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Poly(ADP-ribosylation) *in Vitro*

REACTION PARAMETERS AND ENZYME MECHANISM*

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The reaction catalyzed by poly(ADP-ribose) synthetase from bovine thymus was investigated using the purified enzyme, calf thymus DNA, and histone H1. The rate and extent of poly(ADP-ribosylation), as well as the spectrum of acceptor proteins, changed as reaction conditions were varied. The ionic composition of the buffer greatly influenced the poly(ADP-ribose) synthetase reaction. Auto-poly(ADP-ribosylation) of the enzyme could be varied over 30-fold, from 13 residues per enzyme molecule at low ionic strength to over 400 at high ionic strength in the presence of Mg^{2+}. When histone H1 was present, both the enzyme and H1 were acceptors for poly(ADP-ribose). At low ionic strength, increasing H1 concentration strongly stimulated the poly(ADP-ribosylation) of both enzyme and histone. Under all conditions, H1 molecules not bound to DNA were poly(ADP-ribosylated) in preference to DNA-bound H1.

The results indicate that when the enzyme binds to the activating DNA, poly(ADP-ribosylation) of both enzyme and free histone H1 (if present) takes place. With increasing auto-poly(ADP-ribosylation), there is increasing repulsion between the negatively charged poly(ADP-ribose) covalently linked to the enzyme and the activating DNA. As the enzyme accumulates a progressively higher net negative charge, a "repulsion point" is reached so that activation by the negatively charged DNA no longer occurs. This repulsion point depends on the amount of shielding between the poly(ADP-ribose) and the DNA, and determines the extent of poly(ADP-ribosylation) carried out by each enzyme molecule. Thus, the extent of the reaction varies depending on the ionic strength of the medium, and the presence or absence of divalent cations and histones. The concept of a repulsion point allows a simple rationale for the complex changes in the poly(ADP-ribosylation) reaction as the reaction parameters are altered.

The enzyme poly(ADP-ribose) synthetase, found ubiquitously in the nuclei of eukaryotic cells, polymerizes ADP-ribose residues using NAD as a substrate (1, 2). These polymeric ADP-ribose units are covalently attached either to the enzyme itself (3-5) or to histones (6) and other nuclear proteins (7). Recently, it has been shown that when histones H1 and H2B serve as acceptors, poly(ADP-ribose) is linked to a glutamic acid residue on the protein (8, 9). The biological role of the enzyme has not been firmly established. However, compelling evidence has been presented that inhibition of the enzyme can cause a lesion in DNA repair (10). *In vitro* (11-13) and in permeabilized cells (14-16), the enzyme requires damaged DNA as an activator in the poly(ADP-ribosylation) reaction. On this basis, a role for poly(ADP-ribosylation) in DNA repair events has been proposed. There is no shortage of other postulated biological roles for poly(ADP-ribosylation), although at the present time the evidence for involvement in such processes as replication, transcription, and differentiation is far from definitive (1, 2).

The enzyme has been purified and studied in several laboratories (17-21). Considerable variation in the poly(ADP-ribosylation) reaction has been observed. Thus, it has been reported that in the absence of magnesium, histones are the major poly(ADP-ribosylated) protein (22). On the other hand, a recent report suggests that enzyme auto-poly(ADP-ribosylation) is the major reaction in isolated rat liver nuclei (5). In addition, the amount of poly(ADP-ribosylation) that occurs varies considerably. In this report, the purified enzyme has been studied under simple defined conditions in an effort to understand the enzyme mechanism. From these studies a mechanistic picture of the poly(ADP-ribosylation) reaction has been developed.

EXPERIMENTAL PROCEDURES

Materials

DNA-agarose was made by the method of Schaller *et al.* (23) and the preparation contained 1.6 mg of single-stranded DNA/ml of gel. Agarose (ME) was manufactured by the Marine Colloids Division of FMC Corp. Heifer thymuses, obtained from a local slaughter house, were frozen on dry ice immediately after the animals were killed. Other chemicals were obtained as follows: calf thymus DNA (D-1501) and salmon protamine sulfate (P-4020) (Sigma); Sephadex G-150 and G-25 (Pharmacia); hydroxylapatite (Hypatite C) (Clarkson Chemical Co., Williamsport, PA); [^{32}P]NAD, 23 Ci/mmol (New England Nuclear); [^{14}C]NAD, 265 mCi/mmol (Amersham); special enzyme grade ammonium sulfate (Schwarz/Mann); calf thymus histone H1 (24) was the gift of Professor LeRoy Kuehl, University of Utah. Histone H1, with *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (1 acyl group per H1) and iodinated by the lactoperoxidase method (25) was the generous gift of Ms. Lily Wu and Dr. L. Kuehl, University of Utah.

