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ARTICLE *in* MOLECULAR AND BIOCHEMICAL PARASITOLOGY · JULY 2004

Impact Factor: 1.79 · DOI: 10.1016/j.molbiopara.2004.02.005 · Source: PubMed

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MOLECULAR & BIOCHEMICAL PARASITOLOGY

Purification and properties of poly(ADP-ribose)polymerase from *Crithidia fasciculata* Automodification and poly(ADP-ribosyl)ation of DNA topoisomerase I

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Received 10 December 2003; received in revised form 24 February 2004; accepted 26 February 2004

Abstract

Poly(ADP-ribose)polymerase has been purified more than 160,000-fold from *Crithidia fasciculata*. This is the first PARP isolated to apparent homogeneity from trypanosomatids. The purified enzyme absolutely required DNA for catalytic activity and histones enhanced it 2.5-fold, when the DNA:histone ratio was 1:1.3. The enzyme required no magnesium or any other metal ion cofactor. The apparent molecular mass of 111 kDa, determined by gel filtration would correspond to a dimer of two identical 55-kDa subunits. Activity was inhibited by nicotinamide, 3-aminobenzamide, theophylline, thymidine, xanthine and hypoxanthine but not by adenosine. The enzyme was localized to the cell nucleus. Our findings suggest that covalent poly(ADP-ribosyl)ation of PARP itself or DNA topoisomerase I resulted in the inhibition of their activities and provide an initial biochemical characterization of this covalent post-translational modification in trypanosomatids.

Keywords: *Crithidia fasciculata*; PARP; Poly(ADP-ribose)polymerase; Trypanosomatids; DNA topoisomerase I regulation by PARP; Automodification

1. Introduction

Poly(ADP-ribosyl)ation is a post-translational modification of glutamate, aspartate and lysine residues of nuclear proteins (acceptor proteins) and represents an immediate eukaryotic cellular response to DNA damage. The most abundant and best characterized enzyme catalyzing this reaction is named PARP-1. A number of acceptor proteins have been identified in intact cells, such as histones and DNA topoisomerases, but the main acceptor protein is PARP itself [1–3]. The physiological function of PARP-1 is still under debate. Poly(ADP-ribosyl)ation has been suggested to regulate gene expression and gene amplification, cellular differentiation, malignant transformation, cellular division, DNA repair, DNA replication, and maintenance of genomic stability, as well as apoptosis [4–12]. The ADP-ribose polymer is formed by sequential attachment of ADP-ribosyl moieties from NAD^+ and can reach a length of up to 200 units with multiple branching points, thus containing negative charges. The addition of an anionic polymer on a DNA-binding protein may prevent any interaction with other anionic molecules such as DNA, with physiological consequences on the functional and physicochemical properties of these proteins [12].

Over the past few years, the existence of several additional PARPs has been identified (PARP-2, PARP-3, vault-PARP and tankyrase) in multicellular organisms. These new members of the "PARP family" seem to account for roughly 25% of total cellular poly(ADP-ribose) production. Whereas some of the new PARPs might function in genome protection, others appear to play diverse roles in the cell, including telomere replication and cellular transport [13–16]. The identification of multiple PARPs with distinct primary structures, subcellular localizations and functions in multicellular organisms, illustrates the potential importance of poly(ADP-ribose) in modulating cellular functions and discloses a novel level of complexity in the regulation of poly(ADP-ribose) metabolism.

Abbreviations: DTT, D,L-dithiothreitol; β-Me, β-mercaptoethanol; NAD^+, β-nicotinamide adenine dinucleotide; PARP, poly(ADP-ribose)polymerase; TCA, trichloroacetic acid; topo, DNA topoisomerase I

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doi:10.1016/j.molbiopara.2004.02.005

PARP has been purified from a variety of sources [17–25], but limited information is available about PARP in trypanosomatids. Recently we demonstrated the presence of PARP in the trypanosomatid *Crithidia fasciculata* and some enzyme properties were recognized [26]. We report here the detailed purification method, characterization and nuclear localization of *C. fasciculata* PARP. Furthermore, we investigated the effect of PARP on endogenous proteins. The results here described suggest that the poly(ADP-ribosyl)ation of *C. fasciculata* DNA topoisomerase I and PARP itself, results in an inhibition of topo unwinding and PARP activities and provide an initial biochemical characterization of this process in trypanosomatids.

2. Materials and methods

2.1. Materials

φX 174 DNA, ethidium bromide, polyvinylidene difluoride (PVDF) membranes, anti-rabbit IgG-FITC conjugate, 5-bromo-4-chloro-3-indolyl phosphate, DNA cellulose, phenylmethylsulfonyl fluoride (PMSF), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), Höechst stain solution, N^{α}-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK), nitrotetrazolium blue chloride (NBT), pepstatine-A, β-Me, DTT, EDTA, saponin, Trizma base, histone II-A, NAD^+, nicotinamide, 3-aminobenzamide, activated DNA, bovine serum albumin (BSA), TCA, xanthine, hypoxanthine and adenosine, were purchased from Sigma Chemical Co. (St. Louis, MO, USA); [adenine-2,8-^{3}H]-NAD^+ was from DuPont New England Nuclear; Affigel Blue and BioSil Sec-250 were from Bio-Rad (Richmond, CA, USA).

