ADP-ribosylation in isolated nuclei of *Physarum polycephalum*

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ADP-ribosylation of histones and non-histone nuclear proteins was studied in isolated nuclei during the naturally synchronous cell cycle of *Physarum polycephalum*. Aside from ADP-ribosyltransferase (ADPRT) itself, histones and high mobility group-like proteins are the main acceptors for ADP-ribose. The majority of these ADP-ribose residues is NH_2OH -labile. ADP-ribosylation of the nuclear proteins is periodic during the cell cycle with maximum incorporation in early to mid G_2 -phase. In activity gels two enzyme forms with M_r of 115000 and 75000 can be identified. Both enzyme forms are present at a constant ratio of 3:1 during the cell cycle. The higher molecular mass form cannot be converted *in vitro* to the low molecular mass form, excluding an artificial degradation during isolation of nuclei. The ADPRT forms were purified and separated by h.p.l.c. The low molecular mass form is inhibited by different ADPRT inhibitors to a stronger extent and is the main acceptor for auto-ADP-ribosylation. The high molecular mass form is only moderately auto-ADP-ribosylated.

INTRODUCTION

ADP-ribosylation is a post-translational modification of nuclear proteins which is assumed to be involved in processes such as differentiation, gene expression, DNA repair and DNA replication. This modification is catalysed by ADP-ribosyltransferase (ADPRT) (EC 2.4.2.30), which transfers the ADP-ribose moiety of NAD^+ to acceptor proteins. Histones, high mobility group (HMG) proteins, DNA topoisomerase, an endonuclease, RNA polymerase, DNA ligase, DNA polymerase, lamins and ADPRT itself are known as nuclear acceptor proteins for ADP-ribosylation (for reviews see Hayaishi & Ueda, 1982; Althaus *et al.*, 1985; Gaal & Pearson, 1985; Althaus & Richter, 1987).

ADPRT is found in a variety of eukaryotic cells. Brightwell *et al.* (1975) could show the existence of this enzyme in the lower eukaryote *Physarum polycephalum*. Gröbner & Loidl (1985) demonstrated that ADPRT is a typical peak enzyme which shows maximum activity during the S-phase of the cell cycle of *Physarum*. Poirier *et al.* (1987) showed that the histones H1, H2A, H2B and HMG-like-proteins are the main acceptors for ADP-ribosylation in amoebal nuclei of *Physarum*. These investigations were all performed in isolated nuclei since the substrate NAD^+ cannot pass the cell membrane and it is not possible to permeabilize the cell membrane to NAD^+ uptake in *Physarum* due to the thick slime layer deposited on the surface of plasmodia. For this reason we used the analysis *in vitro* of nuclei isolated with a novel, extremely gentle isolation procedure (Nothacker & Hildebrandt, 1985; Loidl & Gröbner, 1987).

Since very conflicting results have been published about ADP-ribosylation in the cell cycle (e.g. HeLa cell cycle; Kidwell & Mage, 1976; Adolph, 1985; Tanuma *et al.*, 1986), we explored cell cycle characteristics of ADP-ribosylation in *Physarum*. This organism has attracted considerable interest as a model system for studies of cell cycle dependent events, since nuclear division occurs

spontaneously and is naturally synchronous. We could show that maximum ADP-ribosylation takes place in early to mid G_2 -phase. The main acceptor proteins are the core histones and ADPRT. This enzyme exists in *Physarum* in two forms with relative molecular masses of 115000 and 75000, and the low molecular mass form is the main acceptor for auto-ADP-ribosylation.

MATERIALS AND METHODS

Materials

[U- ^{14}C -adenine] NAD^+ (290 $\mu\text{Ci}/\mu\text{mol}$) was obtained from Amersham International plc (Amersham, Bucks., U.K.). [U- ^{14}C -adenine] NAD^+ (555 $\mu\text{Ci}/\mu\text{mol}$) and [^{32}P]- NAD^+ (45 Ci/mmol) were from New England Nuclear (Boston, U.S.A.). NAD^+ , thymidine and hydroxyapatite were from Boehringer Mannheim (Mannheim, West Germany). Nicotinamide and dithiothreitol were from E. Merck (Darmstadt, West Germany). Phosphocellulose P11 was from Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.). 3-Aminobenzamide was from Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.) and benzamide from EGA-Chemie (Steinheim/Albuch, West Germany). DNA (calf thymus, type I; highly polymerized), deoxyribonuclease I (bovine pancreas, type II), hydroxyapatite (type I), phosphodiesterase I (*Crotalus atrox*, types IV and VII), histone (calf thymus, type V-S) and theobromine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydroxyurea was from Serva Feinbiochemica (Heidelberg, West Germany).

