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ADP-ribosylation of core histones and their acetylated subspecies

Georg GOLDERER and Peter GRÖBNER*
Institut für Medizinische Chemie und Biochemie der Universität, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria

ADP-ribosylation of core histones was investigated in isolated nuclei of *Physarum polycephalum*. Core histone species differed in the mode of modification. Whereas ADP-ribosylation of H2A and H2B is sensitive to inhibition by 3-methoxybenzamide, as with most other nuclear acceptor proteins, the modification of H3 and H4 is not inhibited. Cleavage experiments with hydroxylamine indicate a carboxylate ester type ADP-ribose–protein bond for H2A and H2B and arginine-linked ADP-ribose residues for H3 and H4. ADP-ribosylation preferentially occurs on acetylated histone subspecies, as shown for H4. These data are substantiated by the use of n-butyrate, which induces hyperacetylation of core histones; the butyrate-induced shift towards more acetylated H4 subspecies is accompanied by an increase of ADP-ribose incorporation into highly acetylated H4 subspecies.

INTRODUCTION

Core histones are subject to a number of post-translational modifications, among which ADP-ribosylation has attracted considerable interest, since it has been implicated in important nuclear processes, such as DNA repair, DNA replication and transcription (for review see Althaus & Richter, 1987). The ADP-ribosylation reaction involves the transfer of the ADP-ribose moiety of NAD^+ to nuclear acceptor proteins, resulting in mono(ADP-ribose) up to highly branched poly(ADP-ribose) residues.

We have studied ADP-ribosylation of nuclear proteins in the myxomycete *Physarum polycephalum*. In macroplasmodia of *Physarum*, mitosis occurs in a spontaneously synchronous manner. For this reason *Physarum* has become an attractive system in which to study cell-cycle-dependent events. The natural synchronous cell cycle of macroplasmodia (8–10 h duration) consists of the S-phase (3 h), which immediately follows after mitosis, and a G_2-period; no G_1-period has been detected in *Physarum* macroplasmodia. We have shown that ADP-ribosyltransferase activity fluctuates during the cell cycle of *Physarum polycephalum*, with a maximum in the S-phase (Gröbner & Loidl, 1985). On the other hand, maximum incorporation of ADP-ribose into nuclear acceptor proteins is found in the early G_2-period (Golderer *et al.*, 1988) using isolated *Physarum* nuclei as an *in vitro* system.

Little information is available on the parallel occurrence of different post-translational histone modifications. Tanigawa *et al.* (1983) showed that the ADP-ribosylation of H1 and core histones caused a significant depression of phosphorylation. Smulson and coworkers (Malik & Smulson, 1984; Wong & Smulson, 1984) reported that acetylated core histones may also be subject to ADP-ribosylation; Boulikas and coworkers demonstrated that in dimethyl sulphate-treated mouse myeloma cells, acetylated H4 subspecies are predominantly tri- and tetra-(ADP-ribosyl)ated (Boulikas, 1988) and that in activated human lymphoid cells the number of ADP-ribose groups on H4 equals or exceeds by one the number of acetyl groups (Boulikas *et al.*, 1990). DNA strand breaks induce the formation of poly(ADP-ribosyl)ated species of histones (Boulikas, 1989), whereas in the absence of DNA strand breaks histones are mono(ADP-ribosyl)ated.

The aim of this study was to investigate the regulation of histone ADP-ribosylation and to decide whether acetylation and ADP-ribosylation interact with each other in a concerted way. We showed that acetylated H4 subspecies are the preferential substrates for ADP-ribosylation.

MATERIALS AND METHODS

Microplasmodia of *Physarum polycephalum* (strain M3b, a Wis 1 isolate) were maintained in submerged shake culture in semi-defined medium (Daniel & Baldwin, 1964), supplemented with 0.013% haemoglobin instead of haematin. Disc-shaped macroplasmodia (2–5 cm diameter) were prepared by coalescence of exponentially growing microplasmodia and cultivated in Petri dishes on filter paper supported by glass beads.

Nuclei were isolated according to a published procedure (Nothacker & Hildebrandt, 1985), with the modifications outlined elsewhere (Loidl & Gröbner, 1987*a*).

