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Purification and characterization of NAD+:ADP-ribosyltransferase (polymerizing) from *Dictyostelium discoideum*

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A novel affinity-purification scheme based on the tight binding of NAD^+:ADP-ribosyltransferase (polymerizing) [pADPRT; poly(ADP-ribose) polymerase; EC 2.4.2.30] to single-strand nicks in DNA, single-stranded patches and DNA ends has been developed to facilitate the purification of this enzyme from the lower eukaryote *Dictyostelium discoideum*. Two homogeneous forms of the enzyme, with M_r values of 116000 and 90000, were prepared from *D. discoideum* by using poly(A) hybridized to oligo(dT)–cellulose as affinity material. The K_m is 20 μM NAD^+ for the 90000-M_r protein and 77 μM NAD^+ for the 116000-M_r protein. The optimum conditions for the enzyme activity *in vitro* are 6–10 °C and pH 8. The time course is linear during the first 10 min of the reaction only. As in enzymes of higher eukaryotes, the activity is dependent on DNA and histone H1 and is inhibited by 3-methoxybenzamide, nicotinamide, theophylline, caffeine and thymidine.

INTRODUCTION

NAD^+:ADP-ribosyltransferase (polymerizing) [pADPRT; poly(ADP-ribose) polymerase; EC 2.4.2.30], is an enzyme localized in the nucleus of eukaryotic cells. It catalyses the transfer of the ADP-ribose moiety from NAD^+ to proteins and finally forms branched poly(ADP-ribose) chains. This polymer is bound to various nuclear proteins which are involved in DNA metabolism and chromosomal architecture (De Murcia et al., 1988; Althaus et al., 1991). Inhibitor studies have shown that pADPRT plays a role in regulating cell proliferation (Colon-Otero et al., 1987; Kaiser et al., 1992), cell differentiation (Exley et al., 1987; McNerney et al., 1989; Williams et al., 1989; Cesarone et al., 1990; Golderer and Gröbner, 1990) and DNA repair (Shall, 1984; Schweiger et al., 1987; Satoh and Lindahl, 1992). Nevertheless, the ultimate answer to the question of what role pADPRT plays in the various cellular processes has not yet been found. Detection of loss-of-function mutants could possibly clarify this question. However, no mutants of this type have been found in higher eukaryotic systems so far. Negative mutants can be generated with the help of several techniques, such as the introduction of a highly expressed mutant pADPRT gene with a dominant negative function in cultured cells (Küpper et al., 1990). An alternative would be to inactivate the pADPRT gene by means of homologous recombination; however, the mouse is the only mammal in which this is possible. All these techniques are much more easily performed in lower-eukaryotic model systems; in addition, their extensive genetic background is also of advantage. Precise knowledge of the properties and characteristics of pADPRT and its gene in lower eukaryotes is a prerequisite for studies on pADPRT function in these organisms.

Saccharomyces cerevisiae, the lower eukaryote most widely used in cell proliferation, differentiation and DNA-repair studies, proved to be inappropriate, because ADP-ribosylation activity could not be detected in this organism (Scovassi et al., 1986; Kaiser et al., 1992). However, the slime mould *Dictyostelium discoideum* was found to contain pADPRT (Rickwood and Osman, 1979; Rickwood, 1982) and has also proved to be a useful model for studying cellular differentiation. The great advantage is that synchronous differentiation into two types of cells only can be induced in large numbers of these cells. Therefore *D. discoideum* was chosen to study the role of poly-ADP-ribosylation in the differentiation of cells, which is important for understanding the physiological function of this enzyme.

Unlike pADPRT from higher eukaryotes, pADPRT from *D. discoideum* and other lower eukaryotes could not be purified by affinity chromatography on 3-aminobenzamide–Sepharose (Burtscher et al., 1986, 1987a,b; Ushiro et al., 1987); therefore we have developed a novel purification scheme. Purified pADPRT was found to require DNA for its activity, which is a result of the enzyme's binding to strand breaks, nicks or single-strand patches (Benjamin and Gill, 1980). On the basis of this property, poly(A)–oligo(dT)–cellulose was employed as an affinity matrix for pADPRT chromatography. Purification and characterization of pADPRT from *D. discoideum* by this method is described in the present study.