Culture techniques and isolation of nuclei

Physarum polycephalum, strain M_3b , a Wis 1 isolate was used. Microplasmodia were cultivated at 24 °C in submersed shake culture in semi-defined medium (Daniel & Baldwin, 1964).

Giant macroplasmodia (S-shaped) were prepared by coalescence of 3 ml of microplasmodial sediment on filter

Abbreviations used: ADPRT, ADP-ribosyltransferase; HMG, high mobility group; PMSF, phenylmethanesulphonyl fluoride.

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paper in bio-assay dishes as described by Affolter *et al.* (1979). For determination of mitosis (telophase) in ethanol-fixed smears, tiny plasmodial explants were taken from various positions within the plasmodium and inspected under phase contrast. Telophase occurred within 5 min for all plasmodium.

At selected time points between M2 and M3 explants ($\frac{1}{4}$ – $\frac{1}{2}$ of the macroplasmodium) were harvested and homogenized in a Waring blender. Nuclear isolation was performed in the presence of 0.2 mM-phenylmethanesulphonyl fluoride (PMSF) according to a published procedure (Nothacker & Hildebrandt, 1985) with modifications as described by Loidl & Gröbner (1987). Nuclei were counted in a haemocytometer.

ADP-ribosylation of nuclear proteins

Isolated nuclei (standard conditions; 2.5×10^8 /ml) were incubated in incubation buffer (15 mM-Tris/HCl (pH 7.3)/15 mM-MgCl₂/1 mM-CaCl₂/5 mM-EGTA/3 mM-dithiothreitol) and [¹⁴C]NAD⁺ (standard conditions; 10 μ M, 3 μ Ci/ml, 290 μ Ci/ μ mol) for 10 min. After incubation an aliquot was trichloroacetic acid-precipitated, washed and analysed for ¹⁴C radioactivity as described by Gröbner & Loidl (1985). SDS sample buffer (Laemmli, 1970) was added to the remaining incubation mixture and aliquots were subjected to electrophoretic analysis.

Gel electrophoresis

Activity gel. The frozen samples were thawed and aliquots containing 2.5×10^6 lysed nuclei run on an SDS/10%-polyacrylamide slab gel (6 cm) containing 100 μ g of calf thymus DNA/ml activated by the method of Loeb (1969). After electrophoresis SDS was removed and ADPRT reactivated according to Scovassi *et al.* (1984). The gel was incubated at 4 °C with reaction mixture containing [³²P]NAD⁺, washed and dried. Active bands were visualized by autoradiography using an intensifying screen.

Parallel gels were stained with Coomassie Blue and subjected to fluorography as described below.

Standard SDS/polyacrylamide gel. The frozen samples were thawed and heated for 3 min at 95 °C. Aliquots of 5×10^6 lysed nuclei (standard conditions) were run on an SDS/15%-polyacrylamide slab gel (12 cm long) as described by Laemmli (1970). Gels were stained with Coomassie Blue and after destaining fluorography was performed according to a published procedure (Laskey & Mills, 1975) using pre-flashed Kodak X-Omat RP film. Either commercially available non-histone molecular mass standards (Bio-Rad) or [³H]lysine-labelled *Physarum* histones were used as markers for molecular mass determination.

Separation and purification of ADPRT enzyme forms

The plasmodial pellet (70 g) from 40 shake cultures was homogenized in 250 ml of buffer A [100 mM-Tris/HCl (pH 7.3)/20% (w/v) glycerol/25 mM-K₂S₂O₈/12 mM- β -mercaptoethanol/5 mM-EDTA] containing 50 mM-KH₂PO₄, 0.5 M-KCl and 1 mM-PMSF with a Waring blender.

The homogenate was centrifuged at 14000 g for 10 min. The supernatant was filtered through cheesecloth and then shaken with 100 g (dry wt.) of hydroxyapatite (Boehringer Mannheim) for 60 min at 0 °C. The

suspension was centrifuged at 1600 g for 5 min and the hydroxyapatite pellet washed with 500 ml of buffer A containing 50 mM-KH₂PO₄ and 1 mM-PMSF. Elution was performed with 2×150 ml of buffer A containing 0.5 M-KH₂PO₄ and 1 mM-PMSF. The combined eluates were diluted to a final volume of 3.5 litres with buffer A and filtered by suction (0.2 litre/min) through a Buchner funnel containing 100 ml of settled phosphocellulose. The phosphocellulose was washed with 500 ml of buffer A. Elution was performed on the Buchner funnel in several portions by pouring buffer A containing 0.8 M-KCl in aliquots of 10 ml on to the phosphocellulose under suction. ADPRT was eluted in fractions 7 and 8.

