 5.0) was added to a final concentration of 1 M (pH lowered to about 3 as judged by a pH-paper test). This mixture was added to two 10-μl portions of 0.5 M NaBH₄ freshly dissolved in 5 mM NaOH at 30-s intervals at 0°C. The mixture was then dialyzed against five portions (10 μl each) of 0.5 M NaOH at 30-s intervals at 0°C. The dialyzed material was lyophilized and dissolved in 40 μl of 10 mM ammonium acetate (pH 5.8) for 24 h at 4°C; the buffer was changed every 16 h.

Poly(ADP-ribosyl)ation of ADP-ribose-Histone Adducts—The standard reaction mixture and procedure for assaying poly(ADP-ribose) synthetase described above were used to poly(ADP-ribosyl)ate ADP-ribose-histone adducts except that histone in the standard reaction mixture and procedure for assaying poly(ADP-ribosyl)ated ADP-ribose. Histone Adducts except that histone in the standard reaction mixture and procedure for assaying poly(ADP-ribosyl)ated ADP-ribose was replaced by 30 μg of poly(ADP-ribosyl)ated ADP-ribose. Histone H₁ adduct, incubated (or unincubated) with poly(ADP-ribose) synthetase and NAD, was precipitated with 20% Cl₃COOH, suspended in 5% HClO₄, and the mixture was centrifuged; the supernatant was examined for radioactivity. From the large gel, a 1-cm region corresponding to the radioactivity peak in the small gel was cut out. The sample, freed of small molecules by precipitation with 20% Cl₃COOH for additional 12 h, the mixture, supplemented with 2-mercaptoethanol, bromphenol blue, and glycerol, was electrophoresed on a polyacrylamide gel as described by Weber and Osborn (31). 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 dissolved in 32% H₂O₂ at 60°C, and the radioactivity was determined in a mixture of a tolune scintillator and Triton X-100 (32).

Proteinase K Treatment of Poly(ADP-ribosyl)ated Material and Analysis of Products by Electrophoresis—The reaction mixture containing poly(ADP-ribosyl)ated products was dialyzed against 1 liter of 17 mM sodium phosphate (pH 7.2) containing 3.4% SDS. After standing for 12 h at 25°C, the mixture was applied onto an Eastman gel (7.5 cm). Electrophoresis was carried out for approximately 6 h at a constant current of 30 mA/cm² as described by Weber and Osborn (31). After electrophoresis, the sample, freed of small molecules by precipitation with 20% Cl₃COOH for 24 h at 4°C, the buffer was changed every 4 h.

IsoADP-ribose and Analysis—The fractions eluting from the Dowex 1 column were applied to a Whatman No. 1 filter paper. Paper chromatography was carried out in a descending direction; either of the two solvent systems was used; System 1, isobutyric acid, 1 M NH₄OH, 0.1 M EDTA (pH 8.0) (100:60:1.6, v/v/v); System 2, 0.1 M potassium phosphate (pH 6.8), (NH₄)₂SO₄, propanol-1 (100:60:2.5, v/v/v). Strips 1 cm wide were cut out and examined for radioactivity in a toluene-based scintillator.

Determination of Average Chain Length of Poly(ADP-ribose) Average chain length of poly(ADP-ribose) was determined by the method described previously (1) with a slight modification as follows; the sample was freed of small molecules by precipitation with 20% Cl₃COOH, and the mixture was centrifuged; the supernatant was examined for radioactivity. From the large gel, a 1-cm region corresponding to the radioactivity peak in the small gel was cut out. The sample, freed of small molecules by precipitation with 20% Cl₃COOH for 15 min, and the supernatant fraction was collected. The precipitate was resuspended in 5% HClO₄, and the mixture was centrifuged; the supernatant fractions were combined and neutralized with KOH. After removing the precipitate by low speed centrifugation, the supernatant fraction, mixed with marker ADP-ribose, was applied to a Dowex 1-formate (X2, 200 to 400 mesh) column (0.6 x 6 cm). The column was eluted with 100-ml gradients of HCOOH from 0 to 0.3 M and then from 0.3 to 6.0 M. Fractions (1.25 ml) were collected and examined for activity as described earlier.

