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Characterization of poly(ADP-ribose)polymerase from *Crithidia fasciculata*: enzyme inhibition by β-lapachone

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Abstract

Crithidia fasciculata poly(ADP-ribose)polymerase (PARP) has been isolated and partially purified. This is the first PARP isolated from trypanosomatids; it requires DNA and histone for activity, using NAD^+ as substrate. Thiol compounds specially dithiothreitol essentially contributed to PARP stability during purification and to PARP activity during assays. Nicotinamide, 3-aminobenzamide, theophylline, histamine, histidine, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, *p*-chloromercuriphenylsulfonic acid and *o*-iodosobenzoate inhibited PARP, thus confirming enzyme identity. PARP was also inhibited by the $Fe(II)/H_2O_2$ Fenton system. β-Lapachone inhibited PARP, apparently by direct interaction with the enzyme. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Crithidia fasciculata*; Poly(ADP-ribose)polymerase; Trypanosomatids; β-Lapachone; Oxidative damage

1. Introduction

Poly(ADP-ribose)polymerase (PARP) is a nuclear enzyme which catalyzes transfer of ADP-ribose moieties from nicotinamide adenine dinucleotide (NAD^+) to itself and to several other nuclear proteins including histones, p53 and topoisomerases I and II [1]. PARP is activated upon binding to DNA strand breaks thus in response to DNA damaging agents. Moreover, PARP interacts directly with the polymerase α-primase complex and binds to a protein of the base excision repair pathway, which in turn interacts with DNA ligase II and DNA polymerase β. Therefore, PARP may act as a DNA nick sensor recruiting proteins of the base excision repair complex to the site of DNA damage. In that manner, PARP favors access of this complex to DNA, by decondensing chromosomes via ADP-ribosylation of histones [2–7].

Limited information is available about PARP in trypanosomatids. Indirect evidence, suggest its presence in *Trypanosoma cruzi* [8,9], but the enzyme has not yet been isolated and studied. In the present study, we isolated and partially purified PARP from the trypanosomatid *Crithidia fasciculata*. Moreover, we investigated (a) enzyme response to typical PARP inhibitors; (b) PARP activation by thiol compounds (DL-dithiothreitol (DTT) or L-Cys); (c) PARP inhibition by oxygen radicals generating systems; and (d) PARP inhibition by the lipophilic *o*-naphthoquinone β-lapachone. This last effect seemed worthy of special consideration since it induces PARP cleavage in cancer cells [10–14] and PARP inhibition on CHO cells [15]. In addition, β-lapachone has other important biological activities including inhibition of trypanosomatids growth [16–21], inhibition of DNA, RNA and protein synthesis, production of DNA strand breaks [22], inhi-

Abbreviations: BSA, bovine serum albumin; CG 8-935, 3,4-dihydro-2-methyl-2-ethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; DMSO, dimethyl sulfoxide; DTT, DL-dithiothreitol; α-lapachone, 3,4-dihydro-2,2-dimethyl-2*H*-naphtho[2,3*b*]pyran-5,10-dione; β-lapachone, 3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2*b*]pyran-5,6-dione; β-Me, β-mercaptoethanol; NAC, *N*-acetyl-L-cysteine; NEM, *N*-ethylmaleimide; NP-40, (Igepal CA-630) non-ionic detergent; PCMB, *p*-chloromercuribenzoic acid; PCMPS, *p*-chloromercuriphenylsulfonic acid; ROS, reactive oxygen species (superoxide anion radical, hydroxyl radical, hydrogen peroxide); SOD, superoxide dismutase; TCA, trichloroacetic acid.

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bition of tumor cell growth [16,17,23–29], induction of chromosomal alterations [30] and topoisomerases inhibition [31–33].