2.2. Organisms and growth

C. fasciculata (ATCC 11745) was provided by Dr. S. Hutner from Haskins Laboratory, Pace University, New York, USA. Cells were cultured in a medium containing (g l^{-1}): 5.0 NaCl, 2.0 KCl, 0.6 NaH_2PO_4, 0.2 $MgSO_4$, 2.5 sucrose, 0.2 sodium ascorbate, 0.5 MOPS, 5.0 powdered dried liver, 7.5 pluripeptone, 7.5 brain–heart infusion, 0.02 haemin (dissolved in triethanolamine:H_2O (1:1 v/v)). Cells were cultured at 28 °C for 48 h in a New Brunswick Scientific Gyratory Shaker (Edison, NJ, USA) model G-25 at 100 rpm. Cells were harvested by centrifugation at 750 × *g* and 4 °C.

2.3. PARP purification

All steps were carried out at 4 °C. *C. fasciculata* cells were subjected to three freeze–thawing cycles at −20 °C. Disrupted cells were suspended in solution A (50 mM Tris–HCl, pH 8.0, 50 mM $NaHSO_3$, 1.0 mM EDTA, 10% (v/v) glycerol, 10 mM β-Me) supplemented with 0.5 mM DTT, 0.3 M NaCl and protease inhibitors E-64 (1 μg ml^{-1}), pepstatine A (1 mM), PMSF (1 mM), TLCK (0.1 mM). The suspension was sonicated in a Sonifier Cell Disruptor (Model W185, Heat Systems-Ultrasonic Inc., Plainview, IL, NY, USA) by means of five 1-min treatments at 45–50 W, to total cell disruption checked by microscopy. The cell homogenate was centrifuged at 27,000 × *g* for 30 min, and the supernatant (cell-free extract) fractionated by $(NH_4)_2SO_4$ precipitation. The fraction obtained between 30 and 80% saturation was collected by centrifugation at 27,000 × *g* and resuspended in the least volume of solution A containing 1 mM DTT (solution B). The PARP preparation was dialyzed overnight against solution B and loaded onto a DNA cellulose column (2.2 cm × 7.0 cm), equilibrated with solution B. The column was washed with the same solution containing 0.1 M NaCl, until absorbance at 280 nm was negligible. Active PARP fractions were then eluted with solution B containing 0.35 M NaCl. Resultant fractions were pooled and dialyzed against solution B containing 2.5 mM DTT (solution C) and loaded onto an Affigel Blue column (2.2 cm × 6.0 cm) equilibrated with solution C. The column was stepwise eluted successively with 0.2 and 0.4 M NaCl. Active fractions eluted with the last mentioned NaCl concentration were pooled and dialyzed for 2 h against solution D (10 mM phosphate, pH 7.4, 2.5 mM DTT, 0.1 mM PMSF and 10 mM β-Me). The dialyzed pool was loaded onto a hydroxyapatite column (1.3 cm × 2.2 cm), equilibrated with solution D. The column was washed with 0.1 M phosphate, pH 7.4, 5 mM DTT, 0.1 mM PMSF and 10 mM β-Me, and active PARP fractions were then eluted with 0.2 M phosphate, pH 7.4, 5 mM DTT, 0.1 mM PMSF and 10 mM β-Me, and pooled. The pool containing PARP was four-fold diluted with solution A containing DTT 5 mM (solution E), and loaded onto a DNA-cellulose column (1.2 cm × 0.6 cm) equilibrated with solution E. Active PARP fractions were further eluted with solution E containing 0.5 M NaCl.

2.4. Protein content

Protein concentration was measured by Biuret [27] or Lowry [28] method, except for the preparations obtained after Affigel Blue, hydroxyapatite or DNA-cellulose (II), in which cases the protein content was calculated by amino acid analysis of the samples.

2.5. PARP assay

Activity was measured as TCA-precipitable radioactivity incorporated from ^{3}H-NAD^+ [29]. PARP samples (15 μl) were incubated for 5 min at 30 °C in a standard assay mixture (135 μl) containing 100 mM Tris–HCl, pH 8.0, 10% (v/v) glycerol, 1.5 mM DTT, 70 μg ml^{-1} activated DNA (DNAse digested) and 33 μg ml^{-1} histone II-A. The reaction was started by adding 0.5 mM NAD^+ (^{3}H-NAD^+: 1 μCi ml^{-1}). After incubation, TCA (25%, w/v) was added to the assay system to stop the reaction. BSA 2 mg ml^{-1}

was added and after 20 min standing on ice, samples were filtered on 0.45 μm nitrocellulose filters, previously saturated with 25% (w/v) TCA. Filters were then washed twice with ethanol at 96 °C, dried under vacuum and ^{3}H incorporation into TCA-precipitated protein was measured. PARP activity was expressed as U ml^{-1}. One unit is defined as the nanomoles of ADP-ribose incorporated to proteins, per minute, under standard conditions, taking into account that in our case, 4.8×10^3 dpm were equivalent to 1 nmol of ADP-ribose incorporated.