RESULTS
Nonenzymatic ADP-Ribosylation of Histones—Ribose and its derivatives including ADP-ribose were bound nonenzymatically and covalently to various proteins as described by Kun et al. (28). In the presence of histone H₁, the binding, as assayed by the incorporation into acid-insoluble material, was almost a linear function of the concentration of the binding substance (Fig. 1). Ribose was much less reactive than ADP-ribose. Optimal pH for the reaction was around 9 to 10. At pH 9 and 37°C, the reaction proceeded nonlinearly for several hours (Fig. 2). The incorporation of the ribose portion of ADP-ribose (the moiety originally linked to nicotinamide in NAD) exceeded that of the adenine portion (Fig. 2). This difference seems to suggest a partial degradation of bound ADP-ribose to AMP and ribose 5-phosphate since the pyrophosphate linkage in ADP-ribose is unstable under alkaline conditions (35), and ribose 5-phosphate was as reactive as ADP-ribose in the binding (data not shown). On the basis of ³H incorporation upon reduction with NaBH₄, the linkage between ADP-ribose and protein has been reported to be a Schiff base between the terminal ribose and ε-amino group of lysine residue (28); whether an Amadori rearrangement (36) participates or not is not known. In support of the idea of

²AMP was added to inhibit phosphatases in the ADP-ribose treatment used.

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0 20 40 60 80

FIG. 1. Effects of concentrations of ADP-ribose or ribose on non-enzymatic histone H1 adduct formation. Reaction conditions were as described under "Materials and Methods" except that the concentrations of [Ade-\textsuperscript{14}C]ADP-ribose or \textsuperscript{[14]C}ribose were varied and the treatment with NaBH\textsubscript{4} was omitted. After incubation, 20% Cl\textsubscript{3}COOH-insoluble material was collected on a Millipore filter and the radioactivity was determined.

FIG. 2. Time course of nonenzymatic formation of ADP-ribose·H1 or ribose·H1 adduct. Conditions of incubation were as described in Fig. 1 except that either ADP-\textsuperscript{[14]C}ribose, [Ade-\textsuperscript{14}C]ADP-ribose, or \textsuperscript{[14]C}ribose was used as indicated and the treatment with NaBH\textsubscript{4} was omitted.

Schiff base formation, the treatment with NaBH\textsubscript{4} resulted in stabilization of the linkage at alkaline pH values, and the treatment with snake venom phosphodiesterase released AMP from the bound ADP-ribose but no ribose 5-phosphate. Furthermore, when the ADP-ribose·H1 adduct reduced by NaBH\textsubscript{4} was digested with proteinase K (1:20 (w/w) to protein; 37°C, 4 h) and analyzed by paper electrophoresis, no free ADP-ribose was recovered, further supporting the view that the linkage between ADP-ribose and H1 was of a covalent nature. The ADP-ribose adducts used in the following studies were all treated with NaBH\textsubscript{4} and extensively dialyzed in order to remove unreacted ADP-ribose.

Activation of Poly(ADP-ribose) Synthesis by ADP-ribose·Histone Adduct—As previously described (21, 22), poly(ADP-ribose) synthetase purified from rat liver nuclei absolutely required DNA and, in addition, histone for full activity. The extent of activation by histone varied with the preparation of enzyme, the kind of histone as well as DNA, the ratio of histone to DNA, and other conditions, such as salt concentrations and temperature. Under all conditions tested, an ADP-ribose·H1 adduct was more stimulatory than unmodified H1. The time course (Fig. 3) showed that the adduct was approximately 4 times as potent as unmodified H1 at any time points up to 3 h. Furthermore, as shown in Fig. 4, an ADP-ribose·H1 adduct was more stimulatory than unmodified H1 at any given concentrations as well as at respective optima, in both 10- and 60-min incubations, indicating that the difference was not due to a shift of concentration optimum of histone. Free ADP-ribose showed no stimulatory effect on poly(ADP-ribose) synthetase in the absence and presence of H1.