Isolated nuclei (2×10^8) were incubated in 500 μl of incubation buffer (15 mM-Tris/HCl, pH 7.3, 15 mM-$MgCl_2$, 1 mM-$CaCl_2$, 5 mM-EGTA and 3 mM-dithiothreitol) containing [^{14}C]NAD^+ (4 μM, 2 μCi/ml, 500 μCi/μmol) at 4 °C for 15 min in the presence or the absence of 50 μM-3-methoxybenzamide.

After incubation, portions of 10^7 nuclei each were precipitated with 30% (w/v) trichloroacetic acid, then washed three times with cold 20% trichloroacetic acid and three times with cold ethanol. The pellets were resuspended in 1 M-NH_2OH (pH 7.0) and incubated for 0, 0.25, 2 or 4 h at 37 °C. Thereafter the samples were again precipitated with trichloroacetic acid and washed, and the residual radioactivity in the pellet was measured. For gel electrophoresis, the pellets were dissolved in 100 μl of SDS sample buffer (Laemmli, 1970) and 50 μl samples were run on an SDS/15%-polyacrylamide slab gel and subjected to fluorography as described by Laskey & Mills (1975).

Histones were extracted after incubation with [^{14}C]NAD^+ from 10^8 nuclei according to the procedure of Mohberg & Rusch (1969), and precipitated using novobiocin (Loidl & Gröbner, 1986). Histones were analysed on acid/urea/Triton gels (Zweidler, 1978); proteins were blotted on to nitrocellulose sheets and stained with Amido Black. The nitrocellulose sheets were subjected to autoradiography using pre-flashed Amersham Hyperfilm-MP. Protein bands were evaluated for radioactivity by slicing the gel as described (Smolarz *et al.*, 1988).

After incubation with [^{14}C]NAD^+, nuclei of macroplasmodia were directly analysed by SDS/15%-polyacrylamide slab gel electrophoresis with subsequent fluorography.

* To whom correspondence should be addressed.

All experiments shown in the Figures and Tables were repeated two or more times.

RESULTS

Inhibition of ADP-ribosylation by 3-methoxybenzamide in isolated nuclei followed a dose-dependent mode, yielding a maximum inhibition of 80% at a concentration of 50 μM. The remaining 20% of total ADP-ribosylation was resistant to 3-methoxybenzamide, even at concentrations up to 10 mM. 3-Methoxybenzoic acid served as a control, and did not affect ADP-ribosylation (Fig. 1). Other ADP-ribosyltransferase inhibitors, such as benzamide and 3-aminobenzamide, exerted the same effect as 3-methoxybenzamide; treatment with these inhibitors also resulted in approx. 20% residual activity (Table 1). The ADP-ribosylation reaction in the presence and absence of 3-methoxybenzamide was extremely sensitive to heat treatment; even a 15 min preincubation of isolated nuclei at 40 °C decreased the ADP-ribosyltransferase activity to less than 20% (Table 1).

The inhibitor-resistant ADP-ribosylation was not evenly distributed among nuclear acceptor proteins, as shown for 3-methoxybenzamide. Most of the remaining label was due to ADP-ribosylation of histones H3 and H4, which was completely unaffected by 3-methoxybenzamide. This phenomenon was independent of the cell cycle stage (Fig. 2). The ADP-ribosylation of H2A and H2B under these conditions was almost completely inhibited (Fig. 2; see also Fig. 4*b*). We could confirm our previous results (Golderer *et al.*, 1988) that the total ADP-ribose incorporation was considerably higher in the G_2-phase in comparison with the S-phase (Fig. 2). The strong inhibition by 3-methoxybenzamide also affects the 75 kDa ADP-ribosyltransferase enzyme form B, which is the main substrate for automodification (Golderer *et al.*, 1988).