2. Materials and methods

2.1. Organism and growth

C. fasciculata (ATCC 11745) was grown, harvested, and washed essentially as described by Molina Portela et al. [21].

2.2. Materials

DNA cellulose, β-NAD$^+$, phenylmethylsulfonyl fluoride (PMSF), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), pepstatine-A, β-mercaptoethanol (β-Me), DTT, L-Cys, EDTA, Trizma base, D-mannitol, histone II-A, histamine, nicotinamide, 3-aminobenzamide, spermine, theophylline, activated DNA, bovine serum albumin (BSA), NP-40, dimethyl sulfoxide (DMSO), superoxide dismutase (SOD), *N*-acetyl-L-cysteine (NAC), *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoic acid (PCMB), *p*-chloromercuriphenylsulfonic acid (PCMPS), trichloroacetic acid (TCA), L-ascorbic acid were purchased from Sigma Chemical Co. (St Louis, MO); [Adenine-2,8-^{3}H]-NAD$^+$ was purchased from Dupont New England Nuclear; Affigel-Blue was purchased from Bio-Rad; β-lapachone and α-lapachone were obtained from the Program for the Synthesis of Antiparasitic Drugs, Universidad Federal de Rio de Janeiro, Brazil; CG 8-935 was supplied by CIBA-GEIGY NOVARTIS (Basel, Switzerland). Quinones were dissolved in DMSO and stored at -20°C until used.

2.3. Nucleus isolation

Nuclei were isolated as described by Rubio et al. [34]. Essentially, *C. fasciculata* cells (20 g wet weight) were subject to three freeze-thawing cycles, at -20°C, the first freezing step lasting for 12 h. Disrupted cells were suspended in 10 ml of buffer solution A (20 mM Tris–HCl, pH 7.4, 0.15 M KCl, 5.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, supplemented with protease inhibitors E-64 (1 μg ml^{-1}), pepstatine A (0.7 μg ml^{-1}), PMSF (174 μg ml^{-1}), TLCK (37 μg ml^{-1}) and 5.0 mM EDTA), containing 0.5–1.0% (w/v) NP-40 and left for 10 min in the ice-bath. The lysate was diluted 10-fold with solution A. The suspension was sonicated in a sonifier cell disruptor, Model W 185 (Heat Systems-Ultrasonic, Plainview, IL) at 45 W for 20 s. Cell disruption was checked by microscopic observation of fresh and Giemsa-stained extensions. The disrupted *C. fasciculata* suspension was centrifuged at $700 \times g$ for 10 min to discard whole *C. fasciculata* and cell debris, and the supernatant further centrifuged at $2000 \times g$ for 15 min. The resulting pellet, predominantly containing nuclei, was resuspended in 5 ml of solution A and centrifuged twice as above, then resuspended in 2.0–2.5 ml of buffer solution B (100 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1.5 mM DTT, 10% (v/v) glycerol) and protease inhibitors as described above. In order to purify further the nuclei preparation, an equal volume of 2 M sucrose was added to the suspension, then sonicated for 1–2 s and centrifuged at $13\,500 \times g$ in the sw rotor of the Beckman centrifuge. Purity of isolated nuclei samples was checked by microscopic observation of Giemsa-stained extensions.

2.4. Poly(ADP-ribose)polymerase isolation and partial purification

C. fasciculata PARP was partially purified following Zahradka and Ebisuzaki [35] procedure for calf thymus PARP with modifications as described below. All steps were at 4°C. *C. fasciculata* cells (about 30 g) were subjected to three freeze-thawing cycles at -20°C. Disrupted cells were suspended in buffer solution C (50 mM Tris–HCl, pH 8.0, 50 mM $NaHSO_3$, 1.0 mM EDTA, 10% (v/v) glycerol, 10 mM β-Me) containing 0.5 mM DTT, protease inhibitors as described above and 0.3 M NaCl. The suspension was sonicated in a sonifier cell disruptor by means of five 1-min treatments at 45–50 W, to total cell disruption checked by microscopy. The cell homogenate was centrifuged at $27\,000 \times g$ for 30 min; solid $(NH_4)_2SO_4$ was added to the supernatant (cell-free extract). The fraction precipitating between 30 and 70% $(NH_4)_2SO_4$ was left to sediment for 45 min, then collected by centrifugation at $27\,000 \times g$ and resuspended in the least volume of solution C containing 1 mM DTT (solution D). The PARP preparation was dialyzed overnight against solution D and loaded onto a DNA cellulose column (2.2×7.0 cm), equilibrated with solution D. The column was washed with the same solution containing 0.1 M NaCl, until absorbance at 280 nm was negligible. Thereafter, active PARP fractions were further eluted with a 0.35 M NaCl/supplemented solution D. Active PARP fractions were pooled, dialyzed against solution C containing 2.5 mM DTT (solution E) and loaded onto a Affigel-Blue column (2.2×6.0 cm) equilibrated with solution E. Increasing NaCl concentration in the elution buffer up to 0.4 M, produced PARP elution. Active fractions were pooled and concentrated by ultrafiltration at 4°C through membranes of 30 000 MW cutoff using Millipore concentrators. The enzyme preparation was used for the experiments described below.

