Purification and Characterization of the Major Nonhistone Protein Acceptor for Poly(adenosine diphosphate ribose) in HeLa Cell Nuclei†

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ABSTRACT: Previous studies had indicated that the major ADP-ribosylated in vitro acceptor protein in HeLa cell nuclei and in oligonucleosomes was not a histone but rather a specific nonhistone protein which possessed molecular properties similar to those reported for the nuclear enzyme poly(ADP-Rib) polymerase isolated from sources other than HeLa cells. Accordingly, the present study was undertaken to ascertain whether this major acceptor and the HeLa enzyme copurified. Poly(ADP-Rib) polymerase was purified 454-fold from HeLa nuclei and 2200-fold from whole HeLa cells with a 24% recovery of enzymatic activity. The isolated enzyme was found to be a basic 5.2S protein of 112000 daltons. The enzyme possessed an absolute requirement for both DNA and histones for catalytic activity and had an apparent K_m for NAD of 46 μM and a V_{max} of 1470 nmol/(min mg of protein) at pH 8.0 and 25 °C. Though the enzyme was capable of histone ADP-ribosylation in isolated nuclei and polynucleosomes, it failed to ADP-ribosylate histones in a reaction containing exogenous DNA and histones. Data presented in the following paper show that when the purified enzyme is reconstituted with oligonucleosomes stripped of endogenous enzyme, the ability for histone ADP-ribosylation was restored. These data suggest that specific macromolecular interaction between the enzyme and the prospective acceptor protein, i.e., histones, must exist for protein modification. Analysis of the protein-bound product of in vitro poly(ADP-Rib) polymerase activity indicated that the enzyme undergoes automodification to a significant extent. The data supporting this view include the following: (a) the major protein acceptor of ADP-Rib copurified with poly(ADP-Rib) polymerase from nuclei; (b) both the enzyme and acceptor protein had the same molecular characteristics, i.e., 112000 M_r and 5.2 S; (c) protein-bound oligo(ADP-Rib) could be chased from the 112000 M_r protein with [^{3}H]NAD (50 μM). Pulse–chase studies also revealed that poly(ADP-Rib) turns over on the enzyme during catalysis. Product analysis indicated that the enzyme synthesized poly(ADP-Rib) under optimal conditions with an average chain length of 6.1 residues and that the number of chains synthesized per molecule of enzyme increased with time of incubation. Taken together, these data suggest that poly(ADP-Rib) polymerase catalyzes its automodification in vitro during an intermediate step in the synthesis of protein-free poly(ADP-Rib).

Poly(ADP-Rib)[1] polymerase, a tightly bound chromatin enzyme, catalyzes the successive transfer of ADP-Rib units from NAD to nuclear proteins [see reviews by Hayaishi & Ueda (1977), Smulson & Shall (1976), and Hilz & Stone (1976)]. The products of the reaction are chains of oligo- or poly(ADP-Rib) covalently attached to both histones and nonhistone chromosomal proteins (Jump et al., 1979; Giri et al., 1978a; Burzio et al., 1979). We have extensively characterized the internucleosomal location of poly(ADP-Rib) polymerase (Mullins et al., 1977; Giri et al., 1978b) and the association of the enzyme and its acceptor proteins with regions of chromatin undergoing DNA synthesis (Jump et al., 1979). In the course of our studies we reported that the major nonhistone protein acceptor for poly(ADP-Rib) in the HeLa nucleus, accounting for as much as 90% of "activity" measurements in most assays for this enzyme, was an internucleosomal protein (M_r 125000; protein C). This was also found in in vitro assays with octanucleosomes, a form of chromatin shown to possess high specific activity for poly(ADP-Rib) polymerase (Butt et al., 1979). It was therefore of importance to characterize this acceptor and determine its relationship to poly(ADP-Rib) polymerase and the ADP-ribosylation of histones.

Yoshihara et al. (1977) had reported earlier that highly purified calf thymus poly(ADP-Rib) polymerase (130000 M_r) catalyzed its automodification under in vitro conditions. This observation led us to tentatively suggest that protein C represented the automodified or ADP-ribosylated form of the HeLa poly(ADP-Rib) polymerase (Jump et al., 1979; Butt et al., 1979). The present study was therefore undertaken to isolate and characterize the major HeLa acceptor (protein C) and the HeLa poly(ADP-Rib) polymerase. Particular emphasis has been given to establishing whether HeLa poly(ADP-Rib) polymerase catalyzed its automodification in vitro as it appears to do in nuclei.

A second salient issue presented in this and the following report deals with the ADP-ribosylation of histones by different orders of chromatin complexity and by the purified enzyme. Numerous reports have appeared that describe the absence of histone modification by the purified enzyme from rat liver and bovine thymus (Okayama et al., 1977; Yoshihara et al., 1978). Accordingly, we report in the following paper that purified HeLa poly(ADP-Rib) polymerase will covalently modify HeLa nucleosomal histones when the enzyme is reconstituted with oligonucleosomes stripped of the endogenous enzyme. The following report presents preliminary characteristics of oligonucleosomes reconstituted with purified HeLa poly(ADP-Rib) polymerase.

Materials and Methods

Materials. [^{32}P]NAD (256 Ci/mmol) and [*adenine*-2,8-^{3}H]NAD (28 Ci/mmol) were synthesized from [α-^{32}P]ATP (256 Ci/mmol; Amersham) or [*adenine*-2,8-^{3}H] (28 Ci/mmol; Amersham), respectively, and NMN (Sigma Chemical Co.)