2.6. DNA topoisomerase I activity assay

C. fasciculata topo was partially purified as follows. Cell-free extract, prepared as described above for PARP purification, was dialyzed overnight against solution B and loaded onto a DNA cellulose column (2.2 cm × 7.0 cm), equilibrated with solution B. The bulk of proteins containing topo activity were eluted with solution B supplemented with 0.2 M NaCl.

DNA topoisomerase I activity was assayed by the unwinding of a supercoiled φX 174 DNA to its relaxed circular form [30]. Aliquots of preparations, containing two enzyme units, were incubated in a reaction mixture (20 μl) containing 50 mM Tris–HCl, pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 8.0, 0.5 mM DTT, 30 μg ml^{-1}, BSA and 0.2 μg of supercoiled φX 174 DNA (SC), during 20 min at 30 °C. The reaction was stopped by adding 3.5 μl of stop buffer (0.4% (w/v) bromophenol blue, 25% (v/v) glycerol, 50 mM EDTA, and 5% (w/v) SDS). The supercoiled and relaxed circular form (OC) topoisomers were separated by electrophoresis in a 1% (w/v) agarose gel in TAE buffer (40 mM Tris–HCl, pH 7.6, 20 mM acetic acid, 1 mM EDTA). Gels were then stained with 0.7 μg ml^{-1} ethidium bromide during 30 min and destained during 30 min with distilled water. DNA bands were visualized by UV light in a transilluminator, quantitated by scanning and analyzed using the Gel-Pro Analyzer 3.1 (Media Cybernetics, Inc., Silver Spring, MD, USA) program.

One unit is defined as the amount of topo required to relax 50% of the supercoiled DNA under standard conditions.

2.7. SDS–PAGE

SDS–PAGE was carried out under reducing conditions in 12.5% polyacrylamide minigels and proteins were stained with Coomassie Brilliant Blue R250 [31].

A sample from DNA cellulose (II) was precipitated by extensive dialysis against saturated $(NH_4)_2SO_4$ solution buffered with 50 mM borate/boric, pH 7.5, containing 0.1 mM EDTA. The preparation was centrifuged at 105,000 × *g* and the pellet resuspended in the least volume of SDS–PAGE sample buffer (80 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue).

2.8. Western blot

Purified PARP samples were subjected to SDS–PAGE (12.5% gel) followed by transfer to PVDF membrane, and incubation with rabbit polyclonal antiserum against human PARP (1/800). Proteins were visualized with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1/1000), followed by incubation with 0.30 mg ml^{-1} nitroblue tetrazolium (NBT) and 0.15 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

2.9. Immunofluorescence

C. fasciculata cells were suspended in phosphate-buffered saline (PBS) (10 mM phosphate, pH 7.6, 150 mM NaCl) (about 1×10^7 cells ml^{-1}). Cells were spread onto a glass slide and fixed for 15 min with 4% (w/v) paraformaldehyde followed by incubation for 15 min with PBS containing 25 mM NH_4Cl. Cells were blocked and permeabilized for 30 min with PBS containing 2% (w/v) BSA, 5% (v/v) goat serum and 0.5% (v/v) saponin. Samples were incubated with (1/500) rabbit polyclonal antiserum against human PARP, washed with PBS and incubated with (1/500) fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) and Höechst. Signal was visualized using an Olympus BX 50 (Olympus America Inc., Melville, NY, USA) fluorescent microscope.

3. Results

3.1. Poly(ADP-ribose)polymerase purification

In a previous paper, we reported the presence of PARP in *C. fasciculata* homogenates and isolated nuclei [26]. In order to improve the enzyme characterization, in the present work we purified it and determined its physicochemical as well as its enzymological properties. Purification was performed as outlined in Section 2. The results of a typical experiment are summarized in Table 1.

To minimize loss of protein activity by oxidation and protease digestion a number of sulfydryl-reducing agents such as DTT and β-Me, and protease inhibitors were used throughout the purification procedure, as described in Section 2. The presence of 0.3 M NaCl, in the cell-free extract preparation, was essential to separate PARP from nuclear DNA.

The DNA cellulose (I) chromatography resulted in a 2.4-fold increase in total activity recovered, suggesting the removal in this step of an enzyme inhibitor present in the original extract. The DNA cellulose (I) flow-through showed inhibitory action on PARP activity [26]. Moreover, heating such fraction for 3 min at 100 °C failed to change its inhibitory property (data not shown).