The activation of poly(ADP-ribose) synthetase was relatively specific for histones even in the ADP-ribose adduct form; an ADP-ribose adduct of albumin gave little, if any, effect on the synthetase (Table I). A preliminary survey has shown that the capacity to bind to DNA is essential for a
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The standard reaction mixture modified to contain 20 nmol of [Aden.4C]NAD, 0.4 mg of poly(ADP-ribose) synthetase, 14 μg of DNA, and either no protein or 10 μg of histone H1, bovine serum albumin, or their ADP-ribose adducts as specified was incubated for 10 or 60 min at 37°C. Values represent the means of duplicate experiments.

| System | 10 min | 60 min |
|--------|--------|--------|
| DNA    | 0.21   | 0.46   |
| DNA + H1 | 0.89  | 1.27   |
| DNA + ADP-ribose-H1 | 1.12 | 1.75 |
| DNA + albumin | 0.26 | 0.52 |
| DNA + ADP-ribose-H1 + albumin | 0.24 | 0.52 |

4.6 mol of ADP-ribose/mol of H1.
8.7 mol of ADP-ribose/mol of albumin.

protein to stimulate the DNA-activated synthetase.4

The stimulation by ADP-ribose·histone adducts varied with the content of ADP-ribose. In the case of H1 adducts, the adduct having about 2 ADP-ribose residues/mol of protein was most stimulatory.

These observations, together with our previous finding that histones did not accept ADP-ribose in the purified system (22, 27), suggest that an ADP-ribose·histone adduct may play two separate roles in poly(ADP-ribose)lation, one as a general activator as does unmodified histone and the other as a specific activator depending on its ADP-ribose residue.

ADP-ribose·Histone Adduct as ADP-ribose Acceptor—Evidence for the latter activator function of ADP-ribose·histone adducts ascribable to accepting ADP-ribose was obtained by analyses of the reaction products with the polypeptide treatment and polyacrylamide gel electrophoresis. As shown in Fig. 5 (A and B), pronase, a broad spectrum protease, solubilized essentially no poly(ADP-ribose)ated material synthesized in the absence of exogenous protein or in the presence of unmodified histone H1, the result confirming our previous view that purified poly(ADP-ribose) synthetase did not ADP-riboseylate any of histones but modified an endogenous acceptor depending on its ADP-ribose residue (22). The latter acceptor is known to be very resistant to proteases (27). In contrast, the same protease solubilized a majority of the ADP-ribose incorporated in the presence of ADP-ribose·H1 adduct (Fig. 5C). Although there was a difference in the average chain length among these products (about 17, 10, and 4 ADP-ribose units for the products of A, B, and C, respectively), the difference in protease susceptibility did not appear due to the difference in polymer size, since a polymer having about 10 ADP-ribose units is only partly acid-insoluble.5 It seemed, therefore, that the different susceptibilities reflected the different chemical nature of acceptor molecules and that the acceptor in the reaction with an ADP-ribose·histone adduct would be the adduct itself.

This notion was substantiated by a combination of electrophoretic and proteolytic analyses. While the products synthesized with no added protein or with unmodified H1 remained at the top of the gel apart from histone bands (Fig. 6, A and B), a majority of ADP-ribose incorporated in the presence of ADP-ribose·H1 adduct migrated close to the protein bands (Fig. 6C). Our ADP-ribose·histone H1 adduct preparation contained an H1 dimer (as judged by its molecular weight in the SDS gel); this dimer also accompanied a small amount of incorporated ADP-ribose. A minor portion of ADP-ribose remaining at the top (Fig. 6C) was indicative of poly(ADP-ribose)lation occurring also on an endogenous acceptor as in two other cases. This mobility of the endogenous acceptor (Rf ≈ 0) was different from the value obtained earlier (Rf ≈ 0.58) (22). The difference appeared to be due to the different solubility of the poly(ADP-ribose)lated endogenous acceptor in these two experiments since the average chain length of the products differed markedly (>10 (see below) versus ≤3.7 ADP-ribose units (22)), and the acceptor material with longer polymers has been shown to be less soluble.6