The combined fractions 7 and 8 were diluted with the same volume of buffer B [15 mM-Tris/HCl (pH 7.3)/5 mM-EGTA/3 mM-dithiothreitol] and shaken with 1 g (dry wt.) of hydroxyapatite (Sigma) for 5 min at 0 °C. The suspension was centrifuged at 1600 g for 5 min and the pellet washed with 25 ml of buffer B containing 0.05 M-KH₂PO₄, 25 ml of buffer B containing 0.1 M-KH₂PO₄ and 25 ml of buffer B containing 0.15 M-KH₂PO₄. Elution of ADPRT was performed with 3×1.75 ml of buffer B containing 0.6 M-KH₂PO₄ and 10% (w/v) glycerol.

Of the combined hydroxyapatite eluates, 1 ml was chromatographed by gel filtration h.p.l.c. (Bio-Rad Bio-Sil-TSK-250, column 300 mm $\times$ 7.5 mm) in buffer B containing 0.05 M-KH₂PO₄ and 10% (w/v) glycerol (flow rate 0.6 ml/min). Fractions of 0.6 ml were collected.

Other methods

ADPRT enzyme activity was determined at 4 °C in 500 μ l of reaction mixture containing the enzyme preparation, 15 mM-Tris/HCl (pH 7.3), 15 mM-MgCl₂, 1 mM-CaCl₂, 5 mM-EGTA, 3 mM-dithiothreitol, 10 μ g of histone, 10 μ g of activated DNA (Loeb, 1969) and 5 μ M-[¹⁴C]NAD⁺ (40 μ Ci/ μ mol, 0.1 μ Ci/tube) as described by Gröbner & Loidl (1985). Product analysis after digestion with purified phosphodiesterase I by t.l.c. was performed as described in detail by Burtcher *et al.* (1987). Protein analysis was carried out as described by Lowry *et al.* (1951). Extraction of HMG-like proteins from isolated nuclei followed published procedures (Goodwin *et al.*, 1973; Bhullar & Candido, 1981). Fluorograms and autoradiograms were evaluated by densitometry using a Beckman DU-8B spectrophotometer with gel-scanning equipment.

RESULTS

Identification of ADPRT forms and their automodification

We always observed two enzyme forms when a cellular homogenate (results not shown) or a nuclear lysate was analysed on activity gels. Enzyme form A has a molecular mass of 115000 and enzyme form B of 75000. Analysis of this nuclear preparation (preparation time 1 h) after further incubation for 1, 3 and 5 h at 4 °C revealed that the ratio of the enzyme forms (A:B approx. 3:1) remained unchanged (Fig. 1a, lanes 1–4); there was no decrease in total activity during this incubation as revealed by densitometric evaluation.

As an additional control to make sure that form B is not derived from an unspecific proteolysis of form A, a cellular homogenate was incubated for 1 and 3 h at 4 °C and nuclei were subsequently isolated. The activity gel

Fig. 1. Identification of ADPRT enzyme forms and their automodification

Nuclei were isolated from a plasmodial homogenate and aliquots of the nuclei (2.5×10^6) were analysed on activity gels after incubation for 0, 1, 3 and 5 h at 4 °C (lanes 1–4). For lanes 5 and 6 the plasmodial homogenates were incubated for 1 and 3 h at 4 °C and nuclei isolated thereafter (a). Isolated nuclei were incubated with non-radioactive 100 μ M-NAD⁺ for 0, 0.5, 5, 10, 30 and 60 min (lanes 1–6) and analysed on activity gels (b).

(Fig. 1a, lane 1 for 0 h, lane 5 for 1 h, lane 6 for 3 h) shows that the ratio of enzyme forms A to B remains constant, although the total activity was slightly decreased. There was no increase in band B due to a decrease of A.

Fig. 2. H.p.l.c. separation of ADPRT and enzyme forms

Hydroxyapatite eluate (1 ml) (see Table 1) was subjected to gel filtration h.p.l.c. as described in the Materials and methods section. Fractions (0.6 ml) were collected and analysed for ADPRT activity (○); the eluted protein was scanned by absorbance at 280 nm (●).

When isolated nuclei were incubated with 100 μ M-NAD⁺ for various time periods up to 60 min, the ADPRT enzyme form A (M_r 115000) remained almost unaffected on activity gels. In contrast, the activity of enzyme form B (M_r 75000) decreased with the length of incubation and exhibited a shift to higher molecular mass indicating intensive poly-ADP-ribosylation (Fig. 1b).