Purification of Poly(ADP-ribose) Synthetase

All procedures were performed at 0-4 °C. Buffer A (21) contained 50 mM Tris-Cl, pH 8.0, 0.2 M NaCl, 10% glycerol, and 10 mM β-mercaptoethanol. Buffer B contained, in addition, 5 mM dithiothreitol. The purification procedure is a modification of the procedures of Ito *et al.* (21) and Yoshihara *et al.* (20). Individual frozen thymuses were minced with a meat grinder and homogenized in 5 volumes of extraction buffer, as described by Ito *et al.* (21). The homogenates were centrifuged for 10 min at 12,000 × *g*. Only those supernatants that had specific activities greater than 0.5 unit/mg when assayed the next day were pooled. Thymus extracts from certain animals had very

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TABLE I
Summary of the purification procedure for poly(ADP-ribose) synthetase

Fraction	Total protein	Total units	Specific activity
	g	*nmol/min*	*units/mg*
Crude extract	27.1	30,000	1.1
Ammonium sulfate	12.5	21,800	1.7
Sephadex G-25	9.2	27,370	3.0
DNA agarose	0.10	9,660	96.6
Hydroxylapatite	0.025	5,382	215
Sephadex G-150	0.0075	3,637	485
Sephadex G-150[a]	0.0028	1,950	696

[a] Best fraction.

unstable poly(ADP-ribose) synthetase activity due, presumably, to proteolytic degradation. If such extracts were pooled with stable extracts, the whole pool became unstable. The enzyme preparation described here was made from eight thymuses (2.2 kg wet weight). To the supernatant fraction, 440 ml of 3.75% protamine sulfate was added dropwise with stirring (21). After 15 min, the suspension was centrifuged at 12,000 × *g* for 10 min and the resulting supernatant fraction (crude extract) was fractionated with ammonium sulfate, as described by Yoshihara *et al.* (20) except that the pellets were dissolved in buffer A. The 40–80% ammonium sulfate fraction (400 ml) was desalted on a Sephadex G-25 column (6.5 × 55 cm) equilibrated with buffer A. The eluate (1 liter) was applied to a DNA agarose column at 1.4 ml/min (2.5 × 32 cm) containing 320 mg of insolubilized single-stranded DNA and the column was washed with 500 ml of buffer A. A linear gradient formed using 600 ml of buffer A and 600 ml of buffer A containing 2 M NaCl was applied. The enzyme fraction (235 ml) was next chromatographed on hydroxylapatite, as described by Ito *et al.* (21) except that the total volume of the gradient for elution was increased from 120 ml to 400 ml. The enzyme fraction (78 ml) was concentrated by dialysis overnight against buffer B saturated with ammonium sulfate. The resulting suspension was centrifuged at 17,000 × *g* for 15 min, and the pellet dissolved in buffer B (final volume 7 ml). Gel filtration was next carried out on a Sephadex G-150 column (2.7 × 163 cm) equilibrated with buffer B, and the enzyme fraction was again concentrated by dialysis against buffer B saturated with ammonium sulfate. The final preparation, dissolved in buffer B to yield a protein concentration of approximately 10 mg/ml, was stable for up to a year when stored at −70 °C. A summary of the purification procedure is given in Table I. Electrophoresis on SDS[1]-polyacrylamide gels indicated that the G-150 fraction had a single polypeptide component (Fig. 2, 0 min incubation time). The final specific activity is comparable to those reported for larger scale preparations of the homogenous enzyme (17, 20, 21).

Assay for Poly(ADP-ribose) Synthetase

Enzyme Purification—Fractions containing synthetase activity (1–8-μl aliquots) were incubated in a mixture (65 μl) containing 120 mM Tris-Cl, pH 8.0, 90 mM NaCl, 10 mM $MgCl_2$, 0.2 mM NAD, [^{14}C]NAD (178,000 cpm), 1 mM dithiothreitol, 100 μg/ml of DNA, and 100 μg/ml of histone H1. The reaction mixture was incubated for 5 min at 23 °C and quenched by the addition of 2 ml of 10% trichloroacetic acid. The acid-insoluble material was collected on a Whatman GF/C filter and the radioactivity was determined using a toluene-based scintillation mixture. One unit of enzyme activity catalyzed the conversion of 1 nmol of NAD into an acid-insoluble form/min. Specific enzyme activity was expressed as units/mg of protein, as determined by the method of Lowry *et al.* (26).