2.5. Poly(ADP-ribose)polymerase assay

Activity was measured as TCA-precipitable radioactivity incorporated from ^{3}H-NAD^{+} [36]. PARP samples (30 µl) were incubated for 5 min at 30°C in a standard assay mixture (270 µl) containing 100 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10% (v/v) glycerol, 1.5 mM DTT, 70 µg ml^{-1} activated DNA (DNase digested) and 100 µg ml^{-1} histone II-A. The reaction was started by adding 0.3 µCi of ^{3}H-NAD^{+} (2.2 µCi μmol^{-1}). After incubation, TCA (25% w/v; final concentration) was added to the assay system to stop the reaction. Two milligrams per milliliter BSA was added and after 20 min standing on ice, samples were filtered on a 0.45 µm Millipore filter, previously saturated with 25% (w/v) TCA. Filters were then washed twice with 95% ethanol 96°, 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 amount of enzyme required to convert 1 nmol of NAD^{+} min^{-1} under standard conditions, taking into account that 4.44 dpm were equivalent to 1 nmol of metabolized ^{3}H-NAD^{+}. In order to evaluate the possible contribution of monoADP-ribose transferase to PARP assay, specific inhibitor vitamin K_3 [37] was used, to find that 120 µM vitamin K_3, a concentration producing 50% inhibition of monoADP-ribose transferase activity, failed to affect the ADP-ribose incorporation into TCA precipitable protein.

In some experiments, PARP was treated with inhibitors or activators in a preincubation mixture, free of assay components. This procedure was used to prevent interactions with PARP substrates or other assay mixture components.

Protein content was determined by Bensadoun and Weinstein method [38].

2.6. SDS-PAGE

SDS/PAGE was carried out under reducing conditions in 10% polyacrylamide minigels and the proteins were stained with Coomassie Brilliant Blue R250 [39].

2.7. Expression of results

Activity of PARP samples represent the mean ± S.D. Statistical analysis was performed using ordinary ANOVA, Dunnett's multiple comparisons test.

3. Results

3.1. Poly(ADP-ribose)polymerase isolation and characterization

PARP was detected in *C. fasciculata* homogenates and subsequently purified, as summarized in Table 1. Presented results illustrate a typical experiment. Procedure steps deserve the following comments. Step 1 (cell-free extract): Buffer solution C was supplemented with DTT, protease inhibitors and 0.3 M NaCl as described in Section 2. DTT was required to maintain activity throughout the purification procedure, as illustrated later in this paper; protease inhibitors were added to prevent decay of PARP activity that followed cell-free extract preparation, as described for other *C. fasciculata* and *T. cruzi* enzymes purification procedures [40]. Finally, 0.3 M NaCl was essential for separation of PARP from nuclear DNA. Step 3 (DNA cellulose chromatography): This step resulted in a 3.2-fold increase in total activity recovered and a 36-fold increase in specific activity, suggesting the removal of an enzyme inhibitor present in the original cell-free extract. The 'inhibitor' was not adsorbed by DNA cellulose. Assay of such eluate on active PARP fraction eluted from DNA cellulose, demonstrated the former's inhibitory action as indicated by the following PARP activity values (in U ml^{-1}): mean ± S.D. ($n = 3$); (a) active PARP fraction: 2194 ± 340; (b) eluate fraction: 107 ± 45; $a + b$: 567 ± 32 (74% inhibition of PARP activity). Step 4 (Affigel-Blue chromatography): This step produced a remarkable increase in PARP specific activity, namely 83 700-fold. However, yield decreased about 61% and the enzyme became very unstable. SDS-PAGE of the Steps 3 and 4 is shown in Fig. 1.