Preparative separation of ADPRT enzyme forms

To investigate further the two enzyme forms it was necessary to separate them on a preparative scale. We achieved a separation of the forms A and B in four steps with resulting 53- and 171-fold purification respectively (Table 1). To avoid proteolytic degradation we included potassium bisulphite and PMSF as protease inhibitors during the initial two purification steps. The maximum duration of the whole procedure was 4 h. After adsorption on phosphocellulose and hydroxyapatite the enzyme forms were separated by gel filtration h.p.l.c. and ADPRT activity of each fraction was determined (Fig. 2). H.p.l.c. fractions 10 and 11 (A), 14 and 15 (B), respectively, were combined, and frozen at –30 °C. No

Table 1. Separation and purification of ADPRT enzyme forms

70 g of plasmodial pellet was used as starting material and processed as described in detail in the Materials and methods section.

Step	Volume (ml)	Total protein (mg)	Total activity (μunits)	Specific activity (μunits/mg of protein)	Purification (-fold)	Yield (%)
Homogenate	320.0	1008.00	19400	19	1	100.0
Phosphocellulose	24.0	25.00	18400	735	37	94.8
Hydroxyapatite	5.5	11.30	10800	960	51	55.6
H.p.l.c.*						
115000- M_r fraction (A)	6.6	2.18	2200	1007	53	11.3
75000- M_r fraction (B)	6.6	0.62	2000	3225	171	10.3

* H.p.l.c. was performed with 1 ml of the hydroxyapatite fraction, giving 1.2 ml each of fractions A and B. One of these repeated h.p.l.c. runs was multiplied by a factor of 5.5 to obtain the values in the Table.

Table 2. Kinetic properties of ADPRT enzyme forms

Purified ADPRT enzyme forms A and B (20 μ l of h.p.l.c. fraction; for preparation see Table 1) were incubated under different conditions for 10 min. Enzyme activity measured under standard conditions as described in the Materials and methods section was taken as 100 %.

Assay conditions	Relative activity (%)	
	A	B
Standard assay 4 °C	100	100
10 °C	80	80
20 °C	50	50
25 °C	25	25
37 °C	< 2	< 2
–DNA	< 2	< 2
+ 50 μ M-Theobromine	65	37
+ 50 μ M-Thymidine	61	29
+ 50 μ M-Benzamide	43	9
+ 50 μ M-Aminobenzamide	47	9
+ 50 μ M-Nicotinamide	50	24

decrease in activity of both fractions occurred even after repeated thawing.

Characterization of ADPRT enzyme forms

Molecular mass was determined both by SDS/polyacrylamide gel electrophoresis and by h.p.l.c. to be 115000 for enzyme form A and 75000 for B. The pH-dependence exhibited a broad maximum between pH 7 and 8.5 for both enzyme forms. The K_m for NAD⁺ was found to be 30 μ M for both forms. The time course of the enzyme reaction was identical for both forms and resembles the kinetics measured in isolated nuclei (compare with Fig. 3a). Both forms require DNA as a cofactor. The temperature-dependence in the enzyme assay was the same for the two forms and the maximum activity was exhibited at 4 °C. The activity declined with increasing temperature; at 37 °C no activity could be detected. The two enzyme forms differed with respect to their behaviour towards a variety of enzyme inhibitors. Enzyme form B was more strongly inhibited by various inhibitors in comparison with form A (Table 2).

ADP-ribosylation in isolated nuclei

When isolated nuclei were incubated with 10 μ M-[¹⁴C]-NAD⁺ at 4 °C ADP-ribose incorporation followed a hyperbolic curve as shown in Fig. 3a. After lysis of nuclei the analysis of SDS/polyacrylamide gels after fluorography revealed ADP-ribose incorporation into predominantly five bands with comparable kinetics (Fig. 3b, lanes 1–6). If the nuclei, which had been incubated for 15 min, were incubated for another 15 min at 25 °C (low enzyme activity at this temperature) in the absence or presence of 2 mM-nicotinamide (inhibitor of ADPRT), the radioactive label decreased in all five bands (Fig. 3b, lanes 7 and 8). If the nuclei were incubated under the same conditions with phosphodiesterase and nicotinamide, > 90 % of the incorporated radioactivity disappeared (Figs. 3a and 3b, lane 9).