To investigate the nature of the bond, we cleaved the ADP-ribose–protein bonds with neutral hydroxylamine. In controls, which had not been treated with 3-methoxybenzamide, more than 60% of the ADP-ribose was released within 15 min. In contrast, after 3-methoxybenzamide inhibition the remaining ADP-ribose adducts were more resistant with respect to hydroxylamine hydrolysis, with a half-life of approx. 1.5 h (Fig. 3). Since the ADP-ribosylation remaining after 3-methoxybenzamide inhibition is almost exclusively due to modification of histones H3 and H4, we conclude that this ADP-ribose–protein bond is chemically different from that in H2A and H2B.

Our main interest was to study whether acetylated core histone subspecies differ in their degree of ADP-ribosylation. This can be investigated by electrophoretic analysis on acid/urea/Triton gels; in this gel system, H4 splits into five separate bands, mainly due to the decrease of positive charges (Panyim & Chalkley, 1969; Zweidler, 1978), corresponding to non-acetylated up to tetra-acetylated subspecies (Fig. 4*a*). The designation of ADP-ribosylated H4 subspecies was based on the following criteria. ADP-ribosylated subspecies migrate more slowly on acid/urea/Triton gels (Boulikas *et al.*, 1990), due to the decrease in the net positive charge of H4. Histones are mono(ADP-ribosyl)ated in the absence of DNA strand breaks (Boulikas, 1989). We also found more than 90% of ADP-ribosylation as mono(ADP-ribose) in histones in the presence and absence of 3-methoxybenzamide (result not shown). In addition, the sharp H4 band in fluorograms of SDS/polyacrylamide gels (Fig. 2, lanes 2; Fig. 3, inset) is also a strong indication for only mono(ADP-ribosyl)ation of H4; since one ADP-ribose residue increases the M_r of H4 by approx. 500, a multiple ADP-ribosylation would also be clearly seen by a band shift to higher M_r values.

The average distribution of acetylated subspecies has been determined as follows (Loidl *et al.*, 1983): non-acetylated, 31%;

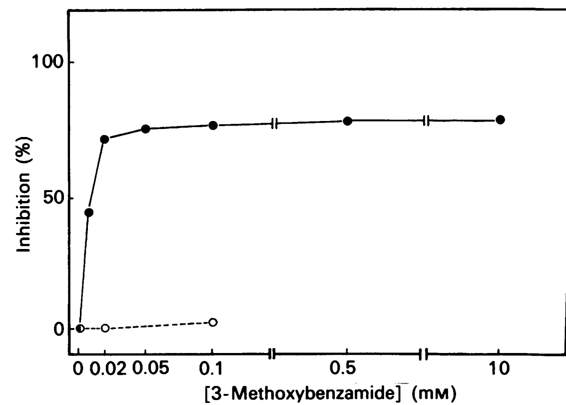

Fig. 1. Inhibition of ADP-ribosylation by 3-methoxybenzamide in isolated nuclei

Different concentrations (up to 10 mM) of 3-methoxybenzamide were added during incubation of nuclei with [^{14}C]NAD$^+$, as described in the Materials and methods section. Inhibition of ADP-ribosylation (●) was calculated in relation to an untreated control. As an additional control, 3-methoxybenzoic acid was added during incubation (○).

Table 1. Effect of heat or inhibitors on ADP-ribosylation in isolated nuclei

Nuclei were preincubated for 15 min at different temperatures, and then 10^7 nuclei were incubated for 15 min with [^{14}C]NAD$^+$, as described in the Materials and methods section. Furthermore, different inhibitors were added during incubation of nuclei with [^{14}C]NAD$^+$.

Incubation conditions (15 min)		Relative activity (%)			
	Preincubation at:	4 °C	40 °C	60 °C	80 °C
4 °C		100*	19	5	< 2
40 °C		< 2	–	–	–
4 °C + 5 mM-3-Methoxybenzamide		23	4	< 2	< 2
4 °C + 5 mM-Benzamide		20	–	–	–
4 °C + 5 mM-3-Aminobenzamide		24	–	–	–

* Identical value was determined without preincubation of nuclei.