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[1] Abbreviations used: ADP-Rib, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NHP, nonhistone protein; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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and purified as previously described (Jump et al., 1979). DNA–agarose was prepared according to the procedure of Schaller et al. (1972). Hydroxylapatite was purchased from Bio-Rad, Inc., and Sephadex G-200 was from Pharmacia, Inc. Protein markers for $NaDodSO_4$–polyacrylamide gel electrophoresis were purchased from Bio-Rad, Inc.: myosin (M_r 200000); β-galactosidase (M_r 116000); phosphorylase *b* (M_r 94000); bovine serum albumin (M_r 68000); ovalbumin (M_r 43000). Protein markers for sedimentation analysis and gel filtration were as follows: IgG (7 S; M_r 156000; Sigma); ovalbumin (3.6 S; M_r 43000; Miles Laboratories); bovine serum albumin (5.1 S; M_r 68000; Miles); catalase (11 S; M_r 270000; Sigma). Calf thymus whole histones (Lot No. 553-8190) were obtained from Sigma. Snake venom phosphodiesterase was purchased from Sigma, and micrococcal nuclease was purchased from Worthington.

Cultivation of HeLa Cells in Suspension. HeLa S_3 cells were maintained at 37 °C in spinner flasks as previously described (Mullins et al., 1977). All cells used in this study were grown asynchronously to a midlog density [(5–8) × 10^5 cells per mL of culture fluid].

Preparation of HeLa Nuclei. HeLa nuclei were prepared from (2–3) × 10^9 cells by resuspending isolated cell pellets in buffer A [50 mM Tris, pH 7.5; 3 mM $MgCl_2$; 0.2 mM $CaCl_2$; 1 mM EDTA; 50 mM $NaHSO_4$ (neutralized with NaOH); 1 mM DTT; 2 mM β-mercaptoethanol; 1 mM NaN_3]. Cells were homogenized in a glass dounce homogenizer (20 strokes) and sedimented at 1500*g* for 10 min. Nuclei were resuspended in buffer A plus 0.25% Triton X-100, dounced, and sedimented as before. Nuclei were then washed again in buffer A. At this stage, nuclei were free of cytoplasmic contaminants as judged by light microscopy. Nuclei prepared in this manner were used immediately for preparation of enzyme. All steps in enzyme purification were carried out at 4 °C unless otherwise stated.

Enzyme Assay. Poly(ADP-Rib) polymerase activity was measured in a reaction mixture that contained 25 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 12.5 μg/mL calf thymus whole histones, 12.5 μg/mL calf thymus DNA, 20 μM [^{3}H]NAD (10 Ci/mol), and enzyme in a total volume of 0.2 mL. The reaction was carried out for 10 min at 25 °C and terminated by the addition of 2 mL of 20% trichloroacetic acid–5 mM pyrophosphate. Radioactivity incorporated as insoluble material was collected on glass fiber filters (Whatman), solubilized in Protosol (0.5 mL; NEN, Inc.), and quantitated by liquid scintillation counting. All steps of the enzyme purification were assayed in this manner. One unit of enzyme activity was defined as being equivalent to 1 nmol of ADP-Rib incorporated into acid-insoluble material per min under the described conditions.

Protein Assays. All protein assays were carried out using the Bio-Rad protein assay reagent and lysozyme as the standard.

Acceptor Assay. Acceptors for poly(ADP-Rib) were determined at various stages of the enzyme purification by incubating an enzymatically active sample (1–20 μg of protein) in the reaction mixture described above with the exception that [^{32}P]NAD (0.5 μCi/mL, 1 Ci/mmol) replaced [^{3}H]NAD as the substrate. Reaction temperatures, times, and volumes were as described above. The reaction, in this case, was terminated with the addition of 4 volumes of absolute ethanol (−20 °C). Radiolabeled proteins and unlabeled proteins (10–200 μg of protein) from corresponding samples which were precipitated with ethanol for 5–12 h (−20 °C) were collected by centrifugation: SW 50.1; 20000*g*; 20 min; 4 °C. Protein pellets were resuspended in 10 mM $NaPO_4$, pH 6.8, 1% $NaDodSO_4$, and 1% β-mercaptoethanol and boiled for 1 min; solubilized protein samples were then separated by a modification of the procedure described by Weber & Osborn (1969) in 7.5% acrylamide gels (acrylamide–*N*,*N*′-methylbis(acrylamide), 100:1) with 50 mM $NaPO_4$, pH 6.8, and 0.1% $NaDodSO_4$ as the buffer. Electrophoresis was for 4–5 h (20 °C) at 60 mA/slab gel (0.15 × 12 × 15 cm). Polyacrylamide gels were stained in 0.2% Coomassie blue, 7% acetic acid, and 50% methanol for 6 h (25 °C) and destained in 20% methanol–7% acetic acid. Destained gels were photographed, dried, and exposed to Kodak SB-5 X-ray film as previously described (Jump et al., 1979).

Product Analysis. Synthesis of [^{32}P]poly(ADP-Rib) was carried out in the enzyme reaction mixture detailed above with the exception that [^{32}P]NAD (80 Ci/mol) replaced [^{3}H]NAD as the substrate. The reaction was performed in 1.0 mL at 25 °C with 342 ng of purified enzyme (3.04 pmol). At the times indicated, aliquots (0.2 mL) were removed and the reaction was terminated with the addition of absolute ethanol (as described above). Samples recovered by centrifugation were digested with snake venom phosphodiesterase (250 μg/mL) as previously described (Mullins et al., 1977). Reaction products were separated on PEI TLC in 1 M acetic acid–0.3 M $LiCl_2$. Regions corresponding to 5′-AMP and phosphoribosyl-AMP were cut from the TLC, plated, and quantitated by liquid scintillation counting.

Buffers for Enzyme Extraction. Buffer A contained 50 mM Tris, pH 7.5, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM EDTA, 50 mM $NaHSO_3$ (neutralized with NaOH), 1 mM DTT, 2.0 mM β-mercaptoethanol, and 1 mM NaN_3. Buffer B contained 10 mM Tris, pH 7.5, 2.0 mM EDTA, 50 mM $NaHSO_3$ (neutralized with NaOH), 1 mM DTT, 2 mM β-mercaptoethanol, and 1 mM NaN_3. Buffer C was equivalent to buffer B with the following changes: 2 M KCl and no EDTA.