Table 2 shows two specific requirements for PARP activity, activated DNA and histone. Omission of DNA almost entirely abolished (> 99%) PARP activity

Table 1
PARP partial purification

Step	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
(1) Cell-free extract	180 187	44	100	1
(2) 30–70% $(NH_4)_2SO_4$	157 437	63	87	1.4
(3) DNA cellulose	575 759	1614	320	36
(4) Affigel-Blue	222 523	3 708 717	123	83 700

PARP isolation and purification was performed using 30 g of *C. fasciculata* (wet weight). Experimental conditions were as described in Section 2.

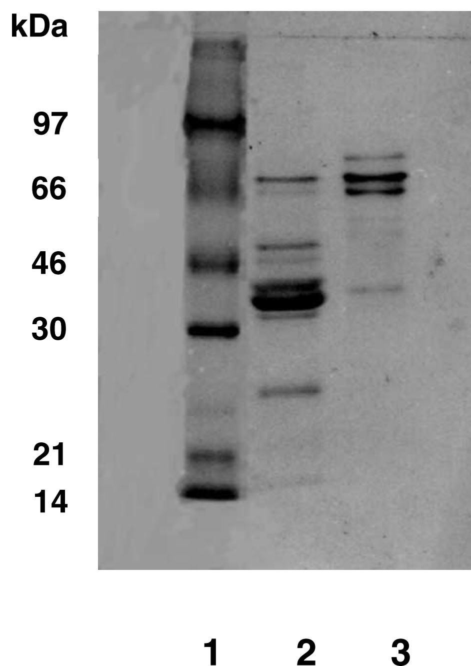

Fig. 1. SDS-PAGE of active fraction obtained after DNA–cellulose (Line 2) and Affigel-Blue (Line 3) chromatography. Line 1, protein markers: phosphorylase b, BSA, ovoalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme (molecular masses indicated on left side).

whereas omission of histone reduced such activity 58%. Thiol compounds were also an essential requirement for PARP activity, both during purification and assay. Accordingly, DTT was added to PARP samples during purification as described in Section 2. The effect of thiol compounds is presented in Table 3, showing that PARP samples dialyzed against thiol-free buffer solution C, displayed a relatively low activity (about 35% of DTT supplemented PARP). The monothiol L-cysteine was somewhat more effective than DTT (Table 3, experiment A). PARP incubation for 30 min at 4°C in the absence of thiol led to a further decrease in PARP activity (roughly 40% of initial activity; Table 3, experiments A and B). Addition of DTT or L-cysteine (0.5 mM) fully prevented PARP activity loss, as shown by comparing corresponding values with PARP activities in experiments A and B (Table 3). Moreover, addition of DTT or L-cysteine to PARP samples preincubated without thiol (Table 3, experiment C) restored PARP activity to experiment A levels, 100 and 63%, with DTT and L-cysteine, respectively. These results suggested that oxidation of PARP thiol groups was largely responsible for PARP inactivation during purification and that thiol oxidation could be prevented or reversed by thiol compounds. Accordingly, sulfydryl reagents such as NEM (1 mM), PCMB (0.01 mM), PCMPS (0.01 mM) inhibited 100% PARP activity, whereas *o*-iodosobenzoate (5 mM) only inhibited 28%.

Table 2
PARP requirements for activity

Assay system	PARP activity ($U\ ml^{-1}$)	Relative activity (%)
Standard	2109 ± 129	100
Same, less activated DNA	1.5 ± 0.9	0.07
Same, less histone	878 ± 147	42
Same, less activated DNA and histone	8 ± 2	0.4

The standard assay mixture contained PARP (total units 600), 0.5 mM ^{3}H-NAD$^+$, 70 μg ml^{-1} activated DNA, 100 μg ml^{-1} histone, 10 mM $MgCl_2$, 10% (v/v) glycerol, 1.5 mM DTT, and 100 mM Tris–HCl, pH 8.0. Other conditions were as in Section 2. Values represent mean ± S.D. ($n = 5$).