Enzymological Studies—In order to prevent the adsorption of macromolecules to surfaces, reactions were carried out in polypropylene test tubes coated with silicone (Sigmacote from Sigma). The incubation mixtures, described in the legends to the figures, contained various concentrations of the highly purified poly(ADP-ribose) synthetase preparation (Sephadex G-150 fraction; "best fraction," see Table I), and were incubated at 22 °C. Unless otherwise indicated, all incorporation is expressed as nanomoles/ml.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was done as described by Weber *et al.* (27). Separating gels were either uniform 5% or gradients of 5–15% in polyacrylamide. Stacking gels were 3.3% in polyacrylamide and were identical, in buffer composition, with the separating gel. Poly(ADP-ribose) synthetase reaction mixtures, which are described in the legends to the figures, were mixed with sample preparation buffer (27) to yield a final concentration of 10 mM sodium phosphate, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.1 mg/ml of bromphenol blue. The mixtures were incubated at 23 °C for 20 min, and 50-μl aliquots were applied to the slots in the slab gel. Electrophoresis was carried out at 35 mA for 14 h at room temperature. Gels were stained with Coomassie brilliant blue G-250 or with the silver staining procedure described by Oakley *et al.* (28).

For autoradiographic analysis, the gels were dried under suction at 75 °C for 2 h.

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

RESULTS

Enzyme Auto-poly(ADP-ribosylation) Is Dependent on the

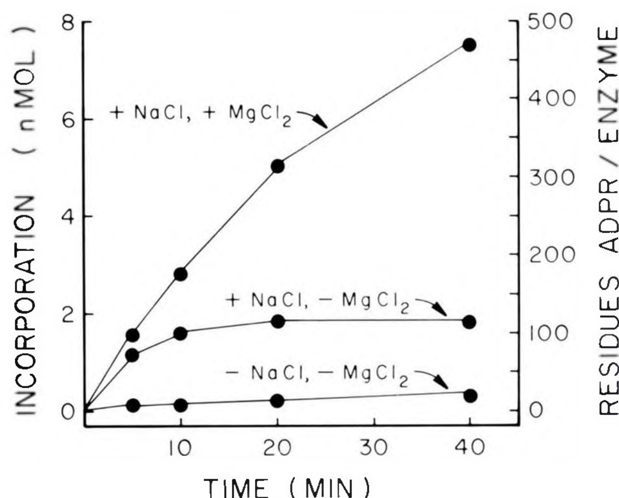

FIG. 1. **Effect of ionic composition on the poly(ADP-ribose) synthetase reaction.** Incubation mixtures (100 μl) contained 2 μg/ml of enzyme, 10 μg/ml of calf thymus DNA, 25 mM Tris-Cl, pH 8.0, 0.2 mM NAD, [^{32}P]NAD (3.5 × 10^6 cpm), 1 mM dithiothreitol, and NaCl (0.15 M) and $MgCl_2$ (10 mM) as indicated. At the times indicated, samples (10 μl) were analyzed for acid-insoluble radioactivity. Incorporation in this and other figures is expressed as nanomoles of ADP-ribose residues incorporated/ml of the reaction mixture.

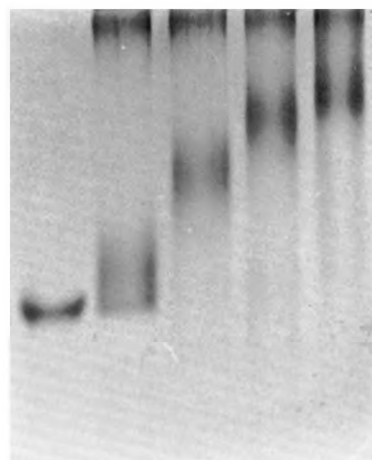

FIG. 2. **Enzyme auto-poly(ADP-ribosylation) as a function of time.** Enzyme (2 μg) was incubated for 0, 0.08, 0.5, 5, or 20 min (*Lanes 1* to *5*, respectively) in 10 mM Tris (pH 8.0), 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 mM [^{32}P]NAD (2.2 × 10^6 cpm), and 4 μg of DNA in a total volume of 0.4 ml after which samples (40 μl) were collected and analyzed by electrophoresis on 5% polyacrylamide gels. Gels were then stained using the silver stain as described under "Experimental Procedures." The silver stain at the top of the gel is due to the DNA and not enzyme. The zero time sample contained the enzyme before the DNA was added.

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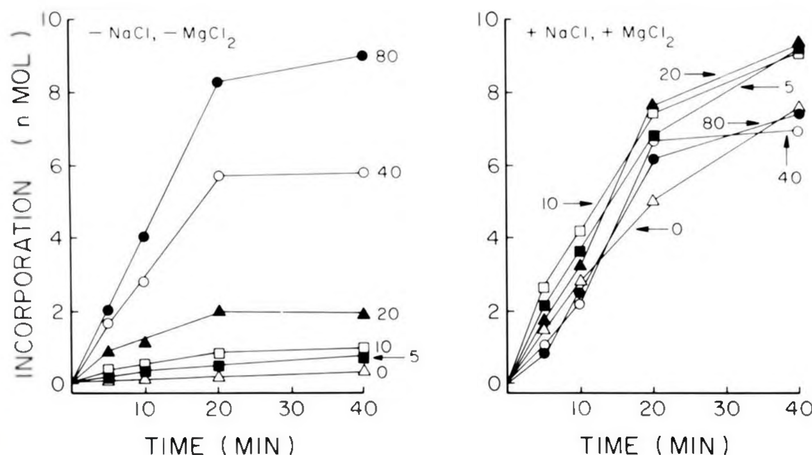