The radioactivity incorporated during a 15 min incubation with [¹⁴C]NAD⁺ can also be removed by subsequent incubation with 1 M-hydroxylamine, pH 7.0. After 15 min

Fig. 3. Incorporation and stability of ADP-ribose in isolated nuclei

Nuclei were incubated with 10 μ M-[¹⁴C]NAD⁺ ((290 μ Ci/ μ mol) at 4 °C. At various times aliquots (5×10^8 nuclei) were analysed for ADP-ribose incorporation (a) by measuring radioactivity in trichloroacetic acid-precipitated nuclei (●), or (b) by fluorographic evaluation of a standard SDS/polyacrylamide gel; lanes 1–6: 0.3, 1, 2.5, 5, 10 and 15 min incubation with [¹⁴C]NAD⁺. After 15 min incubation with [¹⁴C]NAD⁺ at 4 °C, aliquots of the assay mixture were incubated for another 15 min at 25 °C (○; lane 7), in the presence of 2 mM-nicotinamide (□; lane 8), after addition of 1 M-hydroxylamine, pH 7.0 (△) and with 2 munits phosphodiesterase/ml (▲; lane 9).

incubation with hydroxylamine 72 % of the radioactivity disappeared, 81 % after 1 h and 90 % after 2 h (Table 3b). Comparing the radioactivity incorporation after 10 min incubation with [¹⁴C]NAD⁺ (Fig. 4, lane 1) and after an additional 1 h incubation with hydroxylamine (Fig. 4, lane 4) shows a pronounced decrease in label in all bands.

The same result was obtained when nuclei (after 15 min incubation with 10 μ M-[¹⁴C]NAD⁺) were precipitated with 20 % trichloroacetic acid, washed and the pellet was incubated for 1 h at 37 °C with 1 M-hydroxylamine, pH 7.0. During this incubation $\frac{2}{3}$ of the incorporated radioactivity was removed. As a control we incubated the pellet for 1 h with distilled water. No radioactivity was removed. Incubation with 0.1 M-NaOH removed the incorporated radioactivity completely (Table 3a).

Bauer *et al.* (1986) and Huletsky *et al.* (1985) could demonstrate a pronounced dependence of the ADP-

Table 3. Stability of ADP-ribose-protein linkages in the presence of hydroxylamine

Isolated nuclei (1×10^7) were incubated under standard conditions at 4 °C for 15 min with 10 μ M- or 0.5 μ M- [14 C]NAD⁺ as described in the Materials and methods section. Incorporated radioactivity in the trichloroacetic acid-precipitated nuclear pellet was taken as 100%. (a) The precipitated pellet was incubated under different conditions and the residual radioactivity in the pellets expressed as % of the control. (b) After 15 min incubation with [14 C]NAD⁺, 1 M-NH₂OH (pH 7.0) was added to the incubation mixture and after various times aliquots were trichloroacetic acid-precipitated and the residual radioactivity was measured in the pellet and related to the control.

Conditions	Residual radioactivity (%)	
	10 μ M-NAD ⁺	0.5 μ M-NAD ⁺
Control: Pellet after 15 min incubation with NAD ⁺ (4 °C)	100	100
(a) Control pellet incubated with:		
Water (37 °C), 1 h	100	100
1 M-NH ₂ OH (37 °C), 1 h	37	39
0.1 M-NaOH (37 °C), 1 h	< 2	< 2
Phosphodiesterase I (0.1 unit) (37 °C), 12 h*	< 2	< 2
(b) After 15 min incubation with NAD ⁺ (4 °C) 1 M-NH ₂ OH (37 °C) was added for:		
0.25 h	28	22
1 h	19	17
2 h	10	11

* The digestion products were analysed by t.l.c. and found to be 85% 5'-AMP and 15% 2'-(5"-phosphoribosyl)-5'-AMP.

ribose incorporation on the NAD⁺ concentration in mammalian cells; low NAD⁺ concentrations preferentially lead to the initiation of modification, i.e. mono-ADP-ribosylation, whereas higher NAD⁺ concentrations predominantly cause poly-ADP-ribosylation. In *Physarum* we did not observe differences between incubation with 10 μ M-, 0.5 μ M- and 0.1 μ M-NAD⁺ (Fig. 4, lanes 1–3). The same proteins, namely core histones, a band with M_r 45000 and a band with M_r 75000, were preferentially ADP-ribosylated. Since the ADP-ribosylated histones only exhibit a minor shift to higher molecular mass (compare with Fig. 7a) at all NAD⁺ concentrations tested (0.1–10 μ M), this indicates predominantly mono-ADP-ribosylation. The chemical stability of the incorporated ADP-ribose residues is the same for 0.5 μ M- and 10 μ M-NAD⁺ (Table 3).