Table 3
Effect of thiol compounds on PARP activity: protection and reactivation

Thiol (0.5 mM)	PARP activity ($U\ ml^{-1}$)		
	Expt. A	Expt. B	Expt. C
None	1403 ± 287	561 ± 52	561 ± 52
DTT	3985 ± 105*	4275 ± 788*	3808 ± 130**
L-Cysteine	4940 ± 363*	5958 ± 146*	3130 ± 199**

The assay mixture (270 μl) contained 100 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10% (v/v) glycerol, 70 μg ml^{-1} activated DNA, 100 μg ml^{-1} histone II-A and 30 μl PARP plus the following additions: Expt. A: Thiol compounds were added, as indicated above and the reaction was started by adding 0.3 μCi of ^{3}H-NAD$^+$. The reaction rate was measured immediately after thiol addition; preincubation omitted. Expt. B: Thiol compounds were added to assay mixture, as indicated above; after 30 min preincubation at 4°C, ^{3}H-NAD$^+$ was added to complete the standard assay mixture and activity was measured. Expt. C: Samples were preincubated for 30 min at 4°C without thiol, then thiol compounds were added, as indicated above and after 5 min ^{3}H-NAD$^+$ was added to complete the standard assay mixture and activity was measured. Other conditions were as in Section 2. Values represent mean ± S.D. ($n = 3$).

* $P < 0.01$.

** $P < 0.001$.

A characteristic feature of *C. fasciculata* PARP preparations was its inhibition by a series of compounds effective on other well-known PARPs as described in the literature [7,37,41–45]. Thus, histamine, 3-aminobenzamide, theophylline, nicotinamide and thymidine also inhibited *C. fasciculata* PARP (Table 4). Inhibitor concentrations were selected in accordance with their effectiveness on other PARPs. Moreover, PARP inhibitors also affected PARP activity in isolated *C. fasciculata* nuclei although greater concentrations were required than with the purified enzyme (Table 5). In close agreement with the effect on rat liver PARP [46], spermine affected *C. fasciculata* PARP in a concentration dependent manner. Thus, spermine concentration up to 1 mM activated PARP whereas greater

Table 4
Effect of specific inhibitors on PARP enzyme activity

Inhibitor (mM)	PARP activity (U ml^{-1})
None	2109 ± 129
Histamine (0.18)	0 (100)
Theophylline (1.0)	285 ± 33 (86)*
Nicotinamide (1.0)	399 ± 86 (81)*
3-Aminobenzamide (0.01)	668 ± 16 (68)*
Thymidine (0.01)	748 ± 99 (64)*

Standard assay mixture contained PARP (total units 600) and inhibitors as stated above. Other conditions were as in Section 2. Values represent mean ± S.D. ($n = 5$). In parenthesis, inhibition (%) of PARP activity.

* $P < 0.001$.

concentrations (5 mM) proved inhibitory (Table 5). Combined nicotinamide (10 mM) and spermine (1 mM) effects resulted in PARP inhibition (Table 5).

3.2. Effect of Fenton system

Fenton systems such as the $Fe(II)/H_2O_2$ system are effective reactive oxygen species (ROS) generators and therefore, suitable agents for establishing PARP sensitivity towards ROS. The $Fe(II)/H_2O_2$ system was assayed on PARP using an experimental model preincubation of PARP with $Fe(II)/H_2O_2$, in the absence of other additions and subsequently added to the standard assay mixture. Results are summarized in Table 6, showing that Fe(II) and H_2O_2 inhibited PARP 34 and 25%, respectively, but significantly greater inhibition was observed with the $Fe(II)/H_2O_2$ system (94%). Addition of 1.5 mM DTT to the preincubation mixture partially prevented the Fenton system effect.

A similar Fenton system was assayed on PARP in isolated *C. fasciculata* nuclei. Reagents concentration and other experimental conditions were as in Table 6, except preincubation, that was omitted. Fenton system components were added to the assay mixture containing 1.5 mM DTT. Under these conditions PARP was inhibited 30% (15 min incubation experiments).