MATERIALS AND METHODS

Materials

Hydroxyapatite was synthesized by the method of Bernardi (1971). DEAE-cellulose and phosphocellulose (P11) were purchased from Whatman Biosystems (Maidstone, Kent, U.K.). Oligo(dT)–cellulose and poly(A) were from Boehringer Mannheim (Mannheim, Germany). Glass-fibre and nitrocellulose filters were obtained from Schleicher and Schüll (Dassel, Germany). Rainbow protein molecular-mass markers were purchased from Amersham International (Amersham, Bucks., U.K.); [^{32}P]NAD^+ and [*adenine*-2,8-^{3}H]NAD^+ were from Du Pont–New England Nuclear (Boston, MA, U.S.A.). 3-Methoxybenzamide was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Salmon protamine sulphate (grade I), phenylmethanesulphonyl fluoride, unlabelled β-NAD^+, α-NAD^+, calf thymus DNA type I, histone H1 type V-S from calf thymus, thymidine, caffeine, theophylline, theobromine, Coomassie Blue G-250, Coomassie Blue R-250, 5-bromo-4-chloro-3-indolyl

Abbreviation used: pADPRT, NAD^+:ADP-ribosyltransferase (polymerizing).

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phosphate and Nitro Blue Tetrazolium were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Alkaline phosphatase-labelled affinity-purified pig anti-(goat IgG) antibodies were from Tago Inc. (Burlingame, CA, U.S.A.). All other chemicals were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade. The inhibitors, 1,5-dihydroxy-4-phthalazione, 3-aminophthalhydrazide, 3-guanidinobenzamide and 3-hydroxybenzamide were kindly given by Professor S. Shall of the University of Sussex.

Enzyme assay

pADPRT activity was assayed essentially as described previously by Burtscher et al. (1986), except that the reaction was carried out at 6 °C for 10 min.

Protein determination

Protein concentrations were determined by the Coomassie Blue G-250 dye-binding assay (Bradford, 1976), with BSA as the standard.

PAGE

SDS/polyacrylamide (10 %) slab gels were used and electrophoresis was performed by the method of Laemmli (1970). The gels were stained with Coomassie Blue R-250.

Activity gel analysis

Basically, this analysis was carried out as originally described by Scovassi et al. (1984) and modified by Burtscher et al. (1987a), except that the gel was incubated in 5 ml of reaction mixture {100 mM Tris/HCl, pH 8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM NAD^+, 10 μCi/ml [^{32}P]NAD^+ (800 Ci/mmol; 5 mCi/ml} at 6 °C for 12 h.

Immunoblotting

Samples of the two affinity-purified forms of pADPRT eluted together at 100 mM KCl were separated by SDS/PAGE (10 % gel) and transferred to a nitrocellulose membrane [wet blot; 3 g/l Tris/HCl, 14.4 g/l glycine, 0.02 % SDS, 20 % (v/v) methanol; 50–60 V, 2–4 h]. The further procedures were performed as described by Schneider et al. (1987).

Protein purification

Cultivation of cells

Ax-2 wild-type amoebae were grown in an axenic culture at 27 °C (Watts and Ashworth, 1970). Instead of glucose, maltose (18 g/l) was used as a carbon source in the medium. The cells were harvested by centrifugation at 700 **g** for 10 min and then washed by suspension in phosphate-buffered saline and subsequent re-centrifugation. The following procedures were all carried out at 4 °C.

Crude extract

The washed cell pellet (120 g) was homogenized by sonication in 360 ml of buffer A, pH 7.3 [50 mM Tris/HCl, 25 mM $K_2S_2O_5$, 12 mM β-mercaptoethanol, 1 mM phenylmethanesulphonyl fluoride, 20 % (v/v) glycerol] containing 0.5 M-KCl.

Protamine sulphate precipitation

96 ml of a 1 % (w/v) protamine sulphate solution was added slowly by stirring the homogenate for 20 min (the final concentration of protamine sulphate was 0.2 %). The homogenate was centrifuged at 16000 **g** for 20 min.

Hydroxyapatite I

The supernatant was shaken with 120 g of hydroxyapatite (equilibrated in buffer A, pH 7.3, containing 0.5 M KCl) for 60 min. The suspension was centrifuged at 1600 **g** for 5 min. Then the supernatant was discarded, and the hydroxyapatite pellet was washed with 2 × 300 ml of buffer A, pH 7.3, containing 0.5 M KCl. Elution was carried out by shaking the hydroxyapatite in 3 × 100 ml of buffer A, pH 7.3, containing 0.5 M KH_2PO_4 for 20 min. It was then centrifuged as described above.

DEAE-cellulose

The combined hydroxyapatite I eluates were diluted 5-fold with buffer A, pH 8, and filtered through 200 ml of DEAE-cellulose (equilibrated with buffer A, pH 8) settled in a Buchner funnel (10 cm in diameter).