Treatment of all these products with proteinase K, a non-specific protease (37), further disclosed the difference; the treatment converted to smaller products little, if any, of the material synthesized with no addition (Fig. 6D) or with unmodified H1 (Fig. 6E), but totally converted the material which had been synthesized with ADP-ribose·H1 adduct and migrated close to histone bands (Fig. 6F). Since the average chain lengths of the products of A, B, and C, determined in a parallel experiment, were 17.7, 10.5, and 3.9, respectively, and free oligomers and polymers (up to about 15 ADP-ribose units) migrated faster than the marker dye under the present conditions, it did not appear plausible that small peptides accompanying poly(ADP-ribose) remained at the top, but rather that the products in the former cases (A/D and R/F) were resistant, whereas the product in the latter (C/F) was sensitive to the protease. This conclusion was compatible with

4 M. Kawaichi, unpublished results.
5 K. Ueda, unpublished results.
6 H. Okayama and M. Kawaichi, unpublished results.
7 H. Okayama and C. M. Edson, unpublished results.

![Fig. 5. Time course of pronase digestion of the material poly(ADP-ribose)lated under various conditions. Poly(ADP-ribose) products were prepared in the standard mixture (0.6 ml) modified to contain 30 μmol of Tris-Cl, [Aden.4C]NAD (1.8×102 cpn/mol), poly(ADP-ribose) synthetase (2.7 μg of protein), DNA (30 μg), and either none (A), histone H1 (30 μg) (B), or ADP-ribose·H1 adduct (30 μg; 4.6 mol of ADP-ribose/mol of H1) (C). After incubation for 20 min at 0°C, the mixture was made pH 7.0 by addition of 0.11 ml of 0.1 N HCl and heated for 1 min in a boiling water bath. The mixture was divided into 55-ul portions, and to each portion was added either pronase (0.5 μg) or water. Digestion was carried out at 37°C for specified time lengths and terminated by addition of 20% CLCCOOH. Acid-insoluble radioactivity was determined using a Millipore filter.](http://www.jbc.org/Downloadedfrom)
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Fig. 6. SDS-polyacrylamide gel electrophoresis of poly(ADP-ribose)-H1 adduct treated or untreated with proteinase K. Poly(ADP-ribose)-H1 adducts were prepared by incubating (20 min, 0°C) the standard mixture, doubled in scale and modified to contain 20 μmol of Tris-Cl, [Ade-14C]NAD (5.0 × 10⁶ cpm/nmol), 1.8 μg of poly(ADP-ribose) synthetase, 28 μg of DNA, and none (A, D) or 20 μg of histone H1 (B, E) or ADP-ribose-H1 adduct (4.6 mol of ADP-ribose/mol of H1) (C, F). To all mixtures was added 0.1 ml of 2 M ammonium acetate (pH 5.8), and, to the Mixtures A and D, 20 μg of histone H1. Proteinase K treatment (D, E, F) was performed as described under "Materials and Methods"; controls (A, B, C) were with water. Insets show the Coomassie blue staining of the gels. Dotted areas around the position of bromphenol blue (BPB) in Panels D, E, and F indicate the faint bands of digested peptides, which disappeared during the destaining process.

The view that the synthetase-associated acceptor and ADP-ribose-H1 adduct were, respectively, the main acceptors in the absence and presence of the adduct. The ADP-ribose remaining at the top in Fig. 6F was indicative of partial poly(ADP-ribosylation) of an endogenous acceptor, as supposed above.