Table 5
Effect of specific inhibitors and spermine on PARP in isolated nuclei

Addition (mM)	PARP activity (U mg^{-1} protein)
None	200.5 ± 5.5
3-Aminobenzamide (10)	4.7 ± 1.5 (98)**
Nicotinamide (10)	13.2 ± 2.9 (93)**
Theophylline (10)	17.1 ± 1.9 (91)**
Spermine (1)	264.8 ± 28 (−32)*
Spermine (5)	79.0 ± 18 (61)**
Spermine (1) + nicotinamide (10)	48.8 ± 7.5 (76)**

C. fasciculata nuclei (300 μg protein ml^{-1}) in PARP standard assay mixture were incubated for 5 min with inhibitors as stated above. After incubation, ADP ribosylation was measured. Other conditions were as described in Section 2. Values represent mean ± S.D. ($n = 4$). In parenthesis, inhibition (%) of PARP activity.

* $P < 0.05$.
** $P < 0.01$.

Table 6
Effect of Fenton system on PARP activity

Additions	PARP activity (U ml^{-1})
None	1251 ± 65
Fe(II)	827 ± 60 (34)**
H_2O_2	939 ± 67 (25)*
$Fe(II) + H_2O_2$	78 ± 14 (94)**
$Fe(II) + H_2O_2$ + DTT	738 ± 65 (41)**

PARP (~16 000 U ml^{-1}) was preincubated for 15 min at 0°C with additions as stated above. Reagents concentrations: 100 μM Fe(II); 3.0 mM H_2O_2; 1.5 mM DTT; 1.5 mM EDTA in 100 mM Tris–HCl, pH 8.0; 10 mM $MgCl_2$ and 10% (v/v) glycerol. Then 25 μl samples were added to the standard assay mixture (275 μl) without DTT and activity was measured. Other conditions as in Section 2. Values represent mean ± S.D. ($n \geq 3$). In parenthesis, inhibition (%) of PARP activity.

* $P < 0.05$.
** $P < 0.01$.

3.3. Effect of naphthoquinones

Lipophilic *o*-naphthoquinones such as β-lapachone are potent inhibitors of growth and DNA synthesis in trypanosomatids, including *C. fasciculata* and *T. cruzi* [21,22]. Fig. 2 shows that β-lapachone inhibited PARP as a function of quinone concentration and incubation time. In these experiments, PARP was preincubated with the quinone at 4°C, for the time indicated in the figure and then ^3H-NAD^+ was added to complete the assay mixture and start the reaction. β-Lapachone and the *o*-naphthoquinone CG 8-935 inhibited PARP in the 0–20 μM concentration range but further increase in quinone concentration up to 100 μM failed to modify the inhibition level (Fig. 2). PARP inhibition by β-lapachone was reversible since preincubation of PARP with the quinone under inhibitory experimental conditions, followed by 5-fold dilution of the β-lapachone supplemented PARP, yielded PARP samples with activity not significantly different from control PARP activity (experimental data omitted). Interestingly enough, ^3H-NAD^+ addition to the β-lapachone containing preincubation medium significantly prevented PARP inhibition (Fig. 2). It should be pointed out that in these experiments, DTT was added to the assay mixture. This addition posed the possibility of quinone redox-cycling and ROS generation and therefore, ROS could be involved in *o*-naphthoquinone inhibitory effect. However, omission of DTT from PARP assay mixture failed to prevent PARP inhibition by β-lapachone and CG quinone (Table 7). Addition of 2 μM β-lapachone scarcely affected PARP activity (Fig. 2).

Despite that, addition of this concentration of quinone to PARP assay mixture produced roughly 270 μM O_2^- min^{-1}. Accordingly, it is not surprising that addition of SOD, catalase, mannitol, NAC or ascorbate failed to prevent PARP inactivation by β-lapachone 20 μM in the presence of DTT (Table 7). Taken together, these findings support the hypothesis that β-lapachone directly inhibited PARP in a reversible manner.

Under the same experimental conditions, the *p*-naphthoquinone α-lapachone 20 μM was not significantly active on PARP activity (Table 7).