Phosphocellulose (P11)

The filtrate of the DEAE-cellulose was diluted with buffer A, pH 7.3, until a conductivity of 5 mS was achieved, and filtered through a Buchner funnel containing 100 ml of settled phosphocellulose which had been precycled and equilibrated with buffer A, pH 7.3. After washing with 300 ml of buffer A, pH 7.3, the phosphocellulose pellet was packed into a column (5 cm in diameter). Subsequently a linear gradient of 0–0.5 M KCl in buffer A was applied.

Hydroxyapatite II

Active fractions which started to be eluted at 6 mS were pooled and incubated with 10 g of hydroxyapatite. After shaking the suspension for 30 min, the hydroxyapatite was washed with 2 × 100 ml of buffer A, pH 7.3, containing 2.5 M KCl and once with 50 ml of pure buffer A, pH 7.3. The hydroxyapatite was eluted with 3 × 10 ml of buffer A, pH 7.3, containing 0.5 M KH_2PO_4. The pooled eluates were dialysed extensively against buffer B (50 mM Tris/HCl, pH 8.0, 0.5 mM EDTA, 12 mM β-mercaptoethanol, 5 mM $MgCl_2$, 25 mM $K_2S_2O_5$, 1 mM phenylmethanesulphonyl fluoride, 5 % glycerol).

Poly(A)–oligo(dT)–cellulose

The dialysed solution was applied to the affinity column (2 ml, equilibrated with buffer B). After washing the column with 20 ml of buffer B, the enzymes were eluted stepwise with 50 mM and 100 mM KCl in buffer B.

Preparation of the affinity column

Oligo(dT)–cellulose (1 g) was suspended in 10 ml of buffer B containing 1 M KCl for 10 min. A solution of poly(A) (10 mg/ml in 10 mM Tris/HCl, pH 8.0, 1 mM EDTA) was incubated with the oligo(dT)–cellulose suspension [100 A_{260} units/g of dry powder oligo(dT)–cellulose] by shaking it at room temperature for 30 min. After equilibration of the affinity material with buffer B, the amount of bound poly(A) was estimated by measuring the decrease in u.v. absorption (260 nm) of the supernatant.

Catalytic properties

Enzyme kinetics

Samples (10 μl) of the purified enzymes were added to 90 μl of the reaction mixture containing different concentrations of

unlabelled NAD^+ (final concns.: 100, 75, 50, 25, 10 and 1 μM) and incubated at 6 °C for 10 min.

Temperature optimum

Enzyme solution (10 μl) was added to 90 μl of prewarmed/precooled reaction mixture and incubated at 0, 6, 10, 15, 20, 25 or 37 °C for 10 min. The reaction was stopped by adding 100 μl of 40% (w/v) trichloroacetic acid.

Time course

The reaction mixture and enzyme solution were cooled to 6 °C, mixed together and, after incubation for 0, 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 60 or 120 min, 100 μl portions were added to 100 μl of 40% trichloroacetic acid.

DNA and histone H1 dependence

Reaction mixture (90 μl) with or without DNA (20 μg/ml) and histone H1 (20 μg/ml) were incubated with 10 μl of enzyme solution at 6 °C for 10 min. The activity was determined as described above.

Inhibitors

Stock solutions (1 or 10 mM) of nicotinamide, thymidine, caffeine, 3-hydroxybenzamide, 3-guanidinobenzamide, theophylline and 3-aminophthalhydrazide were prepared in water. Methanolic stock solutions (10 mM) of 1,5-dihydroxy-4-phthalazione and 3-methoxybenzamide were diluted. A 10 μl portion of an inhibitor of appropriate dilution (final concn. 1 mM–0.1 μM) were mixed with 10 μl of enzyme solution and subsequently added to 80 μl of reaction mixture. The activity assay was performed as described above.