Results

(A) Purification of Poly(ADP-Rib) Polymerase. A typical purification was achieved on nuclei isolated from HeLa cells (787 mg of protein) according to the following method. The results are summarized in Table I. All steps in the purification were performed at 0–4 °C unless otherwise stated. All buffers used were adjusted to the specific pH at 25 °C with HCl or NaOH.

Preparation of the 0.3 M KCl Extract. Nuclei isolated from logarithmically growing HeLa cells, as described under Material and Methods, were homogenized in buffer A plus 0.3 M KCl by using a dounce homogenizer and then stirred for 60 min. The homogenate was sedimented at 105000*g* for 60 min and enzyme activity was recovered in the supernatant fraction. Note that more units of enzyme are detected in the KCl extract than in intact nuclei (Table I). This is due to the inability of the assay to measure all enzyme activity in nuclei. The routine assay in this study includes both exogenous histones and DNA; however, these components are not required in the usual assay for this enzyme in nuclei. The bulk of the endogenous DNA and histones are removed at this step (Figure 2); hence, the enzyme reaction becomes dependent on the addition of exogenous histones and DNA for activity. It should be noted that even at this early stage in purification, protein C was the major ADP-Rib acceptor in the assay (Figure 2).

DNA–Agarose Chromatography. The nuclear extract containing the 0.3 M KCl soluble nuclear proteins was applied to a DNA–agarose column (12-mL bed volume; 1.5 mg of fixed DNA per mL) which had been equilibrated in buffer

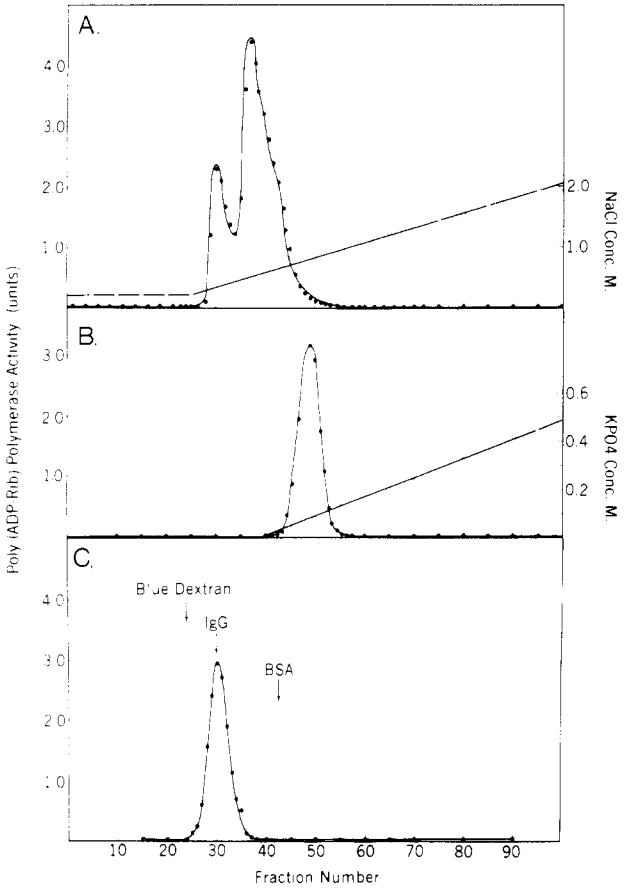

FIGURE 1: Column chromatography profiles of HeLa cell poly(ADP-Rib) polymerase on (A) DNA–agarose, (B) hydroxylapatite, and (C) Sephadex G-200. Enzyme activity was measured by the standard assay (see Materials and Methods) and expressed as enzyme units (●). Molarity of solutions was determined by measuring the conductance in the fractions indicated (○). For details see the text.

B plus 0.2 M KCl. Greater than 95% of the enzyme activity was retained while 90–95% of the protein passed through the column unretained. The column was washed with buffer B plus 0.2 M KCl, and the enzyme was eluted with a linear gradient from 0.2 to 2.0 M KCl in buffer B (Figure 1A). It should be noted that monitoring the column for protein (i.e., A_{280nm}) during the gradient elution was precluded by the low levels of protein in the samples. The enzyme was eluted from the column in two peaks; each was pooled separately and concentrated (Millipore CX ultrafiltration). Nearly 80% of the enzyme activity was recovered in the large peak (fractions 35–45). No further activity could be detected in fractions eluting at higher salt concentrations. Subsequent steps in the enzyme purification were carried out on enzyme recovered from the large peak (fractions 35–45) since this fraction contained fewer contaminating proteins. We have repeatedly observed that enzyme activity could be eluted as two discrete peaks. The major acceptor of ADP-Rib in each case is the same 120 000 M_r NHP, i.e., protein C (see Figure 2). These different affinities for DNA may reflect different populations of enzyme varying in extent of modification.

Hydroxylapatite Column Chromatography. The concentrated enzymatically active preparation from DNA–agarose (1.02 mg) was applied to a hydroxylapatite column (1.5-mL bed volume) equilibrated with buffer C plus 1 mM KPO_4. The column was washed extensively with buffer C plus 1.0 mM KPO_4, and the enzyme was eluted with a linear gradient from 1 to 500 mM KPO_4 in buffer C (Figure 1B). Enzyme activity eluting as a single peak between 45 and 125 mM KPO_4

Table I: Purification of HeLa Cell Poly(ADP-Rib) Polymerase[a]

purifn step	protein[b] (mg)	enzyme act. (units)	sp act. (units/mg of protein)	x-fold purified	% recovered
nuclei	177.00	43.6	0.25	1.0	110
0.3 M KCl extract	88.00	49.5	0.56	2.3	114
DNA–agarose	1.02	34.5	33.82	137.5	79
hydroxylapatite	0.34	19.1	55.85	227.0	44
Sephadex G-200	0.09	10.5	111.70	454.0	24

[a] The enzyme was purified from 2.5×10^9 HeLa cells (787 mg of total protein). [b] Protein content and enzyme activity were assayed as described under Materials and Methods.