FIG. 3. **Effect of histone H1 on poly(ADP-ribosylation).** *A*, reaction mixtures (100 μl) contained 2 μg/ml of enzyme, 10 μg/ml of DNA, 25 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, 0.2 mM NAD, [^{32}P]NAD (3.6 × 10^6 cpm), various amounts of H1 (as indicated in micrograms of H1/ml of reaction). *B*, reaction mixtures contained, in addition, 10 mM $MgCl_2$, 0.15 M NaCl. At the indicated times, samples (10 μl) were withdrawn and analyzed for acid-insoluble radioactivity.

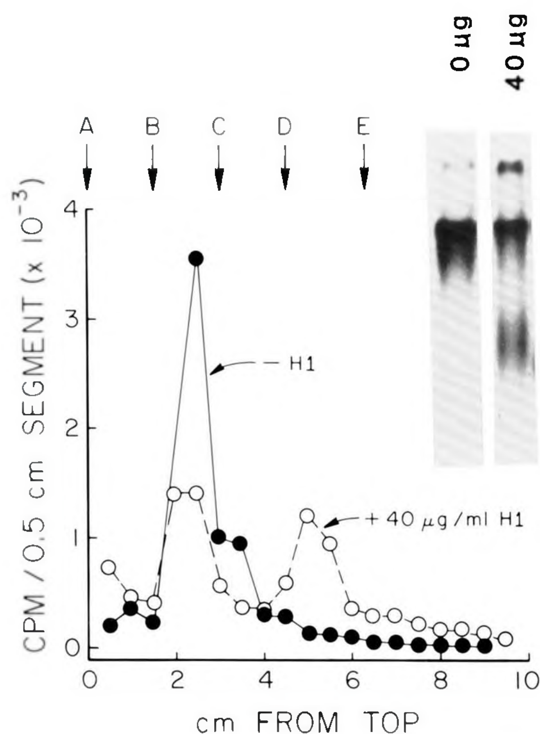

FIG. 4. **Separation of poly(ADP-ribosylated) enzyme and histone H1 using SDS-polyacrylamide gel electrophoresis.** The poly(ADP-ribose) synthetase reaction mixture (100 μl; 20-min incubation) contained 2 μg/ml of enzyme, 25 mM Tris-Cl, pH 8.0, 10 μg/ml of DNA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.15 M NaCl, 0.2 mM NAD, [^{32}P]NAD (3.6 × 10^6 cpm), and 0 or 40 μg/ml of histone H1. Aliquots of the reaction mixture (10 μl) were prepared for electrophoresis, as described under "Experimental Procedures." The dried gels were first analyzed autoradiographically (*inset*) and then cut into 0.5-cm segments and the radioactivity was determined by scintillation counting. *A*, top of stacking gel (cathode); *B*, top of separating gel (5–15% polyacrylamide gradient); *C*, position of unmodified poly(ADP-ribose) synthetase; *D*, position of bovine serum albumin; *E*, position of unmodified H1.

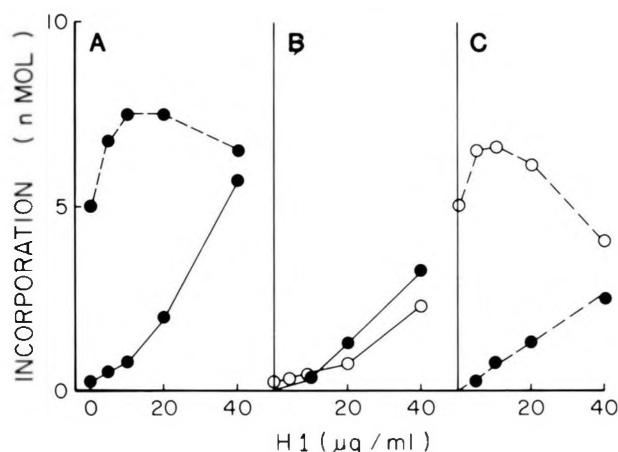

FIG. 5. **Effects of histone H1 on the poly(ADP-ribose) synthetase reaction.** *A*, total incorporation, exactly as described in the legend to Fig. 3 (20-min reaction). *Solid line*, reaction mixture minus $MgCl_2$, NaCl; *dashed line*, reaction mixture containing 10 mM $MgCl_2$, 0.15 M NaCl. *B* and *C*, results obtained when reaction mixtures, described in *A*, were analyzed using the SDS-polyacrylamide gel technique discussed in the legend to Fig. 4. *Open circles*, poly(ADP-ribose) associated with the enzyme fraction; *closed circles*, polymer bound to H1. *B*, reaction mixture minus $MgCl_2$, NaCl; *C*, reaction mixture containing $MgCl_2$, NaCl.