The ADP-ribose residues incorporated in isolated nuclei are sensitive to phosphodiesterase and the product analysis for both investigated NAD⁺ concentrations (0.5 and 10 μ M) revealed 85% to be 5'-AMP and 15% 2'-(5"-phosphoribosyl)-5'-AMP (Table 3). The average chain length was calculated to be 1.2 ADP-ribose residues.

To characterize further the ADP-ribose incorporation we treated isolated nuclei with various concentrations of DNAase I and subsequently incubated these nuclei with 5 μ M-[14 C]NAD⁺. Fluorography of SDS/polyacrylamide

Fig. 4. Dependence of ADP-ribosylation on NAD⁺ concentration in isolated nuclei

Nuclei were incubated with 10 μ M-[14 C]NAD⁺ (290 μ Ci/ μ mol), lane 1, 0.5 μ M-NAD⁺ (555 μ Ci/ μ mol); lane 2, 0.1 μ M-NAD⁺ (555 μ Ci/ μ mol); lane 3, for 10 min at 4 °C. In lane 4 the assay mixture of lane 1 was incubated for a further 1 h with 1 M-hydroxylamine, pH 7.0 at 37 °C. 5×10^6 nuclei (lanes 1 and 4) or 1×10^7 nuclei (lanes 2 and 3) were analysed on standard SDS/polyacrylamide gels with subsequent fluorography.

gels of these samples shows that the overall incorporation is enhanced in all main acceptor protein bands with increasing DNAase I concentrations (Fig. 5).

To identify most of the proteins in SDS/polyacrylamide gels that are modified by ADP-ribosylation, we also extracted HMG-like proteins (Coté *et al.*, 1985) and analysed them for ADP-ribosylation. We could show that besides H1, which is co-extracted with the HMG-like proteins, all four HMG-like proteins (AS1–4) are ADP-ribosylated (Fig. 6).

It was then possible to designate the main acceptor proteins for ADP-ribose in isolated nuclei (see Fig. 3b and also Fig. 7a). In the low molecular mass range these are the core histones H4, H3/H2B and H2A and the HMG-like proteins AS3 and AS4, which migrate in the region of core histones in SDS/polyacrylamide gels. Histones H3 and H2B are not separated on SDS/polyacrylamide gels, due to their almost identical molecular mass, but electrophoresis on acid/urea/Triton-gels (Zweidler, 1978; this gel separates H3 and H2B), with subsequent fluorography showed that H3 as well as H2B are both ADP-ribosylated (results not shown). A heavily labelled band with an apparent molecular mass of 45000 is likely to be AS2. H1, which has an apparent molecular weight of 54000 in *Physarum*

Fig. 5. Effect of DNAase I incubation of nuclei on ADP-ribosylation

Isolated nuclei (1×10^7 in $40 \mu\text{l}$ incubation buffer) were preincubated with increasing DNAase I concentrations at 4°C for 5 min. Subsequently these preparations were incubated with $5 \mu\text{M}$ - ^{14}C NAD $^+$ ($290 \mu\text{Ci}/\mu\text{mol}$) under standard conditions for 10 min and analysed by fluorography of a standard SDS/polyacrylamide gel.

Fig. 6. ADP-ribosylation of HMG-like proteins

Isolated nuclei (4×10^8) were incubated with $10 \mu\text{M}$ - ^{14}C NAD $^+$ ($290 \mu\text{Ci}/\mu\text{mol}$) for 10 min at 4°C . HMG-like proteins were extracted and analysed by fluorography of a standard SDS/polyacrylamide gel.

Fig. 7. ADP-ribosylation of nuclear proteins during the cell cycle

Nuclei from various stages of the cell cycle (0.3–8 h after mitosis 2; 8 h after M2 = 1 h before M3) were isolated from macroplasmidia and incubated for 10 min at 4°C with $10 \mu\text{M}$ - ^{14}C NAD $^+$ ($290 \mu\text{Ci}/\mu\text{mol}$). Nuclear lysates of 5×10^6 nuclei each were analysed by fluorography of a standard SDS/polyacrylamide gel. ^3H Lysine-labelled *Physarum* histones were used as an internal marker (a). In addition, samples (2.5×10^6 nuclei) were analysed on activity gels. One gel was re-activated for enzyme activity determination (b). A parallel gel was subjected to fluorography (c).

Fig. 8. Effect of hydroxyurea on ADP-ribosylation

Macrophasmodia in S-phase (0.3 h after M2) or G₂-period (5 h after M2) were grown for up to 1 h in medium containing 50 mM-hydroxyurea. At various times (S-phase, 0, 0.25 and 1 h; G₂-period, 0 and 1 h) nuclei were isolated and incubated with 10 μ M-[¹⁴C]NAD⁺ (290 μ Ci/ μ mol) for 10 min at 4 °C. Aliquots (5×10^6 nuclei) were analysed for ADP-ribose incorporation by measuring radioactivity in trichloroacetic acid-precipitated nuclei; each 0 h value was set at 100 % (a). In parallel aliquots were analysed by fluorography of a standard SDS/polyacrylamide gel (b).