Affigel Blue chromatography led to a significant increase in PARP specific activity (more than 160,000-fold). How-

Table 1
Purification of *Crithidia fasciculata* PARP

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Cell-free extract	2,613	89	0.034	100	1
30–80% $(NH_4)_2SO_4$ (after dialysis)	1,308	89	0.068	100	2
DNA-cellulose (I) (before dialysis)	120	132	1.10	148	32
DNA-cellulose (I) (after dialysis)	119	218	1.83	245	54
Affigel Blue (before dialysis)	0.02	30	1,364	33	40,118
Affigel Blue (after dialysis)	0.02	114	5,700	128	167,647
Hydroxyapatite	0.01	4	333	5	9,794
DNA-cellulose (II)	0.003	1	332	1	9,772

PARP isolation and purification was performed using roughly 15 g of *C. fasciculata* (wet weight). Experimental conditions and enzyme activity were as described in Section 2.

ever, at this point of purification, the enzyme became very unstable and yield decreased drastically after this step. For that reason, the overall purification achieved was only 9800-fold and final yield 1% (Table 1).

Increasing concentrations of NaCl, KCl and $(NH_4)_2SO_4$ showed inhibitory effect on PARP activity, in which cases 50% inhibition was recorded with 100 mM NaCl or KCl and, 14.5 mM $(NH_4)_2SO_4$. This result is in agreement with the remarkable increase in PARP activity after each dialysis during the purification procedure (Table 1).

Gel filtration of the native enzyme on a BioSil SEC-250 column showed a molecular mass of 111 kDa (Fig. 1), whereas analysis of the purified preparation by SDS–PAGE under dissociating conditions disclosed the presence of a single protein band with an apparent molecular mass of *circa* 55 kDa (Fig. 2A). This result would suggest that the native enzyme is a dimer, formed by two identical monomers of 55 kDa each. When examined by Western blot, the *C. fasciculata* PARP monomers were recognized by a polyclonal antibody against human PARP (Fig. 2B).

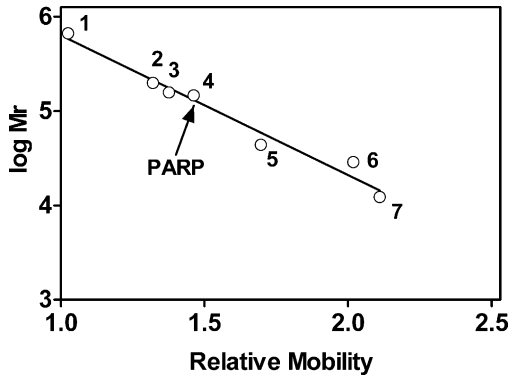

Fig. 1. Determination of molecular mass of *C. fasciculata* PARP. The enzyme (45 U) was applied on a BioSil SEC-250 column equilibrated with 25 mM Tris–HCl, pH 6.8, 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 2.5 mM DTT, 10 mM β-Me, 1 mM PMSF and eluted with the same buffer. Enzyme activity and absorbance at 280 nm of 0.5 ml fractions were determined. Standard proteins were: 1, thyroglobulin (670 kDa); 2, β-amylase (200 kDa); 3, γ-globulins (158 kDa); 4, alcohol dehydrogenase (150 kDa); 5, ovoalbumin (44 kDa); 6, carbonic anhydrase (29 kDa); 7, cytochrome *c* (12.4 kDa).

3.2. Requirements for poly(ADP-ribose)synthesis. Effect of inhibitors

As shown in Fig. 3, PARP activity was absolutely dependent upon the presence of activated DNA, in which case a simple saturation kinetics was obtained. Addition of histone altered this behavior. High concentrations of histone in relation to activated DNA were inhibitory, while concentrations of the same order stimulated the synthesis of poly(ADP-ribose). Maximal activation, up to 2.5-fold, was reached when histone was added in a DNA:histone ratio of 1:1.3 (Fig. 3). When activated DNA was replaced by DNA without nicks, PARP activity decreased up to 50%. In addition to DNA and histone, a sulfydryl reagent was required for full enzyme activity as we reported previously [26].

The requirement for a divalent cation cofactor was further investigated. Neither the presence nor the absence of magnesium, manganese or calcium (1 mM) affected PARP activity. Neither was there any significant effect on enzyme

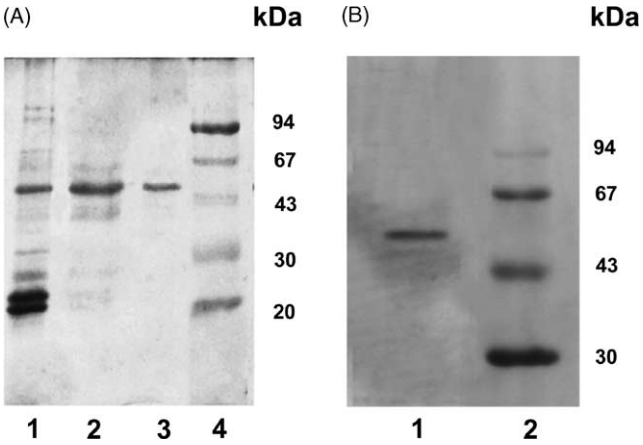

Fig. 2. SDS–PAGE and Western blot of *C. fasciculata* purified PARP. (A) SDS–PAGE (12.5% (w/v) gel) of samples at different purification steps. Active PARP fraction obtained after DNA-cellulose (I) (lane 1), Affigel Blue (lane 2) and DNA-cellulose (II) (lane 3) chromatography. Lane 4, protein markers: phosphorilase b, BSA, ovoalbumin, carbonic anhydrase, soybean trypsin inhbitor, α-lactoalbumin (molecular masses indicated on right side). (B) Western blot of a purified PARP, recognized by a polyclonal antibody against human PARP (lane 1). Lane 2, protein markers, as indicated in (A).