Enzymatic poly(ADP-ribosylation) of ADP-ribose-histone adducts was also implied by a change in the electrophoretic mobility after the synthetase reaction (Fig. 7). The adduct preparation used in this experiment gave one major and several minor bands, all accompanied by ADP-ribose. When this preparation was incubated with purified poly(ADP-ribose) synthetase and [3H]NAD followed by extraction with 0.25 N HCl, a slight shift of the 14C peak to a slowly migrating range or a shoulder of 14C coincident with the peak of 3H emerged, suggesting that a part (approximately 20%) of ADP-ribose-H1 adducts was poly(ADP-ribosylated) and changed the mobility.

In order to determine the size of polymers synthesized on the adducts, the reaction products were extracted with 0.25 N HCl and purified by electrophoresis. Two different conditions of poly(ADP-ribosylation), 0°C for 30 min (Fig. 8B) or 37°C for 30 min (Fig. 8C), were examined. The products synthesized with unmodified H1 were similarly treated for reference (Fig. 8A). Recovery of acid-insoluble ADP-ribose into the HCl extract was quantitative for both products with ADP-ribose-H1 adducts, but only one-third for the products made with unmodified H1. The latter products migrated at a distance from the main band of histone (Fig. 8A) and hardly contributed to the radioactivity around histone. The products

Fig. 7. SDS-polyacrylamide gel electrophoresis of [14C]ADP-ribose-H1 adduct incubated or not incubated with poly(ADP-ribose) synthetase. Chemically prepared [Ade-14C]ADP-ribose-H1 adduct (30 μg of protein; 0.29 mol of ADP-ribose/mol of H1, 26,000 cpm/nmol of ADP-ribose) was incubated for 1 h at 37°C in the standard mixture, triple-scaled and modified to contain 60 nmol of [Ade-3H]NAD (14,000 cpm/nmol), poly(ADP-ribose) synthetase (0.56 μg of protein), and 30 μg of DNA. After the incubation, the mixture was subjected to electrophoresis as described under "Materials and Methods" (bottom), as was unincubated ADP-ribose-H1 adduct (top). Insets show the staining profiles of the gels. BPB, bromphenol blue.

Fig. 8. SDS-polyacrylamide gel electrophoresis of histone H1 and ADP-ribose-H1 adduct incubated with poly(ADP-ribose) synthetase. The standard mixture, 10-fold scaled-up, containing 200 nmol of [Ade-14C]NAD (9,500 cpm/nmol for A and C, or 47,500 cpm/nmol for B), the synthetase (2.7 μg of protein), 100 μg of DNA, and 100 μg of histone H1 (A) or ADP-ribose-H1 adduct (2.6 mol of ADP-ribose/mol of H1) (B and C), was incubated for 30 min at 37°C (A and C) or 0°C (B). The mixture was then extracted with 0.25 N HCl, and the extract was subjected to electrophoresis as described under "Materials and Methods." The recovery of acid-insoluble ADP-ribose was 32% in A and 100% in B and C. Insets illustrate the staining of the gels. Arrows indicate the gel sections used for analyses of average chain lengths (see text). BPB, bromphenol blue.
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synthesized with the adducts at 0°C migrated close to the histone bands (Fig. 8B), whereas those of the 37°C incubation retarded and were distributed in several peaks (Fig. 8C). Analyses of average chain lengths of the peak fractions (indicated by arrows) revealed that the products synthesized at 37°C was 10.7 ADP-ribosyl units long and that of 0°C incubation was 2.5 units long. These results indicated that not only oligomers but also polymers were synthesized on ADP-ribose-histone adducts and that the larger the polymer was, the more slowly the modified adduct moved.

All above results led to the conclusion that an ADP-ribose-histone adduct, but not unmodified histone, served as an acceptor for enzymatic poly(ADP-ribosyl)ation. This conclusion, however, did not necessarily mean that poly(ADP-ribosyl)ation took place at preattached ADP-ribose.