Analysis of ADP-ribose polymers

The size of the pADPRT reaction product was determined on non-denaturing polyacrylamide gels (Panzeter and Althaus, 1990; Simonin et al., 1991). Protein solution (50 μl) was incubated with 450 μl of reaction mixture {100 mM Tris/HCl, pH 8, 1 mM dithiothreitol, 20 μg/ml activated DNA (Loeb, 1969), 20 μg/ml histone H1, 2 μl of [^{32}P]NAD^+ (800 Ci/mmol; 5 mCi/ml)} at 6 °C for 30 min. The reaction product was precipitated with 500 μl of 40% trichloroacetic acid at 0 °C for 2 h. After centrifugation, the pellet was washed with 2 × 1 ml of 1% trichloroacetic acid and once with 1 ml of methanol, diethyl ether (1:1, v/v). The dried pellet was dissolved in 200 μl of 10 mM Tris/HCl (pH 12)/1 mM EDTA and incubated at 60 °C for 2 h. The samples were extracted once with water-saturated phenol/chloroform (1:1, v/v) and evaporated in a vacuum desiccator. The pellets were dissolved in 10 μl of water and 10 μl of stop mixture [50% (w/v) urea, 25 mM NaCl, 4 mM EDTA, pH 7.5, 0.02% xylene cyanole FF, 0.02% Bromophenol Blue]. Then the samples were loaded on to a 20%-acrylamide gel [19.86% acrylamide/0.24% bisacrylamide (w/w), in 0.09 M Tris/HCl (pH 8.3)/0.09 M boric acid/2 mM EDTA] and electrophoresis was carried out at a constant power of 55 W. The gel was dried and the polymers were made visible by autoradiography.

RESULTS

Purification of *D. discoideum* pADPRT

Standard enzyme purification from about 120 g of cells resulted in 0.1 mg of a 1700-fold enriched enzyme preparation. The data from a representative procedure are summarized in Table 1. The first steps are, with slight modifications, the same as those described by Burtscher et al. (1986). A crude extract of *D. discoideum* cells was prepared by sonication, and then the DNA was removed by protamine sulphate precipitation. The supernatant was mixed in one batch with hydroxyapatite, which was washed and eluted with 0.5 M KH_2PO_4. The eluate was diluted and filtered through a DEAE-cellulose column to remove residual DNA, which would interfere with the binding of the enzyme to the chromatography materials used in subsequent steps. Then the filtrate was again diluted and loaded on to a phosphocellulose column, to which a linear gradient of 0–0.5 M KCl was applied. The active fractions, which started to be eluted at 6 mS, were pooled (Figure 1, lane 1) and treated again with hydroxyapatite. At this stage it was possible to wash the hydroxyapatite with 2.5 M KCl to remove most of the protein (Figure 1, lane 2). The elution was carried out as described above. Although at this stage the enzyme is strongly inhibited by 3-methoxybenzamide (see also Figure 6), all attempts to bind the *D. discoideum* pADPRT to 3-aminobenzamide–Sepharose failed.

As a final purification step, chromatography was performed on oligo(dT)–cellulose to which poly(A) was hybridized in a 1 M

Table 1 Purification of ADPRT from *D. discoideum*

Step	Total protein (mg)	Specific activity (pmol/min per mg)	Purification (fold)	Recovery (%)
Crude extract	13000	0.42	1.0	100
Protamine sulphate	6400	1.5	3.5	170
Hydroxyapatite I	1300	5.5	13.1	130
DEAE-cellulose	1200	13	31	286
Phosphocellulose	78	23	55	33
Hydroxyapatite II	27	35	84	17
Poly(A)–oligo(dT)–cellulose	0.1	700	1700	1.3

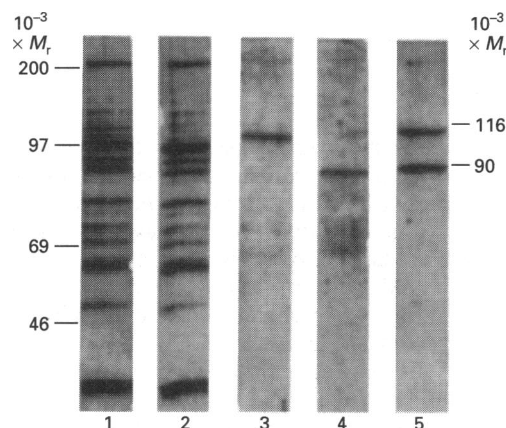


Figure 1 SDS/PAGE analysis of purification steps of pADPRT from *D. discoideum*

Lane 1, phosphocellulose pool; lane 2, hydroxyapatite II after dialysis; lanes 3–5, elution from poly(A)–oligo(dT)–column: with 50 mM KCl (lane 3); with 100 mM KCl after elution with 50 mM KCl (lane 4); directly eluted with 100 mM KCl (lane 5). Electrophoresis was carried out as described in the Materials and methods section. M_r markers were myosin (200000), phosphorylase *b* (97000), BSA (69000) and ovalbumin (46000).