(fractions 45–53) was pooled and concentrated as before.

Sephadex G-200 Column Chromatography. The concentrated enzyme sample (2.0 mL; 300 μg of protein) was applied to a Sephadex G-200 column (200-mL bed volume; 2 × 85 cm) previously equilibrated with buffer B plus 2 M NaCl. The column had been precalibrated with dextran blue, IgG, and BSA. The enzyme eluted from the column in buffer B plus 2.0 M NaCl as a single symmetrical peak near the position of IgG (Figure 1C). The enzymatically active fractions (fractions 27–35) were pooled and concentrated as described above. At this stage, the purified enzyme migrated as a single protein band on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2) with a molecular weight of 112 000. When higher amounts of protein (65 μg) were used, a faint band at 70 000 daltons became evident. No further purification was attempted.

Poly(ADP-Rib) polymerase was purified 454-fold from HeLa nuclei with a recovery of 24% (Table I). Others have reported purifications greater than 1000-fold with similar recoveries (Okayama et al., 1977; Yoshihara et al., 1978; Ito et al., 1979) starting with crude extracts from homogenized tissues. In the present work, if the total cell protein is considered the starting material (crude extract), then a purification of 2200-fold was obtained.

(B) Characterization of HeLa Poly(ADP-Rib) Polymerase. Acceptor Analysis. While fingerprinting ADP-Rib acceptors in relation to simple oligonucleosome complexity, a major nonhistone acceptor was noted only in structures larger than dinucleosomes (Giri et al., 1978a,b). This observation stimulated further characterization of this acceptor, and we subsequently reported that the major acceptor for poly(ADP-Rib) in HeLa nuclei, oligonucleosomes, and octanucleosomes was protein C, a 125 000 M_r NHP (Jump et al., 1979; Butt et al., 1979). It was tentatively suggested that protein C represented the ADP-ribosylated form of the polymerase. Yoshihara et al. (1977) first reported that highly purified calf thymus enzyme (130 000 M_r) undergoes automodification. If the initial step in the synthesis of poly(ADP-Rib) requires a covalent attachment of ADP-Rib to protein, in this case poly(ADP-Rib) polymerase, then this activity should be retained during purification. Consequently, at each step in the purification described above, an acceptor assay (see Methods and Materials) was carried out to determine if poly(ADP-Rib) polymerase and [^{32}P]protein C copurified (Figure 2). The data clearly show that one major protein acceptor copurifies with the enzymatically active fractions. The molecular weight of the NHP acceptor and the purified enzyme is identical, i.e., 112 000.

In this report we have revised the molecular weight of the major NHP acceptor from 125 000 (Jump et al., 1979) to 112 000 (Figure 2). The former value was obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis and was based on

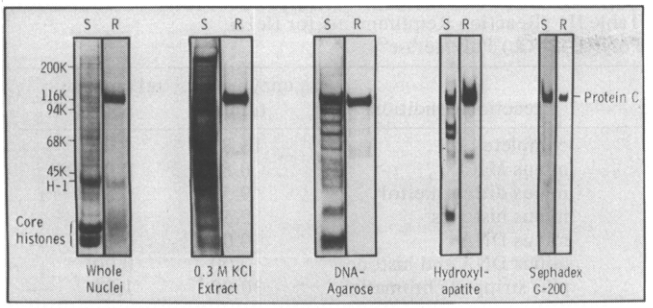

FIGURE 2: Analysis of the protein composition and acceptors for poly(ADP-Rib) at each step of the enzyme purification. Protein samples were prepared and analyzed on $NaDodSO_4$–polyacrylamide gels as described under Materials and Methods. The amount of protein applied to gels was as follows: whole nuclei, 250 μg; 0.3 M KCl extract, 150 μg; DNA–agarose, 100 μg; hydroxylapatite, 69 μg; Sephadex G-200, 20 μg. Each sample was also analyzed for acceptors of poly(ADP-Rib)-utilizing exogenous histones and DNA as described under Materials and Methods. The amount of trichloroacetic acid insoluble radioactivity applied to each gel ranged from 2000 to 10 000 dpm. S = Coomassie blue stain of proteins. R = radioautogram of the corresponding gel. Molecular weight markers: (1) myosin, M_r 200 000; (2) β-galactosidase, M_r 116 000; (3) phosphorylase b, M_r 94 000; (4) bovine serum albumin, M_r 68 000; (5) ovalbumin, M_r 45 000 (6) histone H1; (7) core histones, H3, H2b, H2a, and H4, M_r 11 500–15 000. Direction of electrophoresis is from top to bottom.

the reported molecular weight of β-galactosidase (M_r 130 000). Recently, Fowler & Zabin (1978) determined the amino acid sequence of β-galactosidase and reported the new molecular weight of 116 349. The molecular weight of the purified poly(ADP-Rib) polymerase and NHP acceptor is comparable to that reported for the calf thymus enzyme (M_r 110 000) as determined by the equilibrium sedimentation (Ito et al., 1979).

The modification of the 112 000 M_r protein appears linked to poly(ADP-Rib) polymerase activity. Every preparation so far examined that contains polymerase activity also contains the 112 000 M_r protein as the major acceptor. This includes nuclear proteins in cells made permeable to [^{32}P]NAD, chromatin fragments of varying complexity, HeLa cytoplasm, and purified ribosomes from HeLa cells, in addition to the preparations presented here. Smulson et al. (1975) reported the presence of a cytoplasmic activity in HeLa cells which was elevated during the S phase of the cell cycle. Presumably, this reflects the site of synthesis of the enzyme; both enzyme activity and the 112 000 NHP could be detected by an acceptor assay on partially purified ribosomes (unpublished observations).