Ionic Composition of the Medium—The extent of auto-poly(ADP-ribosylation) that can be achieved is greatly influenced by the ionic composition of the medium (Fig. 1). Little poly(ADP-ribosylation) is seen at low ionic strength; if magnesium ion is added, a striking increase in poly(ADP-ribosylation) is observed. In 20 min, 13 residues of poly(ADP-ribose) are incorporated per enzyme molecule at low ionic strength in the absence of Mg^{2+}. In the presence of Mg^{2+}, 310 residues per enzyme molecule are incorporated. Under these conditions, all enzyme molecules are poly(ADP-ribosylated). Analysis of poly(ADP-ribosylation) using silver-stained SDS-polyacrylamide gels (28) demonstrates that all the enzyme protein is converted into slower moving species (Fig. 2). Under all reaction conditions, the poly(ADP-ribose) formed appears to be bound to the enzyme, and no free polymer is evident (results not shown).

Effects of Histone H1 on the Poly(ADP-ribosylation) Reaction—At low ionic strength, as the H1 concentration increases, the rate and extent of the reaction is increased (Fig. 3). In the presence of Mg^{2+}, in contrast, a relatively small increase is observed in total poly(ADP-ribosylation) as the H1 concentration increases.

The poly(ADP-ribose) associated with enzyme and with H1 can be distinguished using SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 4). Thus, the effects of H1 on poly(ADP-ribosylation) (Fig. 5*A*) can be factored into two components: changes in the rate and extent of enzyme auto-poly(ADP-ribosylation) and H1 poly(ADP-ribosylation) effects. At low ionic strength, histone stimulates enzyme automodification (Fig. 5*B*). However, little histone modification occurs until the histone concentration is increased above a 1:1 mass stoichiometry with the DNA (10 μg/ml) (Fig. 6*B*). At high ionic strength, both histone modification and enzyme

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automodification occur at all concentrations of histone. As the concentration of histone is increased, the relative rate of enzyme auto-poly(ADP-ribosylation) decreases and the histone H1 poly(ADP-ribosylation) increases (Fig. 5*C*). The mobility of the poly(ADP-ribosylated) histones on the gel increases with the histone concentration finally approaching the mobility of unmodified H1 (Fig. 6*A*). Thus, in a 20-min reaction, each individual histone molecule is less extensively poly(ADP-ribosylated) as the number of H1 molecules is increased, although the total amount of poly(ADP-ribose) in the histone fraction increases. It has been shown previously that poly(ADP-ribosylated) H1 migrates more slowly than unmodified H1 in SDS gels, and that the reduced mobility is a function of the polymer chain length (29).

Free Histone Is Preferentially Poly(ADP-ribosylated)—Even under conditions where poly(ADP-ribosylation) is extensive, a fraction of the histone is not poly(ADP-ribosylated) at all. One obvious way to partition H1 into two classes is between histone H1 bound to DNA and histone H1 not bound to DNA. The results at low ionic strength, where no significant poly(ADP-ribosylation) of histone H1 occurs until H1 is present in stoichiometric excess over the DNA (Fig. 6), are strongly suggestive that the enzyme discriminates against histone H1 that is bound to the DNA.

To test whether free histone is preferentially poly(ADP-ribosylated), order of addition experiments were performed. The basic strategy is to make a 1:1 mass complex of histone H1 to the DNA initially ("bound H1") and then to add excess histone H1 with the enzyme ("free H1"). The extent of labeled H1 being converted to a poly(ADP-ribosylated) form was monitored in parallel experiments where "bound H1" was ^{125}I-labeled, and where "free H1" was labeled. A relatively higher percentage of the labeled H1 was poly(ADP-ribosylated) when added with the enzyme, as opposed to being added to the DNA first (Fig. 7). Thus, there is a preferential conversion of "free H1" to a poly(ADP-ribosylated) form compared to "bound H1."