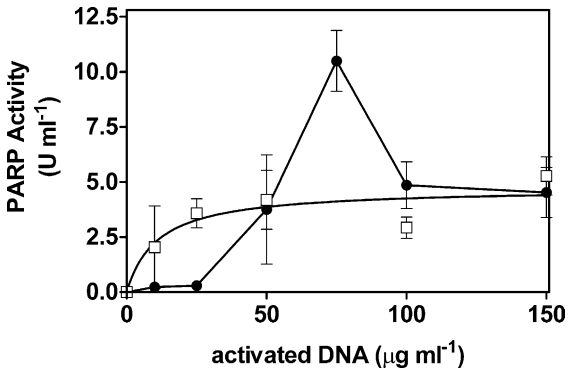

Fig. 3. Effects of DNA and histone on poly(ADP-ribose) synthesis. The reaction was carried out under standard conditions except for the addition of varying amounts of activated DNA in the absence (□) or presence (●) of 100 μg ml^{-1} histone.

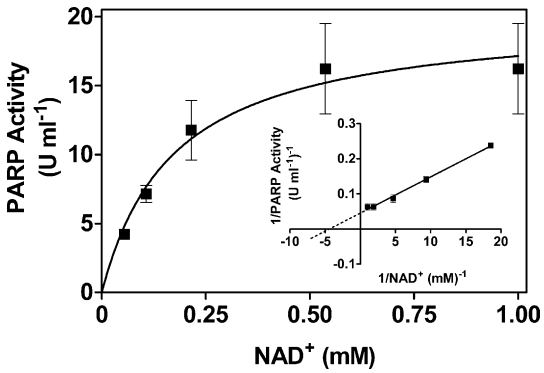

Fig. 4. Michaelis–Menten and Lineweaver–Burk plots of poly (ADP-ribosyl)ation reaction. Purified enzyme preparations were incubated under standard reaction conditions as described in Section 2, except that the concentration of NAD^+ was varied as specified above. Values represent means ± S.D. of at least three independent determinations. *Inset*: Double reciprocal plot of 1/PARP activity (U ml^{-1})$^{-1}$ vs. 1/NAD^+ concentration (mM)$^{-1}$.

activity when the chelating agent EDTA 1 mM was added to the reaction mixture. *C. fasciculata* PARP therefore appears to have no requirement for exogenously added metals. Other metals, such as Zn^{2+} and Cd^{2+} (1 mM), were inhibitory on enzyme activity. The calculated 50% inhibitory concentrations (IC_{50}) were 287 and 220 μM for Zn^{2+} and Cd^{2+}, respectively. Inhibition observed may be due to the ability of these metals to bind to essential sulfydryl groups [23].

In order to characterize *C. fasciculata* enzyme, a series of compounds effective on other well-known PARPs were assayed. Histamine, theophylline, nicotinamide, 3-aminobenzamide (3-AB) and thymidine, were inhibitors on *C. fasciculata* purified enzyme [26]. Purines, such as xanthine and hypoxanthine (10 mM) totally inhibited *C. fasciculata* PARP (100% inhibition), however, adenosine, a purine capable of inhibiting mouse macrophages PARP [32], failed to affect enzyme activity.

3.3. Kinetic constants and optimum pH

The time course of *C. fasciculata* PARP activity was only linear during the first 10 min, then gradually levelled off, and after 20 min there was no further increase in acid-precipitable radioactivity. Poly(ADP-ribosyl)ation reaction followed Michaelis–Menten kinetics with respect to NAD^+, and the K_m value for the substrate estimated from double-reciprocal plot was 226 μM (Fig. 4). In the presence of DNA and histone, optimal pH for PARP activity was 7.5; changes of 1 pH-unit decreased enzyme activity more than 60%.

3.4. Localization of C. fasciculata PARP

To ascertain the intracellular localization of the enzyme, antiserum against human PARP was used in indirect immunoflourescent (IFI) antibody staining as described in Section 2. Differential Höechst staining showed nuclear and kinetoplastic DNA localization, the latter observed as a brighter, more distinct spot than nuclear DNA. When antiserum against PARP was used in IFI, a strong and specific signal which colocalizes with the nuclear region was seen (data not shown). These results agreed with our finding of PARP activity in isolated *C. fasciculata* nuclei reported previously [26].