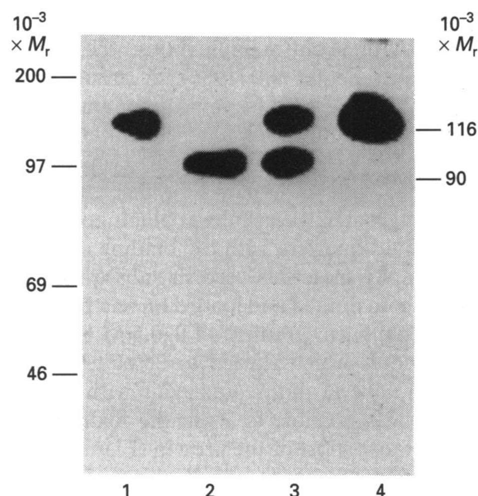


Figure 2 Activity-gel analysis of purified pADPRT

The protein solutions were run on SDS/PAGE (10% gel), re-activated and incubated with [^{32}P]NAD$^+$, and the active bands were detected by autoradiography. Lanes 1–3, purified pADPRT from *D. discoideum*: 750 ng of protein after elution from poly(A)–oligo(dT)–cellulose column with 50 mM KCl (lane 1); 750 ng of protein eluted with 100 mM KCl after elution with 50 mM KCl (lane 2); 1.5 μg of protein directly eluted with 100 mM KCl (lane 3); lane 4, purified human pADPRT (50 ng). M_r markers were as for Figure 1.

Table 2 Histone and DNA requirements for 116000-M_r and 90000-M_r pADPRTs from *D. discoideum*

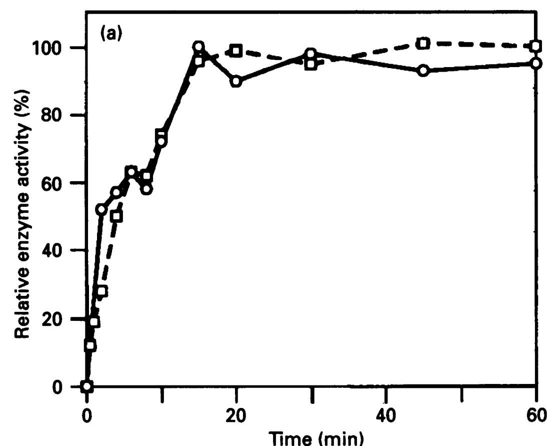
For assay conditions see the Materials and methods section.

Histone (20 μg/ml)	DNA (20 μg/ml)	Activity (%)	
		116000-M_r protein	90000-M_r protein
+	+	100	100
–	+	24.0	23
+	–	10.1	5
–	–	7.3	5.6

KCl buffer. The active hydroxyapatite fractions were dialysed and applied to the affinity column. Elution with 100 mM KCl yielded a fraction with a 1700-fold-enriched enzyme activity; 1.3% of the enzyme activity in the crude extract was recovered (Table 1; Figure 1, lane 5). As shown by Coomassie Blue R-250 staining after PAGE (Figure 1), stepwise elution with 50 mM KCl and 100 mM KCl resulted in the isolation of an active protein of M_r 116000 (Figure 1, lane 3) and a second active protein of M_r 90000 (Figure 1, lane 4). Activity-gel analysis (Figure 2) showed that equal amounts of either protein produced signals of the same intensity (Figure 2, lane 3).

Characterization of the enzyme

The purified enzymes require DNAase I-treated DNA for optimum activity. In the presence of DNA the enzymes can be further stimulated 5-fold by addition of histone H1 (Table 2). Without activated DNA, however, the enzyme activity is decreased to 5% of the maximum even if histone H1 is added.



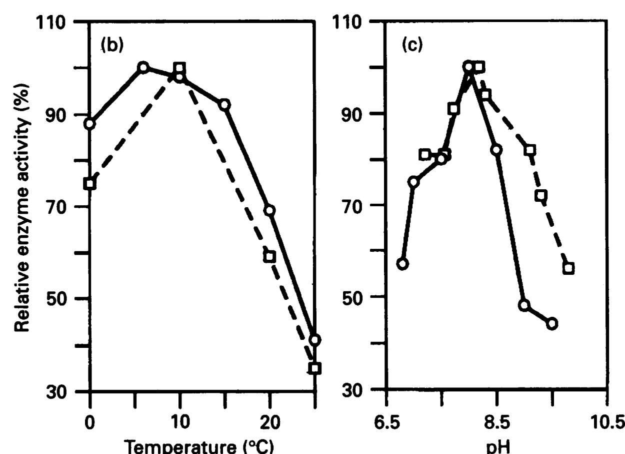


Figure 3 Properties of purified pADPRTs from *D. discoideum*

Enzyme: ○, 116000-M_r; □, 90000-M_r. (**a**) Time course during the enzyme reaction; (**b**) temperature-dependence; (**c**) pH optimum. The experimental conditions are described in the Materials and methods section.