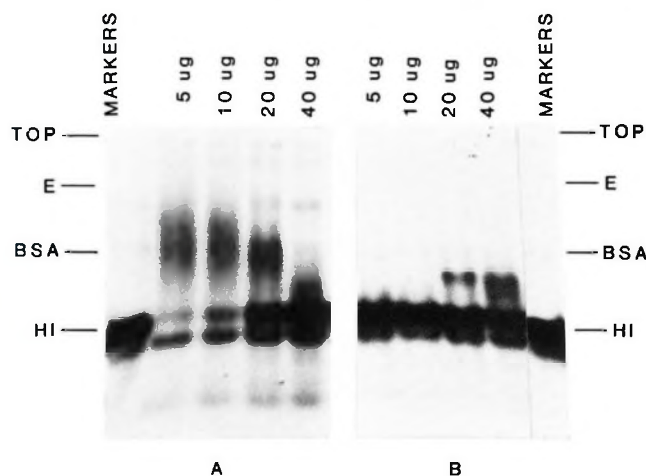

FIG. 6. **Autoradiogram of ^{125}I-labeled H1 after poly(ADP-ribosylation) at high (*A*) and low (*B*) ionic strength.** Reaction mixtures (100 μl) containing 2 μg/ml of enzyme, 25 mM Tris-Cl, pH 8.0, 10 μl/ml of DNA, 1 mM dithiothreitol, 0.2 mM NAD, 5–40 μg/ml of histone H1, ^{125}I-labeled H1 (5 ng, 50,000 cpm), and, for the set of reactions shown in *A*, 10 mM $MgCl_2$ and 0.15 M NaCl, were incubated for 20 min. Samples (11 μl) of each reaction were analyzed by electrophoresis on 5–15% polyacrylamide gels and by autoradiography as described under "Experimental Procedures." Concentrations of H1 (micrograms/ml) in the reaction mixtures are indicated at the top (cathode) of each lane. *Top*, top of separating gel; *E*, unmodified poly(ADP-ribose) synthetase; *BSA*, bovine serum albumin, *H1*, unmodified histone H1. The lanes labeled *markers* show the position of the unmodified ^{125}I-labeled histone H1. The fastest moving ^{125}I-labeled band is contaminating ^{125}I-labeled high mobility group protein in the H1 preparation.

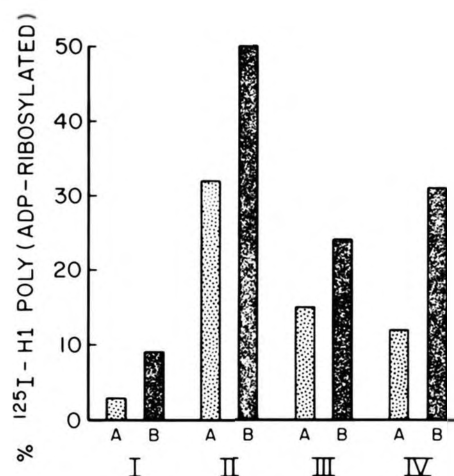

FIG. 7. **Order of addition experiment to determine if free histone is preferentially poly(ADP-ribosylated).** For each reaction, two solutions were separately prepared and then mixed: a stoichiometric *DNA-H1 solution* and an excess *H1-enzyme solution*. In *A*, ^{125}I-labeled H1 (63,000 cpm, 10,000 cpm/ng) was added to the DNA-H1 solution and the H1-enzyme solution was unlabeled. In *B*, ^{125}I-labeled H1 solution was added to the H1-enzyme solution and the DNA-H1 solution was unlabeled. The DNA-H1 solution contained 6.25 mM Tris-Cl, pH 8.0, 1.25 mM dithiothreitol, 0.25 mM NAD, 12.5 μg/ml of H1, and 12.5 μg/ml of DNA, with the following additions: condition I, none; condition II, 0.19 M NaCl; condition III, 12.5 mM $MgCl_2$; condition IV, 12.5 mM $MgCl_2$, 0.19 M NaCl. The mixtures were preincubated at 23 °C for 15 min. The enzyme-H1 solutions (20 μl) contained 10 μg/ml of enzyme and 50 μg/ml of H1. Incubation times were: condition I, 20 min; condition II, 10 min; condition III and IV, 5 min. After incubation, the samples were analyzed by SDS-polyacrylamide gel electrophoresis to separate poly(ADP-ribosylated) H1 from unmodified H1 (see "Experimental Procedures"). The fraction of ^{125}I-labeled H1 poly(ADP-ribosylated) is shown in each case.

However, poly(ADP-ribosylation) of labeled H1 occurs even when it is added to the DNA first. Even at low ionic strength with H1 and DNA present in stoichiometric ratio, if enough enzyme is added, much of the H1 can eventually be poly(ADP-ribosylated). This reaction, however, is extremely slow. This does not necessarily mean that the enzyme can slowly poly(ADP-ribosylate) histone bound to DNA. The enzyme may preferentially (and perhaps exclusively) poly(ADP-ribosylate) those histone H1 molecules not bound to DNA. Since histone H1 rapidly associates and dissociates from the DNA and can exchange between different DNA molecules, every molecule of H1 becomes a potential target for poly(ADP-ribosylation) if enough enzyme is added.