3.5. Automodification reaction

Regulation of mammalian PARP activity is established through different mechanisms [33–35], the best characterized being down-regulation of enzyme activity through auto-poly(ADP-ribosyl)ation [36,37]. To determine the influence of the automodification process on *C. fasciculata* PARP activity, the enzyme was incubated in the presence of ^{3}H-NAD^+ and activated DNA as described in Section 2. At the indicated times samples were withdrawn, and total ^{3}H-ADP-ribosylated proteins were measured. In parallel, a similar experiment was performed using non-labeled NAD^+. At the same time points samples were withdrawn, ^{3}H-NAD^+ was added, and PARP activity measured. More than 90% of maximal incorporation of ^{3}H-ADP-ribose into TCA insoluble proteins was obtained after 10 min incubation. Longer incubation, up to 120 min, resulted only in a slight increase in TCA precipitable radioactivity, indicating a fast rate of ADP-ribosylation at short times. The extent of protein modification was reflected by loss of enzyme activity, since PARP activity decreased significantly in the same period of time (65% after 10 min incubation) (Fig. 5). As previously reported ADP-ribose polymers can be released from proteins by alkaline treatment [37,38]. We found that roughly 65% of ^{3}H-ADP-ribose-protein linked to *C. fasciculata* PARP was hydrolyzed by alkaline treatment at 0 °C during 3 h. Table 2 shows that hydrolysis against bicarbonate buffer, restored lost PARP activity in the modified enzyme. Nevertheless, it should be highlighted that enzyme activity, both in control and modified enzyme, was partially diminished by alkaline treatment (Table 2).

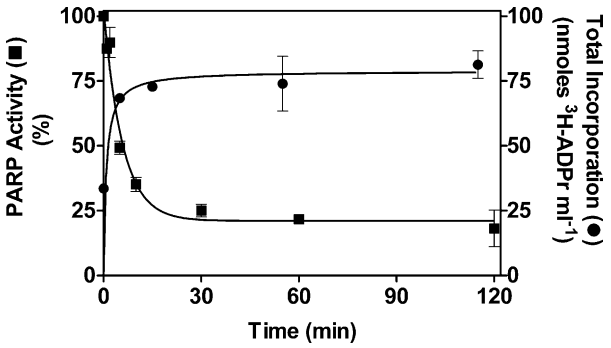

Fig. 5. Time-dependent incorporation of ADP-ribose and PARP activity of auto (ADP-ribosylated) enzyme. PARP activity of auto (ADP-ribosylated) enzyme: reaction mixture containing 100 mM Tris–HCl (pH 8.0), 1.5 mM DTT, 70 μg ml^{-1} activated DNA, 33 μg ml^{-1} histone II-A, 0.5 mM NAD$^+$, and PARP (1.70 U ml^{-1}) was incubated at 30 °C. At the indicated time aliquots were withdrawn, ^{3}H-NAD$^+$ (1 μCi ml^{-1}) added and PARP activity measured as indicated in Section 2. Time dependent incorporation of ADP-ribose: reaction mixture was as described above except that ^{3}H-NAD$^+$ was added at 0 min and total ^{3}H-ADP-ribose incorporated to proteins was measured at each time. Values represent means ± S.D. of at least three independent determinations.

3.6. Inhibition of DNA topoisomerase I activity by poly(ADP-ribosyl)ation

To test the effect of poly(ADP-ribosyl)ation on topo activity, both proteins were incubated in the presence of NAD$^+$ and DNA under the conditions described in Section 2. The incorporation of 23.0 ± 0.74 nmol ml^{-1} min^{-1} ^{3}H-ADP-ribose to TCA insoluble proteins was obtained, which led to nearly 50% topo inhibition, when compared with control value (Fig. 6, lanes 2 and 5).

In order to rule out the possibility that the inhibition observed may be due to poly(ADP-ribose)polymers themselves, produced in the reaction mixture, *C. fasciculata* topo was added to the reaction mixture after the reaction of PARP was stopped by 2.6 mM nicotinamide addition. In this case no inhibition of topo activity was observed (Fig. 6, lanes 2 and 4). DNA topoisomerase I inhibition required incubation conditions where PARP, topo and PARP substrates were present and ADP-ribosylation was allowed (Fig. 6, lanes 2, 3 and 5). The effect of poly(ADP-ribosyl)ation on topo unwinding activity was confirmed by incubation of PARP and topo, in the presence of PARP substrates and the inhibitors, 1 mM 3-AB or 2.6 mM nicotinamide, in which case PARP was 100% inhibited. In such conditions topo inhibition was not observed. Nicotinamide or 3-AB alone failed to affect topo activity (data not shown). When poly(ADP-ribose)polymers were hydrolyzed by dialysis against bicarbonate buffer, topo unwinding activity was totally recovered (Fig. 7, lanes 2–5). DNA topoisomerase I activity was not affected by alkaline treatment as shown in Fig. 7 (lanes 4 and 5).