The data in Figure 2 show that histones are not acceptors of ADP-Rib once the enzyme has been extracted from the nucleus even though exogenous histones and DNA are required for activity. However, in the following paper (Jump et al., 1980), using reconstitution protocols with nucleosomes, we have begun to characterize those conditions required for histone modification by the purified enzyme. Both the calf thymus enzyme (Yoshihara et al., 1978) and the rat liver enzyme (Okayama et al., 1977) have shown a similar absence of histone modification. Ueda et al. (1979) reported that histones, particularly H1, must first be primed with ADP-Rib before poly(ADP-Rib) can be formed on the histones. These data and the results reported here point to two separate enzymes: one that covalently attaches ADP-Rib to histones and another that forms the polymer, poly(ADP-Rib), though 2″–1′ glycosidic linkages. The enzyme we have isolated appears to mainly carry out the latter function when assayed with DNA and histones in solution.

Other proteins (M_r 50 000–100 000) are seen as acceptors during purification [Figure 2, DNA–agarose and hydroxylapatite fractions, and Figure 2 of Jump et al. (1980)]. These cryptic acceptors represent less than 10% of the total protein-bound [^{32}P]ADP-Rib. It should be noted that the molecular weights of these acceptor proteins are similar to those reported previously (Jump et al., 1979). Nevertheless, these data show that many prospective nonhistone acceptor proteins for ADP-Rib have similar affinities for chromatin and DNA (DNA–agarose) as poly(ADP-Rib) polymerase. This suggests that the enzyme and its acceptors may recognize and bind to similar domains in chromatin. This issue has been addressed in a previous report (Jump et al., 1979). These minor acceptors might also be structurally related to the enzyme per se.

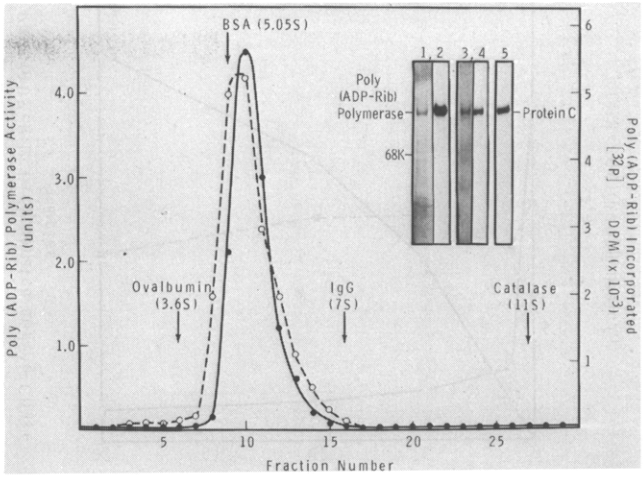

FIGURE 3: Velocity sedimentation of poly(ADP-Rib) polymerase and [^{32}P]protein C. Velocity sedimentation analysis of poly(ADP-Rib) polymerase (i.e., fractions 28–33, Figure 1C) and [^{32}P]protein C was carried out by a modification of the procedure detailed by Martin & Ames (1961). Poly(ADP-Rib) polymerase (34 μg) (●) and protein C (8.6 μg of protein, 2525 dpm/μg) (○) were applied to separate 5–20% sucrose gradients in buffer B plus 1.0 M KCl. Protein markers were applied to separate gradients [bovine serum albumin (M_r 68 000; 5.02 S), ovalbumin (M_r 45 000; 3.6 S), IgG (M_r 156 000; 7.0 S), and catalase (M_r 256 000; 11 S)] in 5–20% sucrose in buffer B plus 0.3 M KCl. Sedimentation was for 24 h in a SW 40 rotor at 38000g, 4 °C. Fractions (0.5 mL) were collected as previously described (Mullins et al., 1977). Position of protein markers was determined by 280 nm of individual fractions. Enzyme activity was analyzed in each fraction as described under Materials and Methods. The position of the ^{32}P-labeled protein was determined by trichloroacetic acid precipitation and liquid scintilation counting. Acceptor analysis was performed on fraction 10 of the gradient containing active enzyme as described earlier. The labeled material recovered from the gradient was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. (Insert) Poly(ADP-Rib) polymerase (5–7 μg of protein) was applied to the sucrose gradient (lane 1) and recovered from the sucrose gradient (lane 3). Corresponding acceptor analysis is shown in lanes 2 and 4, respectively. Radioautogram of the ^{32}P-labeled protein recovered from the sucrose gradients is shown in lane 5.

Velocity Sedimentation Analysis of the Poly(ADP-Rib) Polymerase and [^{32}P]Protein C. Velocity sedimentation of the purified enzyme was carried out to characterize further the relationship between the enzyme and protein C. The data in Figure 3 show that the purified enzyme sediments much slower in sucrose gradients than expected from its molecular weight as obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis. The shape of the enzyme apparently deviates from a globular structure in solution and has an apparent s value of 5.2 S. The calf thymus enzyme has been reported to be 5.8 S with a frictional coefficient of 1.39 (Ito et al., 1979).