The time course of the enzyme activities of the two proteins is linear during the first 10 min only. After 12 min the activity gradually levels off, and after 20 min there is no further increase in acid-precipitable radioactivity (Figure 3a). The optimum temperature is 6–10 °C for both enzymes (Figure 3b).

In the presence of histone H1 and DNA the pH optimum, at pH 8 (Figure 3c), is rather narrow. pH changes by 1 unit in either direction decreased the enzyme activity by about 20%. Analysis of ADP-ribose polymers produced *in vitro* by a sequencing gel showed chains of length up to 50 ADP-ribose units (Figure 4). There was no difference in length between the poly(ADP-ribose) chains produced by human pADPRT and those originating from *D. discoideum* enzymes (Figure 4).

Kinetic constants

Lineweaver–Burk analysis of enzyme kinetics revealed different Michaelis–Menten constants for both enzymes. The 90000-M_r species showed a K_m of 20±2 μM NAD$^+$, whereas the K_m of the 116000-M_r protein was 77±1 μM NAD$^+$ under optimum conditions (Figure 5). pADPRT from *D. discoideum* was found to be sensitive to several NAD$^+$ competitors, such as 1,5-dihydroxy-4-phthalazione, 3-methoxybenzamide (Figure 6), 3-aminophthalhydrazide, 3-guanidinobenzamide and nicotinamide, used at concentrations of 0.1–100 μM. 3-Hydroxybenzamide, α-NAD$^+$,

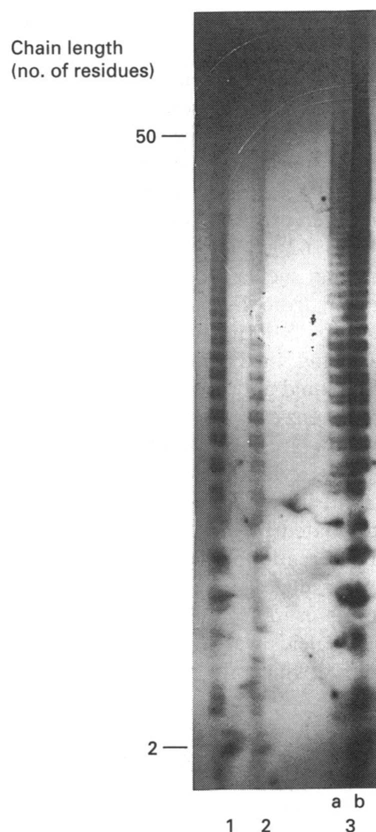


Figure 4 Analysis of the reaction product from pADPRT

pADPRT was incubated with 100 μM NAD$^+$ and 5 μCi of [^{32}P]NAD$^+$. The labelled reaction product was precipitated, washed and detached from proteins. The polymers of ADP-ribose were loaded on to a 20% non-denaturing sequencing gel. Lane 1, crude extract from *D. discoideum* (10 μg); lane 2, purified 116000-M_r protein from *D. discoideum* (100 ng); lanes 3a, 3b, purified human pADPRT (a, 1 ng; b, 10 ng).

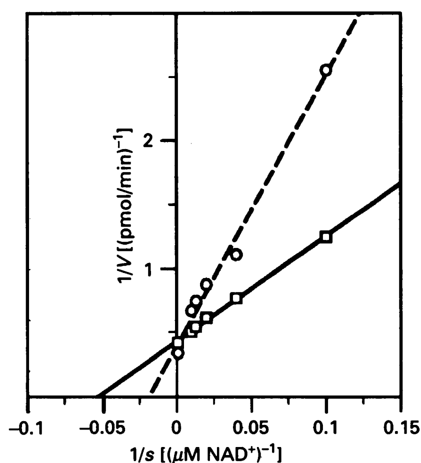


Figure 5 Lineweaver–Burk plots of pADPRT reaction

Purified enzyme preparations (○, 116000-M_r; □, 90000-M_r) were incubated under standard reaction conditions as described in the Materials and methods section, except that the concentration of NAD$^+$ was varied as specified above. The data from a representative experiment are depicted. The K_m values of both enzyme classes were calculated as the mean of two experiments with four replicates for each concentration (77 ± 1 μM NAD$^+$ for 116000-M_r, 20 ± 2 μM NAD$^+$ for 90000-M_r).