Elongation from ADP-ribose Attached to Histone by Terminal Addition Mechanism—In order to investigate whether the ADP-ribose residue bound to histone is the very site of enzymatic modification (that is, elongation), we examined the fate of radioactivity of [Ade-14C]ADP-ribose-histone adduct in the poly(ADP-ribose) synthetase reaction. The principle of the experiment is illustrated in Fig. 9. If the ADP-ribose transfer from NAD by poly(ADP-ribose) synthetase does not occur to chemically bound [14C]ADP-ribose ("not elongated"), the radioactivity is expected to be recovered in AMP upon digestion of the reaction products with snake venom phosphodiesterase. On the other hand, if the enzymatic ADP-ribosylation with unlabeled NAD takes place on the prebound [14C]ADP-ribose ("elongated"), radioactive isoADP-ribose will be produced by the phosphodiesterase digestion; hence the formation of [14C]isoADP-ribose provides an estimate of elongation from prebound ADP-ribose.

The experimental results (Fig. 10) indicated the latter idea to be the case. Among the phosphodiesterase digests of [14C]ADP-ribose-H1 adduct incubated with poly(ADP-ribose) synthetase and NAD, 6.1% of the radioactivity eluting from a Dowex 1 column was recovered in the fractions of isoADP-ribose preceding the marker ADP-ribose. From the adduct not incubated, no radioactivity was recovered in the corresponding fractions. Other labeled products were AMP and adenosine; the latter was probably produced from AMP by phosphatases contaminating the phosphodiesterase preparation used (30). Radioactive isoADP-ribose was identified by paper chromatography in two solvent systems (Fig. 11, A and C) and further by conversion to ribosyladenosine by treatment with alkaline phosphatase (Fig. 11, B and D). These results provided unequivocal evidence for the elongation of the ADP-ribose residue preattached to histone.

Above analyses also indicated that chain elongation proceeded by a terminal addition mechanism, i.e. the ADP-ribose units being transferred to free, adenosine termini. Whether...
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This was the only mechanism for chain elongation or other mechanisms, such as basal addition (an insertion of ADP-ribose between histone and bound ADP-ribose) or a transfer of prebound ADP-ribose to other sites, might also work, was examined by similar analyses using the ADP-ribose-H1 adduct labeled in the opposite position, ribose (Fig. 12). Among the phosphodiesterase digests of incubated as well as not incubated ADP-[3H]ribose-H1 adduct, neither radioactive isoADP-ribose nor ribose 5-phosphate (which should elute out at about 1.5 n HCOOH) was recovered. These results excluded the possibility of alternative mechanisms mentioned above and strongly supported the terminal addition mechanism.

Quantitative estimation of chain elongation from adducts was performed by incubating [α-32P]ADP-ribose-H1 adduct with [Adε-3H]NAD (Table II). Elongation and phosphodiesterase digestion yielded double-labeled isoADP-ribose (Figs. 13 and 14). Approximately 7.4% of original 32P was recovered in isoADP-ribose, and the average chain length of 3H-labeled portion was calculated to be 4.0. From these values, about one-half of new ADP-ribose chains was estimated to have origin on the adducts.

Per cent elongation from adducts, estimated by the produc-

![Diagram](http://www.jbc.org/)

**Table II**

| Step | [32P]-labeled portion | [3H] labeled portion |
|------|-----------------------|--------------------|
| I. Enzymatic poly(ADP-ribose)lation | 60.2 nmol<sup>a</sup> | 34.2 nmol |
| ADP-ribose-H1 adducts incubated | | |
| ADP ribose incorporated from [3H]NAD | | |
| II. Phosphodiesterase digestion | 7.4% | |
| Yield of [32P]isoADP-ribose (= per cent elongation) | 4.5 nmol<sup>b</sup> (A) | 8.6 nmol<sup>b</sup> (B) |
| Amount of elongated chains | 4.0 | |
| Average chain length of synthesized poly(ADP-ribose) | | |
| Total amount of poly(ADP-ribose) | | |
| Per cent utilization of pre-bound ADP-ribose for poly-(ADP-ribose) synthesis (A/B) | 52% | |

<sup>a</sup> As ADP-ribose.