(Fischer & Laemmli, 1980), is also ADP-ribosylated (Fig. 6), but in a nuclear lysate it exhibits only weak label compared with the core histones. The twin-band in the high molecular mass range at approx. 75000 and 115000 most likely resembles the ADPRT enzyme forms A and B. It is again obvious that enzyme form B is more heavily ADP-ribosylated than enzyme form A.

ADP-ribosylation during the cell cycle

Nuclei from various stages of the cell cycle (0.3–8 h after mitosis 2) were incubated in the presence of 10 μ M-[¹⁴C]NAD⁺. Nuclear lysates were analysed on standard SDS/polyacrylamide gels with subsequent fluorography (Fig. 7a) and densitometric analysis of the core histone region. In all stages of the cell cycle the same proteins are ADP-ribosylated. The ADP-ribose incorporation into core histones rises during S-phase (until 3 h after mitosis 2; *Physarum* lacks a G₁-period) to reach a maximum in early to mid G₂-period (4–6 h after mitosis 2). In late G₂ a decrease of the ADP-ribose incorporation was observed.

Analysis of the same samples (lysates of 2.5×10^6 nuclei) on activity gels revealed the presence of both enzyme forms A and B during the entire cell cycle with an almost constant A/B ratio of 3:1 (Fig. 7b). In early S-phase the total enzyme activity is still low, whereas in mid S-phase maximum activity is reached. When such an activity gel was analysed by fluorography, instead of reactivation of enzyme activity, a high ADP-ribose incorporation was observed at the position of enzyme form B (M_r 75000; Fig. 7c). In the position of enzyme form A (M_r 115000) we did not observe a pronounced ADP-ribosylation. This finding again shows that enzyme form B is preferentially ADP-ribosylated. This

automodification of form B does not seem to display cell cycle dependent fluctuations, since the ratio of enzyme activity to ADP-ribose incorporation remains constant throughout the entire cell cycle (compare Figs. 7b and c).

In addition we have grown synchronous S-phase macroplasmodia in the presence of the DNA-synthesis inhibitor hydroxyurea. Subsequently nuclei were isolated and incubated for 10 min with 10 μ M-[¹⁴C]NAD⁺. ADP-ribose incorporation decreased considerably within 1 h of hydroxyurea treatment (Fig. 8a). Analysis of the nuclear lysates by SDS/polyacrylamide gel electrophoresis with subsequent fluorography revealed that the ADP-ribose incorporation into all nuclear acceptor proteins is diminished to the same extent (Fig. 8b). When plasmodia were grown on hydroxyurea in G₂-period, neither the amount of incorporated ADP-ribose nor the distribution to the various nuclear proteins in SDS/polyacrylamide gels was affected (Fig. 8).

DISCUSSION

The present report demonstrates that in the lower eukaryote *Physarum polycephalum* nuclear ADPRT exists in two enzyme forms with molecular masses of 115000 and 75000. These two enzyme forms could best be detected in activity gels at 4 °C, since the enzyme is inactive at 37 °C. This fact might have been the reason why Scovassi *et al.* (1986) found ADPRT activity in higher eukaryotes, but not in a number of lower eukaryotes, that were scanned for ADPRT in activity gels at 37 °C. Maximum activity at lower temperatures could be a common feature of ADPRT in lower eukaryotes.

Nuclear ADPRT from mammalian cells has a molecular mass of around 115000. In HeLa cells Scovassi *et al.* (1986) demonstrated a single active enzyme band at M_r 115000; immunoblot analysis revealed a number of lower molecular mass bands apart from the 115000 form. These had no enzymic activity. It has already been shown by Holtlund *et al.* (1983) that enzymically inactive fragments of ADPRT from calf thymus were generated by proteolytic degradation; these fragments were efficient acceptors of ADP-ribose. Limited proteolysis with different proteases yielded three distinct degradation fragments for ADPRT (Kameshita *et al.*, 1984, 1985, 1986); the first of 54 kDa possesses the NAD^+ binding site, the second of 46 kDa contains the DNA binding site, and the third of 22 kDa contains the acceptor sites for ADP-ribose. The isolated fragments did not exhibit enzymic activity. This indicates that these proteolytically generated fragments have no functional significance *in vivo*. Furthermore, enzymically inactive endogenous proteolytic fragments (M_r 80000 and 64000) were seen. These fragments served as acceptors for ADP-ribose. The authors proposed a biologically significant function of these fragments in nuclear metabolism. Interestingly in amphibians and gastropods, active ADPRT forms with lower molecular mass have been reported (Scovassi *et al.*, 1986). An active form with M_r 75000 is also present in trout (Burtscher *et al.*, 1987), which also shows maximum activity at lower temperatures.