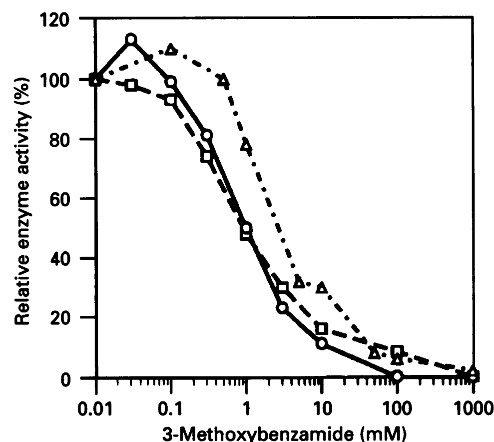


Figure 6 Inhibition of the activity of pADPRT from *D. discoideum* by 3-methoxybenzamide

Purified enzyme preparations (○, 116000-M_r; □, 90000-M_r; △, 116000-M_r human pADPRT) were incubated with various concentrations of 3-methoxybenzamide in standard assay mixture as described in the Materials and methods section.

Table 3 Effect of pADPRT inhibitors on 116000-M_r and 90000-M_r pADPRTs from *D. discoideum*

Inhibitors were added to the standard enzyme assay at the concentrations described in the Materials and methods section. Abbreviation: n.d., not determined.

		Activity (%)	
Inhibitor	Concn.	116000-M_r protein	90000-M_r protein
Control	–	100	100
3-Methoxybenzamide	1 mM	2	0
	10 μM	15	16
	1 μM	50	48
Nicotinamide	1 mM	4	5
	100 μM	28	25
	10 μM	n.d.	63
3-Hydroxybenzamide	100 μM	11	n.d.
	10 μM	64	n.d.
	1 μM	82	n.d.
3-Guanidinobenzamide	100 μM	29	n.d.
	10 μM	86	n.d.
	1 μM	98	n.d.
3-Aminophthalhydrazide	100 μM	2	n.d.
	10 μM	12	n.d.
	1 μM	53	n.d.
1,5-Dihydroxy-4-phthalazione	10 μM	0	n.d.
	1 μM	5	n.d.
	0.1 μM	64	n.d.
α-NAD$^+$	500 μM	60	56
	100 μM	n.d.	76
Thymidine	1 mM	6	12
Theophylline	1 mM	32	61
Caffeine	1 mM	14	43
KCl	100 mM	n.d.	15

thymidine, caffeine and theophylline also inhibited both enzymes (Table 3). Like the 116000-M_r pADPRT from human tissue, the 116000-M_r enzyme from *D. discoideum* was significantly stimulated by concentrations as low as 50 nM 3-methoxybenzamide (Jones et al., 1988). Three independent experiments with four

replicates each revealed an increase to 114±4.58 % (mean±S.D.) of the activity of the untreated enzyme. Interestingly, the 90000-M_r protein (99.3±1.15 %; mean±S.D.) was found to be unable to be activated by very low concentrations of inhibitor.

DISCUSSION

pADPRTs from several higher eukaryotes were purified to homogeneity by affinity chromatography on 3-aminobenzamide–Sepharose (Burtscher et al., 1986, 1987a,b; Ushiro et al., 1987). Unlike pADPRT from higher species, pADPRT from *D. discoideum* did not bind to the immobilized form of 3-methoxybenzamide, although both enzymes were inhibited by the same concentration of this compound. Therefore, we have made use of the DNA-binding property of pADPRT in an attempt to develop an effective purification scheme. pADPRT was found to bind strongly to double-stranded DNA containing a variety of strand breaks, by which it is activated. However, single-stranded DNA as well as poly(dA) and poly(dT) homopolymers proved to be ineffective inductors. As expected, pADPRT did not bind to oligo(dT)–cellulose. On the other hand, poly(dA) is very effective in activating pADPRT, if it is hybridized to poly(dT) (Benjamin and Gill, 1980). Therefore, we used poly(A) hybridized to oligo(dT)–cellulose for the purification of pADPRT by affinity chromatography. In contrast with all other proteins from the active fractions yielded by phosphocellulose chromatography, pADPRTs from *D. discoideum* and human placenta (results not shown) bound tightly to this affinity matrix. Stepwise elution resulted in the separation of two homogeneous active pADPRT proteins with M_r values of 116000 and 90000. This indicates a somewhat increased affinity of the shorter pADPRT species for poly(A)–oligo(dT)–cellulose and demonstrates its efficiency as an affinity-chromatography support for pADPRT purification.