The β-lapachone derivative CG 8-935 was also effective as PARP inhibitor in *C. fasciculata* nuclei. In this experiment, nuclei suspended in standard assay medium lacking ^{3}H-NAD$^+$ were incubated with 10 μM CG 8-935 for 30 min at 30°C. ^{3}H-NAD$^+$ was added and incubation continued for 5 min. Measurements of PARP activities in control and quinone supplemented samples yielded the following values (U mg^{-1} protein; mean ± S.D., $n = 5$): 90 ± 18 (control) and 35 ± 7 (quinone-treated samples) thus showing 61% PARP inhibition by the quinone in isolated nuclei.

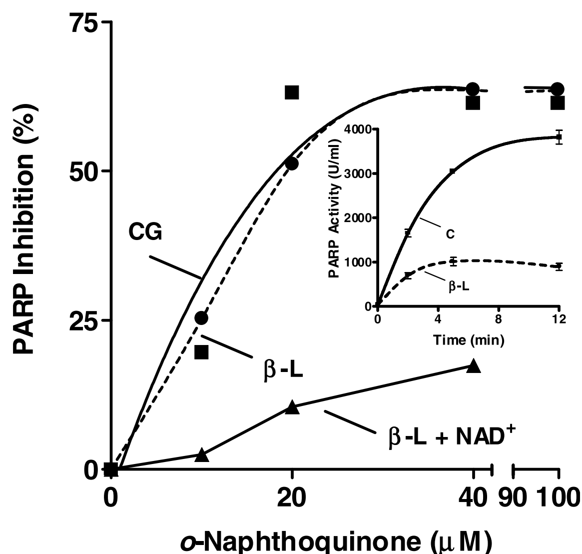

Fig. 2. Effect of *o*-naphthoquinones on PARP activity. β-Lapachone (β-L) or CG 8-935 (CG) were added to the standard assay system at the concentration indicated on the abscissa. Samples were preincubated for 12 min at 4°C, and ^{3}H-NAD$^+$ was added to complete the assay mixture, which was further incubated at 30°C for 5 min. (β-L + NAD$^+$) indicate samples preincubated with β-lapachone in the presence of H^3-NAD$^+$. Control activity: 3045 ± 50 U ml^{-1}. Representative data shown ($n = 4$). Inset: Standard assay mixture containing 20 μM β-lapachone (β-L) or DMSO (C), as indicated in the figure. Samples were preincubated for 12 min at 4°C, then ^{3}H-NAD$^+$ was added and incubated at 30°C for the time indicated on the abscissa. Other conditions as indicated under Section 2. Representative data shown ($n = 3$).

Table 7
Effect of naphthoquinones on PARP activity; lack of action of antioxidants

Naphthoquinone and additions	PARP relative activity (%)
A	
α-Lapachone	90
CG 8-935	38
β-Lapachone	41
B	
α-Lapachone	84
CG 8-935	48
β-Lapachone	50
β-Lapachone + D-mannitol	55
β-Lapachone + SOD + catalase	51
β-Lapachone + NAC	50
β-Lapachone + L-ascorbate	50

Expt. A: PARP preparations were dialyzed against buffer solution C lacking DTT for 2 h. Expt. B: PARP preparations in standard conditions as described in Section 2. In both experiments PARP (total units 1200) was preincubated for 12 min at 0°C in standard assay mixture without DTT (Expt. A) or with 1.5 mM DTT (Expt. B) in the presence of 20 μM quinone, 0.3 mM D-mannitol, 80 μg ml^{-1} (20 U ml^{-1}) SOD and 40 μg ml^{-1} (130 U ml^{-1}) catalase, 0.5 mM NAC or L-ascorbate as indicated above. Naphthoquinones were added in DMSO and the same volume (< 10% v/v) of solvent was added to the control samples. Then ^{3}H-NAD was added to complete the assay mixture and activity was measured. Values represent relative activities, as compared with control sample activity (100%; samples without naphthoquinone). Control activity (U ml^{-1}): 1403 ± 287 (Expt. A); 3985 ± 105 (Expt. B). Values represent mean ± S.D. ($n \geq 4$).