Table 2
PARP reactivation after hydrolysis of ADP-ribose polymers

Condition	PARP activity (U ml^{-1})	
	Before hydrolysis	After hydrolysis
Control	1.610 ± 0.004	0.920 ± 0.110
Automodified	0.650 ± 0.053	0.970 ± 0.130

Automodified: PARP was preincubated for 10 min under standard conditions except for ^{3}H-NAD$^+$, control: PARP was preincubated for 10 min under standard conditions except for ^{3}H-NAD$^+$ and activated ADN. The reaction was stopped by the addition of 2.6 mM nicotinamide. Control and automodified PARP were subjected to dialysis for 3 h at 0 °C against 25 mM bicarbonate buffer, pH 10, 10% glycerol, 100 mM NaCl, 10 mM β-Me and 1 mM DTT. Aliquots of both samples were measured for PARP activity before and after hydrolysis of ADP-ribose polymers, as described in Section 2. Values represent means ± S.D., $n = 4$.

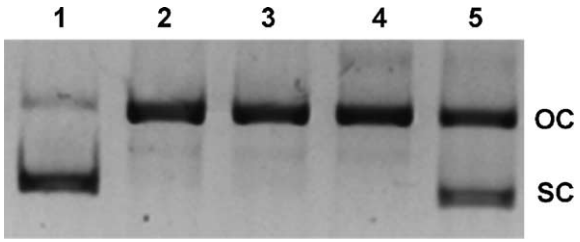

Fig. 6. DNA topoisomerase I inhibition by ADP-ribosylation. Samples containing 100 mM Tris–HCl (pH 8.0), 1.5 mM DTT and PARP substrates (35 μg ml^{-1} activated DNA, 0.5 mM NAD$^+$), were incubated at 30 °C, as follows: (A) topo and PARP, without PARP substrates; (B) PARP, with PARP substrates; (C) topo and PARP, with PARP substrates. After 20 min incubation, an aliquot was quenched by the addition of 2.6 mM nicotinamide, then to complete the system, topo was added to sample (B). Topo activity was assayed in samples as described in Section 2. PARP activity: 23 U ml^{-1}; topo control activity: 2 U. Lane 1: control DNA; lane 2: topo control activity; lane 3: sample (A) topo activity in the presence of PARP; lane 4: sample (B) topo activity in the presence of ADP-ribose polymers; lane 5: sample (C) ADP-ribosylated topo activity.

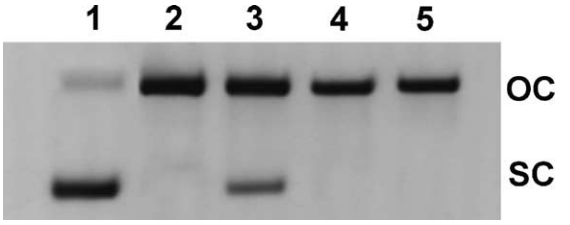

Fig. 7. DNA topoisomerase I reactivation after hydrolysis of ADP-ribose polymers. Incubation mixtures contained topo and PARP in the presence or absence of PARP substrates (activated DNA and NAD$^+$) as indicated in Fig. 6. After 20 min at 30 °C, the reaction was stopped by the addition of 2.6 mM nicotinamide, and the samples dialyzed against bicarbonate buffer for 3 h at 0 °C, as indicated in Table 2. Samples were analyzed for topo activity. Lane 1: control DNA; lane 2: topo control activity; lane 3: ADP-ribosylated topo activity; lane 4: same as lane 2 after alkaline hydrolysis; lane 5: same as lane 3 after alkaline hydrolysis.

4. Discussion

The presence of PARP activity in *C. fasciculata* choanomastigote isolated nuclei as well as some properties of the partially purified enzyme have been already reported

by our laboratory [26]. In this paper we described enzyme purification to homogeneity more than 160,000-fold. The purification was based on the method previously reported [26], which employs DNA-cellulose affinity chromatography instead of a competitive inhibitor affinity column, thus avoiding potential problems associated with inhibitor carryover to the final product [39]. Elution of DNA-cellulose column (I) with 0.1 M NaCl in this step, was essential to eliminate some proteins that otherwise, co-purify with PARP in subsequent purification steps. The high recovery yield of 2.4-fold obtained after this step may be due to the removal of a thermo-resistant inhibitor, whose nature remains to be elucidated. Following the Affigel Blue step, the enzyme proved to be quite unstable and exhibited considerable loss of activity after freezing and thawing. The overall process yield was only 1% (Table 1). The molecular mass of purified enzyme estimated by a gel filtration method was 111 kDa, but the molecular mass determined under dissociating conditions yielded a value of 55 kDa (Figs. 1 and 2), suggesting a dimer structure for *C. fasciculata* PARP. The 55-kDa value, was similar to those obtained for the enzyme from pig thymus [40], the thermophilic archaeon *Sulfolobus sulfactaricus* [18] and other PARP proteins identified in plant cells of *Arabidopsis thaliana* [41] and was significantly lower than the ones obtained for mammalian cells (112–135 kDa) [17,19,23,24] and the nematode *Caenorhabditis elegans* [42]. However, in such cases the enzyme was reported as monomeric. The monomer mass of 55 kDa is still compatible with the presence of a PARP catalytic domain (C-terminal polypeptide), highly conserved during evolution, a DNA binding domain (N-terminal polypeptide), that is involved in the DNA regulation of activity and might include a structural region of automodification as discussed below.