The high recovery rate of 286 % observed during the first steps of the purification scheme may be due to the removal of an inhibitor, probably *D. discoideum* DNA. Similar rates have been reported for the human and the trout enzyme (Carter and Berger, 1982; Burtscher et al., 1987b). During the subsequent purification steps the enzyme is rather unstable, resulting in an overall yield of 1.3 % and a considerable loss of activity due to freezing and thawing of the enzyme solution. The maximum activity of the enzyme is observed at 6–10 °C, which is 10–15 °C below the optimum temperature for culturing *D. discoideum*. This was found to be a common feature of pADPRTs in all species examined (Althaus and Richter, 1987). The temperature optimum for the purified active proteins is somewhat lower than the optimum required for the enzyme activity in nuclei of *D. discoideum* (Rickwood and Osman, 1979). Conversely, the pH optimum in the alkaline region is the same as in nuclei (Rickwood and Osman, 1979) and is similar to that found in other species (Ito et al., 1979; Burtscher et al., 1987a,b; Golderer et al., 1988). Product analysis demonstrated that the polymeric products obtained from the purified 116000-M_r enzyme *in vitro* are identical in length with those synthesized from the purified human enzyme. Despite these similarities in enzyme properties between pADPRT from *D. discoideum* and human pADPRT, immunoblot analysis using antibodies against the purified human enzyme shows no cross-reaction with the two forms of pADPRT from *D. discoideum*. This is in agreement with the results obtained when using antibodies against calf thymus pADPRT, which react with the extracts of several higher eukaryotes, but not with those of lower eukaryotes (Scovassi et al., 1986).

Although the purification scheme was optimized with regard to time (32 h) and proteolytic degradation (phenylmethanesulphonyl fluoride, $K_2S_2O_5$), the appearance of the 90000-M_r protein could not be prevented. Nevertheless, when the serine protease inhibitor phenylmethanesulphonyl fluoride was added, the yield of the 116000-M_r protein increased significantly as compared with the 90000-M_r species (results not shown). This indicates that a specific proteolytic processing step may exist in *D. discoideum*, similar to that described for the human enzyme, which results in different pADPRT fragments, with M_r values of 96000, 79000 and 62000–60000 (Surowy and Berger, 1983, 1985). Assuming that such a processing step exists, the 90000-M_r pADPRT fragment from *D. discoideum* would lack 200 amino acids either from the N-terminal or from the C-terminal end. If the latter were lacking, the NAD^+-binding domain would be lost and the fragment would not show any enzyme activity, supposing the *D. discoideum* enzyme and the various pADPRT species analysed up to now are homologous. Regarding the inhibitor kinetics of 3-methoxybenzamide (Figure 6), the 90000-M_r pADPRT fragment from *D. discoideum* appears to have lost the ability to be stimulated by low doses of inhibitor. This may be due to loss of interaction between multiple binding sites for NAD^+, which were suggested to cause activation of pADPRT at low concentrations of inhibitors (Jones et al., 1988).

If the 200 amino acids were cleaved from the N-terminal end, the two zinc fingers, which are supposed to represent the DNA-binding domain (Kameshita et al., 1986; Gradwohl et al., 1990) would be lost, and binding to poly(A)–oligo(dT)–cellulose would not occur. In fact, the 90000-M_r protein binds to poly(A)–oligo(dT)–cellulose more strongly than the 116000-M_r species. In human pADPRT there is probably a unique sequence motif, located at the amino acids 207–213 and 346–352, which specifically recognizes the DNA ligand and can therefore replace the function of the two zinc fingers (Kurosaki et al., 1987). Interestingly enough, the 90000-M_r pADPRT from *D. discoideum* is also activated by damaged DNA, as was previously shown for the 75000-M_r pADPRTs from *Helix pomatia* and trout (Burtscher et al., 1987a,b). Moreover, the K_m (20 μM NAD^+) of the 90000-M_r pADPRT from *D. discoideum* was found to be in the same range as the K_m (26 μM and 24 μM respectively) of the 75000-M_r enzymes from *Helix pomatia* and trout (Burtscher et al., 1987a,b). The values are in agreement with the K_m (18 μM NAD^+) reported for pADPRT from *D. discoideum* nuclei (Rickwood and Osman, 1979). The K_m of the 116000-M_r enzyme from *D. discoideum* (77 μM NAD^+), however, was slightly higher than the respective values found for purified 116000-M_r enzymes from human (52 μM, Burtscher et al., 1986; 61.7 μM, Ushiro et al., 1987) and calf thymus pADPRTs (55 μM; Ito et al., 1979).

An answer to these discrepancies may be found by analysing the structure of the *D. discoideum* pADPRT gene. However, purification of a sufficient amount of the enzyme by the procedure described in the present study is a prerequisite for this endeavour, which will also provide new insights into the evolution of pADPRT and its functional domains.

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