<sup>b</sup> As polymer (not as ADP-ribose).
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Taking advantage of the specificity of isoADP-ribose production from polymerized ADP-ribose, the present study showed that poly(ADP-ribose) synthetase purified from rat liver transfers ADP-ribose from NAD to the ADP-ribose residue of a chemically prepared ADP-ribose-histone adduct and elongates it into a polymer. The purified enzyme is not able to utilize histone as an acceptor but modifies exclusively an endogenous acceptor (22, 27). Our analysis on the latter acceptor using poly(ADP-ribose) glycohydrolase and snake venom phosphodiesterase has revealed that it has ADP-ribose or alike structure at the ADP-riboseylation site (39).

Recently, Yoshihara et al. reported that poly(ADP-ribose) synthetase purified from calf thymus was also incapable of modifying histones (24). In this case, however, ADP-ribose appeared to be transferred on to the enzyme itself. Whether this means only a very tight association of an endogenous acceptor similar to ours with the enzyme protein or represents a reaction intermediate of poly(ADP-ribose) synthetase is not clear at present.

These results taken together suggest that there may be two distinct enzymes working for poly(ADP-ribose)ylation in rat liver, one for a transfer of ADP-ribose to a protein acceptor (chain initiation) and the other for subsequent transfers of ADP-ribose to the protein-bound ADP-ribose (chain elongation), and probably, that the former enzyme is lost during the purification of the latter synthetase. However, other possibilities such as a loss of factor(s) or structure or conditions necessary for chain initiation also remain to be tested.

Although nonenzymatically prepared ADP-ribose-histone adducts are elongated by poly(ADP-ribose) synthetase, the possibility that nuclear NAD glycohydrolases (32, 40, 41) participate in chain initiation by affording free ADP-ribose appears improbable since unphysiologically high concentrations of ADP-ribose (Fig. 1) and a long incubation at alkaline pH values (Fig. 2) are necessary for such adduct formation. A preliminary comparison of the effects of NaB3H4 treatment on alkali stabilities of ADP-riboseyl histone bonds formed enzymatically and nonenzymatically have supported this view; no stabilization is effected by the treatment of enzymatic products in contrast to complete one with nonenzymatic products.

The enzymatically formed ADP-riboseyl histone linkage has been supposed to be an ester bond between the terminal ribose and a glutamic acid residue (2, 42, 43). This idea, however, has not been fully substantiated yet; other possibilities such as ADP-ribosylation at serine-phosphate (6) or a mediation of the linkage by an unknown small molecule (44) have also been suggested. Structural analyses of ADP-riboseyl histone fragments (42, 43, 45) will help elucidation of the chain initiation mechanism.

Another aspect examined in this study was the mode of ADP-ribose polymerization. By employing variously labeled ADP-ribose adducts and NAD, it was shown that the terminal ribose portion of ADP-ribose moiety of NAD is linked to free, adenosine terminus of the adduct. As judged by the identity of degradation products (Figs. 13 and 14), the first and subsequent stages of chain elongation appear to proceed by the same mechanism. This mechanism, terminal addition, is similar to primer-oriented DNA synthesis but dissimilar to tRNA-mediated polypeptide synthesis or coenzyme A-linked fatty acid synthesis. The same mechanism has also been proposed by Yoshihara et al. on the basis of preliminary evidence with an enzyme-bound early product (24).

These observations appear to indicate that partially degraded poly(ADP-ribose) may be re-elongated in vitro and, most probably, such an event occurs very frequently (46). For a polymer to be re-elongated it will be necessary that the polymer remains bound to an acceptor at one end and preserves the adenosine terminus at the other. Poly(ADP-ribose) glycohydrolase, the enzyme responsible for in vitro degradation of the polymer (47, 48), meets these requirements. A preliminary experiment in our laboratory has suggested that mono- as well as oligo(ADP-ribosyl) histones (H1 and H2B) that are synthesized in rat liver nuclei are elongated by poly(ADP-ribose) synthetase as are the nonenzymatic adducts described in this paper (45).

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