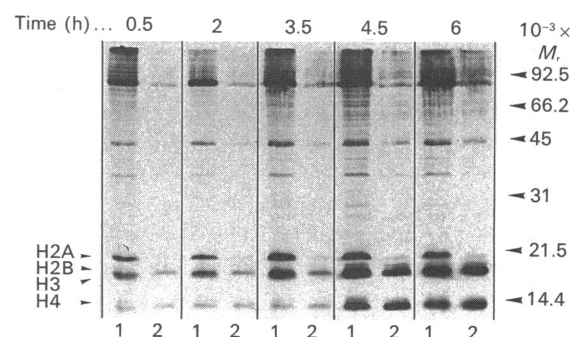

Fig. 2. Inhibition of ADP-ribosylation with 3-methoxybenzamide during the cell cycle *in vitro*

Macroplasmodia were harvested at various stages of the cell cycle (0.5–6 h after mitosis 2); thereafter nuclei were isolated and incubated with 4 μM-[^{14}C]NAD$^+$ at 4 °C for 15 min in the absence (lanes 1) or in the presence (lanes 2) of 50 μM-3-methoxybenzamide. Aliquots (5×10^6 nuclei) were analysed by fluorography of SDS/15%-polyacrylamide slab gels.

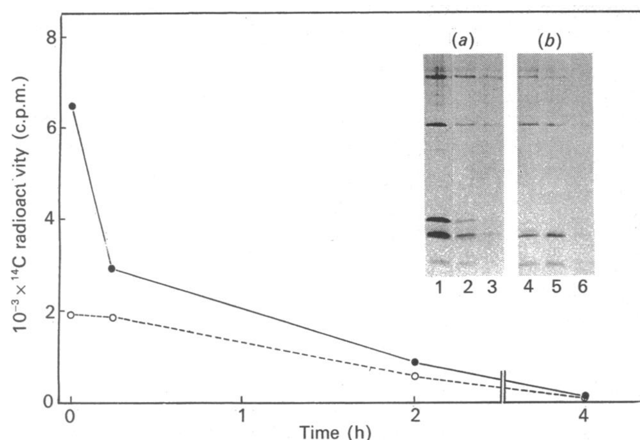

Fig. 3. Stability of ADP-ribose–protein linkages in the presence of hydroxylamine

Isolated nuclei of microplasmodia were incubated with 4 μM-[^{14}C]NAD$^+$ at 4 °C for 15 min in the presence (○) or in the absence (●) of 50 μM-3-methoxybenzamide. Aliquots of 10^7 nuclei were precipitated with trichloroacetic acid, washed and incubated with 1 M-NH_2OH (pH 7.0) for different time intervals. The samples were again precipitated and the residual radioactivity in the pellet was measured. Inset: the same samples {in the absence (lanes 1–3) and in the presence (lanes 4–6) of 3-methoxybenzamide during incubation with [^{14}C]NAD$^+$} were analysed by SDS/polyacrylamide gel electrophoresis and subsequent fluorography (lanes 1 and 4, 0 h; lanes 2 and 5, 0.25 h; lanes 3 and 6, 2 h incubation with 1 M-NH_2OH).

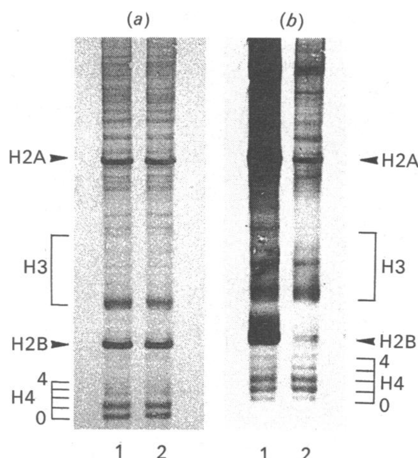

Fig. 4. Electrophoretic analysis of histones on acid/urea/Triton gels

Isolated nuclei of macroplasmodia were incubated with 4 μM-[^{14}C]NAD$^+$ at 4 °C for 15 min in the absence (lanes 1) or in the presence (lanes 2) of 50 μM-3-methoxybenzamide. Extracted histones were analysed on acid/urea/Triton gels and blotted on to nitrocellulose sheets as described in the Materials and methods section. (*a*) Amido Black stain; (*b*) autoradiography. The five H4 subspecies (non-, mono-, di-, tri- and tetra-acetylated), with the non-acetylated being the fastest band, are indicated in the margin (0, non-acetylated, to 4, tetra-acetylated).