4. Discussion

In the present study, we isolated and purified PARP from the trypanosomatid *C. fasciculata* and some of the enzyme properties were recognized. PARP purification was based on Zahradka and Ebisuzaki's procedure [35], however, we replaced Red Agarose by Affigel-Blue in the corresponding step. The high recovery of PARP activity after the third purification step may be due to removal of an inhibitor, whose nature remains to be established. Similar effects have been reported with rat and *Dictyotelium discodeum* PARP [41,47]. After subsequent purification steps the enzyme was rather unstable, resulting in 61% loss of PARP activity as compared with the previous step (Table 1).

PARP preparations showed properties characterizing other representatives of the PARP group, including mammalian PARP, particularly the requirement of activated DNA, histone (Table 2) and thiols for PARP activity, as well as its sensitivity to specific inhibitors [7,37,41,44,48–51]. The effect of activated DNA and histone fits in well with the action of PARP in DNA repair. It has been suggested that histones act as an allosteric activator of PARP or as an ADP-ribosyl acceptor, thus regulating enzyme activity [44,48,50,51].

As illustrated by DTT, thiol compounds were an essential requirement for PARP stability and activity. Thiol compounds exerted the following actions on PARP purified preparations: (a) increased their initial activity 2.8–3.5-fold; (b) prevented PARP activity decay that occurred with PARP samples incubated in absence of thiols; and (c) restored PARP activity after activity decay in the absence of thiols (Table 3). The observed effects of thiol compounds suggested that *C. fasciculata* PARP is a thiol enzyme, and that thiols oxidation suppresses activity. This hypothesis was supported by the inhibitory effect of sulfydryl reagents NEM, PCMB, PCMPS and *o*-iodosobenzoate.

C. fasciculata PARP was inhibited by a series of compounds effective on other PARP preparations. Some similarities were remarkable such as nicotinamide, 3-aminobenzamide, theophylline, histamine and histidine inhibited PARP (Table 4) at concentrations near those effective on mammalian [7,37,44,47,50,51] and *D. discodeum* [41] PARP. The same inhibitors were effective on isolated *C. fasciculata* nuclei (Table 5), in close agreement with PARP distribution on cells. Spermine activated or inhibited PARP in isolated nuclei as a function of its concentration (Table 5), as rat liver PARP [46]. PARP was sensitive towards ROS, specially oxygen radicals. The Fe(II)/H_2O_2 Fenton system inactivated PARP more effectively than Fe(II) or H_2O_2 alone. DTT prevented the Fenton system effect to a limited extent (Table 6). H_2O_2 inhibition was prevented by EDTA, presumably by chelation of metal cations present in the PARP assay system. This result suggests that H_2O_2 effect was due to a Fenton system dependent on heavy metals contaminating PARP (experimental data omitted). β-Lapachone and relative *o*-naphthoquinones are effective inhibitors of trypanosomatids growth and macromolecules synthesis in these organisms [21,22]. Therefore, PARP inhibition by *o*-naphthoquinones (Fig. 2) was compatible with the effect of such quinones in cells. PARP inhibition by β-lapachone and the CG quinone allows at least two possible explanations: (a) quinone–PARP interaction leading to loss of enzyme activity, as occurs with other enzymes, including oncornavirus reverse transcriptase, eukaryotic DNA polymerase [52], topoisomerase I, and topoisomerase II [31,32]; or (b) quinone redox cycling and ROS production as agents of PARP inactivation. The direct inhibition mechanism is supported by (a) the absence of a quinone reductase and its corresponding substrate NAD(P)H in the PARP assay system, essential for quinone redox cycling and ROS production; (b) similar kinetics of PARP and topoisomerases inhibition by β-lapachone (Fig. 2[31,32]); (c) the similar effect of β-lapachone, irrespective of DTT addition (Table 7); and (d) the negative effect of ROS scavengers on PARP inhibition (Table 7). In conclusion, the isolation and characterization of PARP from *C. fasciculata* confirms the presence of this enzyme and suggests the existence of DNA repair mechanisms in this organism. Further studies need to be done to find special characteristic intrinsic to trypanosomatids that could make PARP a suitable target for chemotherapy [53,54].

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