Reactivation of Poly(ADP-ribose) Synthase after Automodification—To compare the enzymatic activity of poly(ADP-ribosylated) *versus* unmodified enzyme, enzyme auto-poly(ADP-ribosylation) was carried out to the point of saturation in the presence of Mg^{2+} (*inset*, Fig. 8). The extensively poly(ADP-ribosylated) enzyme was then dialyzed against one of two buffer solutions: one at pH 6.5 and the other at pH 10. Poly(ADP-ribose) is released from protein by mild alkali (30), probably because poly(ADP-ribose) is generally attached to proteins through an ester-glycosidic bond (8, 9). Thus, upon dialysis at pH 10, we expect the poly(ADP-ribose)-protein linkage to be hydrolyzed. At pH 6.5, such hydrolysis should be negligible. Dialysis of the non-poly(ADP-ribosylated) enzyme was carried out at both pH values as an experimental control. These results are shown in Fig. 8. It is clear that the unmodified enzyme is equally active after dialysis at pH 6.5 and at pH 10. In contrast, inactive auto-

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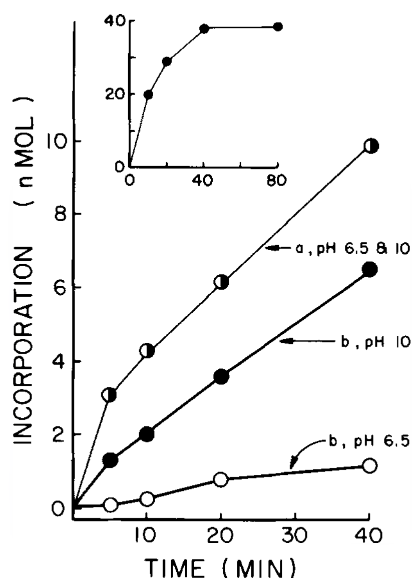

FIG. 8. **Reactivation of poly(ADP-ribose) synthetase after automodification.** Two reaction mixtures (1 ml) containing 10 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 μg/ml of DNA, and 6 μg/ml of enzyme and either no NAD (*a*) or 0.2 mM NAD (*b*) were incubated for 80 min. In addition, an incorporation mixture (0.1 ml) identical with *reaction b* was used to monitor the extent of the reaction and contained [^{14}C]NAD (290,000 cpm) (*inset*: *ordinate* time in minutes; *abscissa*, nanomoles of NAD incorporated per ml of reaction). *Mixtures a* and *b* were each divided into two 0.5-ml aliquots and dialyzed against 0.2 M NaCl, 2.5 mM dithiothreitol, 10% glycerol, and either 25 mM $NaHCO_3$ buffer at pH 6.5 or 25 mM $NaHCO_3$ buffer, pH 10.0, for 3 h at 23 °C. Samples (45 μl) of each of the dialyzed mixtures were then assayed at pH 8.0 in an incubation mixture (100 μl) containing 150 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 10 μg/ml of DNA, 0.2 mM NAD, and [^{14}C]NAD (290,000 cpm). ●, assay of mixtures dialyzed at pH 10.0; ○, assay of mixtures dialyzed at pH 6.5; ◑, indication that the values for the two different samples were not distinguishable within experimental error. The apparent activity per mg of protein in each case was: the starting preparation of enzyme (*inset*), 330 units; *mixture a, pH 10.0*, 160 units; *mixture a, pH 6.5*, 160 units; *mixture b, pH 10.0*, 70 units; *mixture b, pH 6.5*, 10 units. Thus, although non-poly(ADP-ribosylated) enzyme (mixture *a*) showed the same activity when dialyzed at pH 6.5 and pH 10, enzyme which had been extensively poly(ADP-ribosylated) (mixture *b*) showed 7-fold greater activity after dialysis at pH 10 than at pH 6.5.

poly(ADP-ribosylated) enzyme remains largely inactive after dialysis at pH 6.5, but after dialysis against pH 10 buffer, very significant reactivation is observed. These results indicate that removal of poly(ADP-ribose) from the enzyme results in reactivation.

DISCUSSION

We have studied the poly(ADP-ribosylation) reaction catalyzed by the enzyme poly(ADP-ribose) synthetase in reaction mixtures containing only DNA, histone H1, and the enzyme. Even under these carefully defined conditions, the reaction appears on the surface to be very complex. The amount of poly(ADP-ribosylation) of histone and of the enzyme varies depending on the composition of the buffer, the relative proportion of histone to DNA, and the amount of enzyme. Thus, it is not surprising that a unified picture of enzyme activity has not emerged so far in the literature, since in many previous cases, even more complex mixtures (chromatin or whole nuclei) were used.