A second sample applied to sucrose gradients was previously incubated with [^{32}P]NAD in an acceptor assay (see Materials and Methods). As already noted in Figure 2, the primary labeled product under these conditions is protein C (112 000 M_r). Analysis of the sedimentation profile shows that the

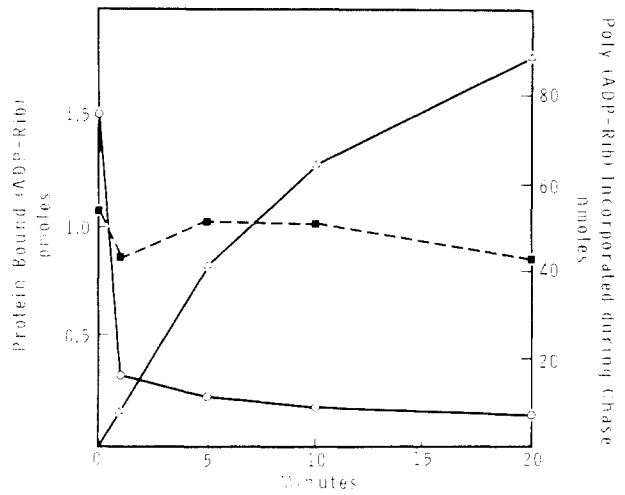

FIGURE 4: Pulse–chase of poly(ADP-Rib) covalently attached to the 112000 M_r protein. Purified poly(ADP-Rib) polymerase (7.5 μg/mL) was incubated with [^{32}P]NAD (0.1 μCi/mL; 256 μCi/mmol) in the standard acceptor assay for 10 s at 25 °C. A 0.2-mL aliquot was removed for ethanol precipitation. (△, ○) The remaining solution was adjusted to 50 μM [^{3}H]NAD (10 Ci/mmol), and 0.2-mL aliquots were removed and precipitated at the times indicated. An aliquot of this sample was trichloroacetic acid precipitated and quantitated by liquid scintillation counting: (△) trichloroacetic acid insoluble [^{3}H]ADP-Rib synthesized during the chase period. Precipitated protein samples were recovered and separated by electrophoresis as described under Materials and Methods. Regions of the gel that correspond to the 112000 protein (visible by Coomassie blue staining) were excised and solubilized in perchloric acid (0.2 mL) and hydrogen peroxide (0.4 mL) overnight in a sealed vial at 60 °C. The samples were cooled, scintillation cocktail was added (Aquasol-2, 10 mL), and radioactivity (as ^{32}P) associated with the 112000-dalton protein during the period of chase with [^{3}H]NAD was quantitated by liquid scintillation counting (○). (■) Enzyme was labeled as described above for 10 s. The enzyme solution was then incubated for various times in the presence of 50 μM thymidine. Radioactivity (as ^{32}P) associated with the 112000-dalton protein during the chase period with thymidine was analyzed as described above (■).

migration of the ^{32}P-labeled protein was coincident with enzyme activity. Thus, protein C and the purified enzyme have essentially the same sedimentations characteristics.

An acceptor assay of the enzyme purified by sedimentation velocity (fraction 20, Figure 3) was carried out as described under Materials and Methods. In addition, the ^{32}P-labeled protein sedimenting coincident with the enzyme activity was analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis and radioautography. Figure 3 (insert) shows the proteins applied to (lane 1) and recovered from (lane 3) the sucrose gradients separated in NaDodSO$_4$–polyacrylamide gels. In addition, acceptor assays were carried out on these two samples and the ^{32}P-labeled products were analyzed (lanes 2 and 4, respectively). The ^{32}P-labeled protein recovered from the sucrose gradient is shown in the radioautogram (lane 5). In each of these cases, the data show that the only labeled product noted was a protein of 112000 M_r. The results reported here, in conjunction with those of Yoshihara et al. (1977), suggest that both the HeLa and calf thymus enzyme similarly undergo automodification. The enzyme product ratio [i.e., radioactivity (picomoles) recovered from the NaDodSO$_4$–polyacrylamide gel electrophoresis divided by the mass of protein (picomoles) applied to the gels] was 0.02, indicating that the automodification is a restricted event. Analysis of the ^{32}P-labeled protein using snake venon phosphodiesterase indicated that chains of 1 to 2 residues of ADP-Rib [oligo(ADP-Rib)] are covalently attached to the protein (unpublished observations).

Pulse–Chase Studies. Pulse–chase studies were carried out to determine if poly(ADP-Rib) turned over on protein C. The enzyme was incubated with [^{32}P]NAD (0.1 μCi/mL, 236 Ci/mmol) for 10 s and then chased with [^{3}H]NAD (50 μM, 10 Ci/mol) for various times. The labeled proteins were prepared and separated by NaDodSO$_4$–polyacrylamide gel electrophoresis. Regions of the gel corresponding to the enzyme (112000 M_r) were excised, hydrolyzed, and radioactivity quantitated (see the legend of Figure 4). The data in Figure 4 show that [^{32}P]ADP-Rib was chased from the protein after addition of [^{3}H]NAD. At the same time, an increase in trichloroacetic acid insoluble [^{3}H]ADP-Rib was observed, indicating the enzymatic synthesis of poly(ADP-Rib). No alteration in the migration of the protein in this gel system was detected during the pulse–chase study.

A control study was carried out to measure poly(ADP-Rib) degrading activity in the enzyme sample (Figure 4). The enzyme protein was labeled as before for a designated time. Thymidine, a potent inhibitor of poly(ADP-Rib) polymerase activity, was added and the incubation was allowed to proceed for an additional 20 min. The results show that no appreciable loss of protein-bound radioactivity occurred during the 20-min chase period. In the absence of new poly(ADP-Rib) synthesis, no evidence of poly(ADP-Rib) degrading activity could be detected in our enzyme sample. These observations, taken together, suggest that oligo(ADP-Rib) continually turns over on the enzyme during poly(ADP-Rib) synthesis. Product analysis studies presented later will reinforce this notion.

Table II: Reaction Requirements for HeLa Poly(ADP-Rib) Polymerase[a]

reaction conditions	enzyme act. (units)	rel enzyme act.[b]
complete	16.82	1.00
minus $MgCl_2$	0.84	0.05
minus dithiothreitol	9.59	0.57
minus histones	2.52	0.15
minus DNA	0.00	0.00
minus DNA and histones	0.00	0.00
plus stripped chromatin[c]	30.90	1.84

[a] The enzyme assay was performed as described under Materials and Methods. [b] The enzyme activity in the standard reaction was set at 1.00 for comparison of enzyme activities in incomplete and substituted reactions. [c] Stripped chromatin (Jump et al., 1980) replaced exogenous histones and DNA in the complete reaction.