It should be pointed out that we obtained in some preparations an additional protein isolated by gel filtration that exhibited a molecular mass of 37 kDa, still preserving PARP activity (data not shown). Similar results have been described by other groups working with PARPs from *Dictyostelium discoideum* and calf thymus, and ascribed to proteolytic PARP fragments [21,25].

Enzyme activity was entirely dependent on DNA presence. When an appropriate concentration of activated DNA is added, the reaction seems to proceed at an appreciably high rate, even without histones. Histones increase the activity, and the amount of histone to achieve full activation was dependent on the amount of DNA used, proving maximal when the DNA:histone ratio was 1:1.3 w/w (Fig. 3), similar results were obtained for mouse testicle and calf thymus synthetase [17,19]. It has been suggested that besides acting as an acceptor for attachment of ADP-ribose moieties, histones behave as allosteric enzyme activators [17,19, 23,41].

C. fasciculata PARP exhibited no metal ion requirement. Neither magnesium nor manganese nor calcium stimulated enzyme activity and chelating agents failed to inhibit its activity. It has been reported that bovine thymus PARP is magnesium dependent [43]. However, other authors have shown that Mg^{2+} is a positive activity effector [23,24,44]. On the other hand, Zn^{2+} and Cd^{2+} proved to be inhibitors of *C. fasciculata* PARP activity.

We have previously reported that PARP activity, both in *C. fasciculata* isolated nuclei and the partially purified enzyme, was inhibited by a series of compounds effective on other PARPs. Thus, the enzyme was inhibited by nicotinamide, 3-aminobenzamide, theophylline, histamine and histidine [26]. We also tested the effect of some purines on purified PARP, since these metabolites were implicated in down-regulation of PARP activity [32], to find that PARP was sensitive towards xanthine and hypoxanthine but not to adenosine, as reported for mouse macrophages [32].

Our results indicate that *C. fasciculata* PARP has an apparent K_m for NAD^+ of 226 μM (Fig. 4); such micro molar K_m is comparable to those of mammalian (lamb thymus 160 μM) [23] and archeabacteria (*S. sulfactaricus*: 154 μM) [18] and is somewhat higher than the apparent K_m of 50–60 μM published earlier by other investigators for calf and bovine thymus polymerase [19,43], 47 μM for mouse testicle [17] and 20–77 μM for *D. discoideum* [21].

The sub-cellular localization of *C. fasciculata* PARP has been investigated employing indirect immunofluorescence. Although an heterologous antibody was used in this experiment, the nuclear localization observed is in close agreement with our previous finding of PARP activity in isolated *C. fasciculata* nuclei [26].

The results shown (Table 2 and Fig. 5) indicate that when *C. fasciculata* PARP ADP-ribosylation was allowed, enzyme activity decreased, due to automodification. ADP-ribosylating activity was recovered after removing ADP-ribose residues from the protein by alkaline treatment, as shown for other PARPs [37,38]. Such findings support the concept that the automodification domain could also be present in *C. fasciculata* PARP. It has been suggested that the large increase in net negative charge upon poly(ADP-ribosyl)ation eventually causes repulsion between activated DNA and automodified synthetase [12,37,38].

In this work we demonstrated that topo from *C. fasciculata* is a target for poly(ADP-ribosyl)ation, resulting in attachment of poly(ADP-ribose) chains to topo and therefore its inactivation (Figs. 6 and 7). A similar explanation for PARP inactivation by the concept of “repulsion point” seems applicable to topo inhibition by PARP. It has been well established by other authors that in the presence of NAD^+, PARP poly(ADP-ribosyl)ates topo, resulting in enzyme inhibition [45,46]. In contrast, activation of topo by a direct binding to PARP-1, in the absence of NAD^+, has been described for topo from calf thymus [47,48]. However, we have not been able to demonstrate such direct interaction in *C. fasciculata* enzyme.

The modulation of topo activity by poly(ADP-ribosyl)ation provides one possible biological function of this synthetase in trypanosomatids.

To sum, *C. fasciculata* PARP exhibits properties characterizing other representatives of the PARP group, although some major differences are reported here.

Acknowledgements

We are grateful to Drs. Maria del Pilar Molina Portela and Gustavo Daniel Capelluto for their collaboration in the early stage of this work. This work was supported by grants from the University of Buenos Aires and CONICET. M.A. Veron, M.C. Lincon and S. del Valle lent able technical assistance. D. Podestá and M.I. García-Herreros are Research Fellows of the University of Buenos Aires (UBA). S. Fernández Villamil and J. Cannata are Career Investigators of CONICET, Argentine. Dr. Andrés Stoppani passed away on March 18, 2003.

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