In the defined system which we have studied, the apparently complex results obtained can be rationalized by a relatively simple mechanistic picture. The enzyme is activated by DNA and can carry out either auto-poly(ADP-ribosylation) or H1 poly(ADP-ribosylation) when activated. However, since the poly(ADP-ribose) that is being covalently attached to the enzyme is making the enzyme progressively more negative, eventually a point will be reached where the poly(ADP-ribosylated) enzyme can no longer be activated by the highly negatively charged DNA. This "repulsion point" would be expected to be a function of the ionic strength. At low ionic strength, a minimal level of auto-poly(ADP-ribosylation) can occur before the repulsion point is reached (under our conditions, ~13 ADP-ribose moieties/enzyme). Increasing the ionic strength with sodium ions causes a greater shielding of charges; in 0.15 M NaCl, a 10-fold higher level of poly(ADP-ribosylation) of the enzyme is possible (see Fig. 1). The addition of magnesium results in an even lower net charge on the DNA (~0.1 per phosphate moiety according to theory) (31). Furthermore, the interaction of Mg^{2+} with the phosphate groups in poly(ADP-ribose) is probably even tighter than with DNA (Mg^{2+} is an excellent precipitating agent of poly(ADP-ribose)) (32). Thus, in the presence of Mg^{2+}, extensive poly(ADP-ribosylation) of the enzyme is possible before the repulsion point is reached. This simple picture accounts for the effect of incubation conditions on poly(ADP-ribosylation) when only the enzyme and DNA are present. Consistent with the above hypothesis, Yoshihara *et al.* (33) recently reported a decreased affinity of poly(ADP-ribose) synthetase for DNA after automodification.

Histone H1 also affects the shielding between the DNA and the poly(ADP-ribose). Especially at low ionic strengths, the addition of histone would be expected to greatly change the repulsion point between the poly(ADP-ribosylated) enzyme and the DNA. The extent of enzyme auto-poly(ADP-ribosylation) under these ionic conditions increases 25-fold if an excess of histone is present (see Fig. 5*B*).

However, histone H1 not only stimulates enzyme auto-poly(ADP-ribosylation) by changing the repulsion point at low ionic strength, but also serves as an acceptor for poly(ADP-ribose). We have established that histone H1 is preferentially poly(ADP-ribosylated) when it is free from the DNA. Yoshihara and co-workers (22) previously presented evidence which was consistent with bound histone not being poly(ADP-ribosylated), although more recent work with DNA-cellulose columns by the same group (33) has led them to modify this conclusion. At high ionic strength, the overall rate of poly(ADP-ribosylation) remains roughly constant as histone H1 levels are increased (Fig. 5*A*), although the fraction of poly(ADP-ribosylation) on histone H1 increases (Fig. 5*C*). This is consistent with the postulate that the enzyme can either poly(ADP-ribosylate) itself or free histone H1 using the same active site. The extent of histone H1 poly(ADP-ribosylation) therefore becomes a function of how many histone H1 molecules are able to enter the active site before the enzyme poly(ADP-ribosylates) itself to the repulsion point. Thus, changing the proportion of enzyme and histone H1 will affect the relative fraction of poly(ADP-ribose) on the enzyme and on histone H1. Thus, the major determinants of the poly(ADP-ribosylation) reaction are: 1) shielding effects of the medium and positively charged proteins, which determine when the repulsion point is reached and, 2) competition between auto-poly(ADP-ribosylation) and ADP-ribosylation of other acceptor proteins (in this study, free histone H1). These considerations allow us to predict how changing any reaction parameters will change the rate and extent of reaction, and the spectrum of acceptors.

Clearly, in chromatin, nuclei, and *in vivo* there are additional factors that need to be considered. The activating structures on DNA that occur *in vivo*, the role of poly(ADP-ribose) breakdown enzymes, and the shielding by other nu-

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clear proteins are all additional factors that have not been considered in the simple mechanistic picture that we have described above. Nevertheless, this scheme simplifies the interpretation of much of the data that have been published in the literature so far, and is a prerequisite for rationalizing the role of the enzyme in more complex systems.

These studies raise one question regarding the role of the enzyme in DNA repair. Presumably when a lesion is present in DNA, a poly(ADP-ribose) synthetase molecule binds at the site and both auto-poly(ADP-ribosylation) as well as poly(ADP-ribosylation) of other protein molecules would continue until the repulsion point is reached, causing dissociation of the enzyme from the DNA. It has been found that agents which inhibit poly(ADP-ribosylation) also inhibit the sealing of DNA strand breaks (10); this observed inhibition may arise for one of two reasons. First, production of poly(ADP-ribose) may be required for a step in the DNA repair process (for example, to unwind chromatin, to inhibit some enzymes, etc.). However, an alternative explanation is that when inhibitors of poly(ADP-ribosylation) are added, the enzyme would be unable to go through its normal binding and dissociation cycle: the failure to auto-poly(ADP-ribosylate) would prevent normal dissociation, since the repulsion point would not be reached, and the enzyme would remain bound to the DNA break. The bound poly(ADP-ribose) synthetase may well inhibit the activity of enzymes normally involved in sealing the DNA. Sealing of DNA breaks may require poly(ADP-ribose) synthetase dissociation. This explanation is tenable since direct evidence that the poly(ADP-ribose) polymer *per se* plays a role in DNA repair has not yet been obtained.

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