Reaction Requirements and Kinetics. The data in Table II show the minimal requirements for the enzyme activity. In our system there was an absolute requirement for DNA and partial requirement for histones. In addition, a divalent cation (Mg^{2+}) and a thiol reagent (DTT) were necessary for complete activity. The pH and temperature optima were determined to be 7.8–8.0 and 22–25 °C, respectively. These observations are typical of those reported by others (Ito et al., 1979; Okayama et al., 1977; Yoshihara et al., 1978) for the purified poly(ADP-Rib) polymerase from various systems.

Though the enzyme has a strict requirement for histones and DNA, these components do not represent the native binding substrates in vivo. Previous studies from our laboratory have shown that the enzyme binds to internucleosomal regions in chromatin (Giri et al., 1978b). We have used chromatin stripped of endogenous enzyme in place of exogenous calf thymus histones and DNA to examine the influence on enzyme activity. The results in Table II show that a twofold increase in enzyme specific activity was seen by using stripped chromatin compared to exogenous DNA and histones. Studies that deal with reconstitution of purified enzyme with stripped chromatin will be addressed in greater detail in the following paper (Jump et al., 1980).

Table III: Product Analysis of Purified Poly(ADP-Rib) Polymerase[a]

min of reaction	product synth[b] (pmol)	products of venom phosphodiesterase digestion[c] 5'-AMP	PR-AMP	chain length, (PR-AMP/5'-AMP) + 1	stoichiometry,[d] chains of poly(ADP-Rib)/mol of enzyme[e]
0.5	110	15.9	94.1	6.9	5.2
2.0	369	45.0	324.0	8.2	14.4
5.0	690	150.0	540.0	4.6	49.3
10.0	1077	211.2	865.8	5.1	69.4
20.0	1449	268.3	1180.7	5.4	88.3

[a] Poly(ADP-Rib) was synthesized in a standard reaction that includes [^{32}P]NAD (20 μM, 80 Ci/mol; see Materials and Methods). Digestion of synthesized products by SV phosphodiesterase was as described previously (Mullins et al., 1977). [b] The amount of product synthesized was determined by measuring the trichloroacetic acid insoluble radioactivity at each time point. [c] Average chain length = 6.1. The average chain length of the product was calculated from the radiolabeled 5'-AMP and phosphoribosyl-AMP recovered from PEI TLC and calculated by the method of Nishizuka et al. (1967). [d] The stoichiometry of product synthesis was calculated from the ratio of picomoles of 5'-AMP to picomoles of enzyme used in the reaction (342 ng, 3.04 pmol) by using 112 000 as the molecular weight of the enzyme. [e] Average turnover number of chains = 7.8/min. Turnover number was calculated by determining the number of chains synthesized per minute per mole of enzyme.

The time course of the enzyme reaction was found to be very rapid and could not be studied during a strictly linear period; therefore, the kinetic parameters obtained reflect only rough estimates. The apparent K_m for NAD was 46 μm and the V_{max} was 1470 nmol/(min mg of protein) by using the standard assay (see Materials and Methods). These values are similar to those reported for rat liver (Okayama et al., 1977) and calf thymus (Yoshihara et al., 1978; Ito et al., 1979). However, it should be noted that these parameters reflect protein C modification, not histone ADP-ribosylation. We favor the view that the latter reaction(s) is the important biological role of the enzyme and that kinetic parameters of this step must be addressed through a precise analysis of histone modification.

Product Analysis. In order to characterize the oligo(ADP-Rib) synthesized by poly(ADP-Rib) polymerase, we incubated the enzyme with [^{32}P]NAD (see Figure 2) and subsequently hydrolyzed the product with snake venom phosphodiesterase and analyzed it as described under Materials and Methods. Nearly 98% of the product synthesized by the enzyme was hydrolyzed to either 5'-AMP or phosphoribosyl-AMP. These two compounds are the characteristic digestion products found in venom phosphodiesterase hydrolysates of poly(ADP-Rib) (Matsubara et al., 1970). The average chain length of 6.1 reported in Table III was calculated by the procedure of Nishizuka et al. (1967). The data in Table III show several points of interest: (a) the product of poly(ADP-Rib) polymerase synthesis was poly(ADP-Rib) with an average chain length of 6.1; (b) the average size of the polymer did not increase with time; (c) the number of chains of polymer synthesized per molecule of enzyme, however, did increase with time. The data suggest that the polymer did not remain bound to the enzyme but became dissociated after the average chain length reached 6 to 7 residues. Therefore, the covalent attachment of ADP-Rib to the enzyme, as revealed in Figure 2 and as described by others (Yoshihara et al., 1977), may represent only a transient event for initiation of chain synthesis.

Discussion

Poly(ADP-Rib) polymerase transfers ADP-Rib from NAD to both histones and nonhistone proteins (Giri et al., 1978a). We favor the view that the former reaction relates more directly to the actual biological function of the enzyme. Regions of histones subject to ADP-ribosylation have recently been studied in detail by Riquelme et al. (1979) and Burzio et al. (1979). In addition, Stone et al. (1978) have made the interesting observation that, in nuclei, poly(ADP-Rib) polymerase catalyzes a reaction whereby two molecules of histone H1 are cross-linked by a single chain of 15 to 16 ADP-Rib units. While histone modification has significant importance, recent data utilizing a variety of experimental approaches (Jump et al., 1979; Butt et al., 1979) indicated to us that the major ADP-Rib acceptor in most assays for this enzyme was a single nonhistone protein ("protein C") rather than histones.