In *Physarum* two distinct enzyme forms exist *in vivo*. We could show that there is no unspecific conversion of the high molecular mass form to the low molecular mass form *in vitro*. Given the assumption that the enzyme form with M_r 75000 is derived from the high molecular mass form, ADPRT is probably regulated by highly specific proteolysis *in vivo*. Such a highly specific proteolysis has already been reported for another chromatin-associated enzyme, DNA topoisomerase (Liu & Miller, 1981). The low molecular mass ADPRT form could have a specific function as the main acceptor for poly-ADP-ribose. The different behaviour of the two enzyme forms towards inhibitors could have a distinct, but yet unknown, biological function.

As already shown by Poirier *et al.* (1987) the pattern of ADP-ribosylation does not change within a wide range of NAD^+ concentrations (0.1–10 μM) in isolated nuclei of *Physarum*. In this respect *Physarum* differs from other systems for which correlation between the mode of ADP-ribosylation (mono- or poly-adducts) and increasing NAD^+ concentrations within this μM range was found (Huletsky *et al.*, 1985; Kirsten *et al.*, 1985; Bauer *et al.*, 1986). Within 2 h 90% of the *Physarum* ADP-ribose adducts are cleaved by neutral hydroxylamine. However, the kinetics of hydroxylamine cleavage of the ADP-ribose protein bonds obviously follows two time courses. Approx. 70% of the bonds are cleaved within 15 min, indicating 'carboxylate ester-linked' ADP-ribose residues; these are known to have a short half-life ($t_{1/2}$ approx. 5 min). A further 20% of the bonds are cleaved within 2 h, indicating 'arginine-linked' ADP-ribose residues, with a half-life of approx. 1.5 h (Payne *et al.*, 1985). These results prove that our experiments deal with a specific ADP-ribose incorporation, not with the non-enzymic formation of Schiff's bases, which are stable towards hydroxylamine with a half-life of a couple of hours.

We could identify core histones, H1, ADPRT and the HMG-like proteins AS1–4 as the main acceptor proteins for ADP-ribosylation. Among these proteins the ADPRT enzyme form with M_r 75000 and the core histones exhibit the most pronounced modification. This partly contradicts a recent report (Poirier *et al.*, 1987) where H1, H2A and H2B were found to be the main ADP-ribose acceptors in *Physarum* amoebae. These authors could not detect ADP-ribose incorporation into ADPRT. Their failure to observe ADPRT as acceptor could be explained by the fact that the authors only looked for ADP-ribose incorporation at M_r 115000, since they did not know that *Physarum* ADPRT exists in two forms, the low molecular mass form being the main acceptor for automodification.

Adolph (1985) showed that all core histones are ADP-ribosylated during the entire cell cycle with a constant distribution of label among the individual histone species. He found maximum ADP-ribose incorporation during mid and late S-phase in HeLa cells. In contrast, Kidwell & Mage (1976) reported a biphasic pattern for the HeLa cell cycle with high incorporation during S-phase, but a second even more pronounced elevation at the S–G₂-transition. Other investigators observed maximum ADP-ribose incorporation in HeLa cells into metaphase chromosomes (Tanuma *et al.*, 1986). These conflicting results within the same experimental system are obviously caused by differences in experimental protocols.

It therefore seems necessary to investigate such cell cycle phenomena in a naturally synchronous organism, like the myxomycete *Physarum polycephalum*. In this organism we saw maximum ADP-ribose incorporation into isolated nuclei in early- to mid-G₂-period. Since ADPRT has been induced by the time of mitosis and reaches maximum activity in early- to mid-S-phase (Gröbner & Loidl, 1985), maximum ADP-ribose incorporation in G₂-period cannot be attributed to an elevation of enzyme activity in nuclei. The interesting result, that hydroxyurea inhibits ADP-ribosylation in S-phase but not in G₂-period, indicates that ADP-ribosylation has some function in DNA replication. The synchronous *Physarum* cell cycle offers the advantage that phase-specific observations can be attributed to certain biochemical events. In G₂-period, for instance, no DNA synthesis occurs, but the bulk of transcription takes place. Therefore it may well be that maximum ADP-ribosylation in G₂-period is somehow involved in the process of transcription.

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