It was of obvious importance for us to characterize this acceptor. We have approached this by exploiting the observation that the molecular weight of purified poly(ADP-Rib) polymerase from a variety of sources closely correlated with the molecular weight of the above-mentioned putative nonhistone acceptor. In addition, the purified enzyme from calf thymus has been reported to undergo automodification (Yoshihara et al., 1977); it was this fact that suggested a possible relationship between extensive protein C ADP-ribosylation and poly(ADP-Rib) polymerase. Accordingly, we have purified poly(ADP-Rib) polymerase from HeLa nuclei 450-fold and 2200-fold from HeLa cells, with a 24% recovery of enzyme activity (Table I). The molecular weight of the enzyme as determined by gel filtration was 150 000 (Figure 1). Electrophoresis in NaDodSO$_4$–polyacrylamide gels indicated that the enzyme was a single polypeptide of 112 000 M_r (Figure 2). The latter value is in good agreement with that recently reported for the calf thymus enzyme, M_r 110 000 (Ito et al., 1979). The protein is soluble in 0.4 N H_2SO_4, indicating that the enzyme is a basic protein similar to the histones (unpublished observation). The sedimentation coefficient of the purified HeLa enzyme, as determined by sedimentation velocity, was 5.2 S (Figure 3). These data, in conjunction with the recent extensive study by Ito et al. (1979), indicate that the HeLa and calf thymus enzymes are basic chromatin proteins (110 000–112 000 M_r) that deviate from a globular structure in solution. Further detailed characterization of the molecular properties of the HeLa enzyme per se is complicated by the expense of obtaining large quantities of HeLa cells in tissue culture for extraction of enzyme.

The catalytic and kinetic characteristics of the HeLa enzyme appear remarkably similar to those reported for rat liver (Okayama et al., 1977), calf thymus (Yoshihara et al., 1978; Mandel et al., 1977; Ito et al., 1979), and Ehrlich ascites cells (Kristensen & Holtlund, 1976). The apparent K_m for NAD and V_{max} were estimated to be 46 μM and 1470 nmol/(min mg of protein), respectively. In addition, the enzyme is also inhibited by thymidine. With the assumption that none of these studies has obtained kinetic properties solely on the transfer of ADP-Rib to one single class of acceptors (histones), it would seem of importance to obtain ultimately such parameters with a purified enzyme in a defined system. An approach to this problem is provided in the following paper (Jump et al., 1980).

The HeLa enzyme has an absolute requirement for DNA (Table II). Others have reported that histones serve only to stimulate enzyme activity twofold over the activity seen in the absence of histones (Okayama et al., 1977). However, the HeLa enzyme has a strict requirement for the addition of exogenous histones. Though the histones are required for full activity of the purified enzyme, they are not acceptors of

ADP-Rib in vitro as they are in vivo (Figure 2). This dicotomy has been recognized by earlier workers (Okayama et al., 1977; Yoshihara et al., 1978, 1977). However, data to be shown subsequently (Jump et al., 1980) indicate that almost 30% of total protein-bound ADP-ribosylation can be demonstrated in vitro on histones when enzymatic activity on polynucleosomes of sufficient size was analyzed.

The data presented here suggest that HeLa poly(ADP-Rib) polymerase, like the calf thymus enzyme (Yoshihara et al., 1977), undergoes automodification under in vitro conditions. The data that support this view include the following: (a) the purified enzyme and the nonhistone acceptor have the same molecular weight, 112 000 (Figure 2) and s value, 5.2 S (Figure 3); (b) oligo(ADP-Rib) appears to turn over on the 112 000 M_r protein; [^{32}P]ADP-Rib can be chased from the protein with no concomitant alteration in electrophoretic mobility of the protein (Figure 4); (c) all preparations thus far examined that possess poly(ADP-Rib) polymerase activity also contain the 112000 NHP acceptor (unpublished results); (d) product analysis indicated that the enzyme synthesized poly(ADP-Rib) with an average chain length of 6.1 residues (Table III). The results in Table III clearly show that the number of chains of polymer increase with time, in excess of the mass of the enzyme protein, indicating that in vitro the purified enzyme functions to synthesize poly(ADP-Rib) with water as the terminal acceptor. In addition, studies (unpublished observation) have shown that oligo(ADP-Rib) covalently bound to the enzyme could not be chased to nucleosomal histones but was found to be liberated from the enzyme as free polymer. Synthesis of free polymer in nuclei has been previously reported (Rickwood et al., 1977). The function of the free polymer in nuclei like the function of ADP-ribosylated histone and nonhistone proteins remains to be determined.

Another interpretation of the results presented here is that an endogenous acceptor protein with essentially the same molecular properties as poly(ADP-Rib) polymerase copurified with the enzyme. Because the extent of automodification is quite limited (product/enzyme ratio = 0.02), this possibility cannot be ruled out. The pulse–chase studies tend to argue against an acceptor protein copurifying with poly(ADP-Rib) polymerase.

Automodification of enzyme protein is not unique to poly(ADP-Rib) polymerase. Rabbit muscle cAMP-dependent protein kinase I has been shown to autophosphorylate the catalytic unit of the protein kinase complex. This phosphorylation does not appear to be an intermediate step in the phosphorylation of other protein substrates, e.g., histones. The function of this automodification event also remains undetermined (Chiu & Tao, 1978).

When experimentally approachable, the reconstitution of components purified from their parent complex has often yielded a better understanding of biological function of these components in vivo. In the case of the purified poly(ADP-Rib) polymerase, an appreciation of the requirements necessary for histone modification seemed desirable. In the following communication (Jump et al., 1980), the purified HeLa enzyme was reconstituted with oligonucleosomes stripped of endogenous enzymatic activity. Experiments will show that when the polymerase is reconstituted with nucleosomes restoration of nucleosomal histone ADP-ribosylation was observed under the appropriate assay conditions.

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