monoacetylated, 45%; diacetylated, 12%, triacetylated, 8%; tetra-acetylated, 4%. The distribution of ADP-ribosylation among H4 forms is entirely different. The major ADP-ribosylated subspecies were the mono- and in particular the di-acetylated forms (Figs. 4*b* and 5). Hyperacetylation of H4 by butyrate causes a shift of ADP-ribose incorporation into highly acetylated

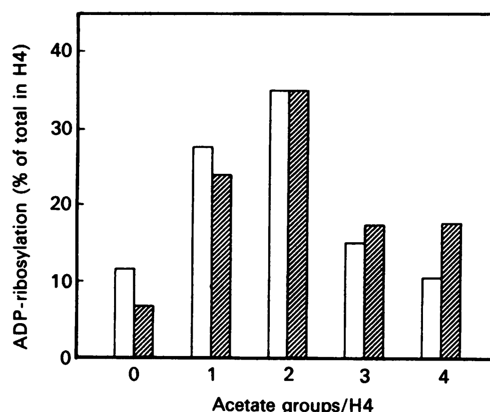

Fig. 5. Distribution of ADP-ribosylation among the acetylated H4 subspecies

Microplasmodia were grown for 1 h in the presence or the absence of 1 mM-n-butyrate; nuclei were then isolated and incubated with 4 μM-[^{14}C]NAD$^+$ at 4 °C for 15 min, and histones were analysed on acid/urea/Triton gels. After fluorography bands from the original gel were excised and counted for radioactivity by liquid scintillation spectrophotometry. Radioactivity in the individual acetylated subspecies was calculated as a percentage of the total ADP-ribose of H4. □, Control; ▨, butyrate-treated microplasmodia.

subspecies, due to a corresponding decrease in non-acetylated and monoacetylated H4 (Fig. 5).

DISCUSSION

In our system, core histones are the main acceptors for ADP-ribosylation, whereas H1 only represents a minor acceptor protein (Golderer *et al.*, 1988). The core histones differ with respect to their behaviour towards the ADP-ribosylation inhibitor 3-methoxybenzamide. The fact that H2A and H2B are sensitive to inhibition, in contrast with H3 and H4, may be due to several reasons. We assume that H3 and H4 are modified by a specific enzyme which is resistant to 3-methoxybenzamide inhibition (up to concentrations of 10 mM). Such an assumption is supported by the finding that a nuclear mono(ADP-ribosyl)transferase exists which is less sensitive to the inhibitor benzamide than poly(ADP-ribosyl)transferase (Rankin *et al.*, 1989). Furthermore, we previously reported on the existence of two forms of ADP-ribosyltransferase (A and B) in *Physarum* (Golderer *et al.*, 1988); form A is considerably less sensitive to ADP-ribosyltransferase inhibitors. For this reason, it seems possible that H2A and H2B are modified by enzyme form B (M_r 75000), whereas H3 and H4 are substrates of enzyme form A (M_r 115000). The type of ADP-ribose–protein linkage is also obviously different for H2A/H2B and H3/H4. The linkage in H2A/H2B is readily cleaved by neutral hydroxylamine, as is characteristic for most other ADP-ribosylated nuclear proteins, indicating carboxylate-ester-linked ADP-ribose residues. The behaviour of ADP-ribosylated H3/H4 towards hydroxylamine (half-life approx. 1.5 h) is indicative of arginine-linked ADP-ribose residues (Payne *et al.*, 1985). This type of linkage would suggest the action of a mono(ADP-ribosyl)transferase, which is known to preferentially modify arginine residues. Non-enzymic ADP-ribose incorporation by the formation of Schiff bases, which are stable towards hydroxylamine with a half-life of several hours, can be excluded, as can the existence of a hydroxylamine-resistant, still unidentified, bond, described for H1 and H2B (Kreimeyer *et al.*, 1985). In addition, the pronounced heat-sensitivity of the reaction is a strong indication of enzymic ADP-ribose incorporation. However, it also seems possible that

different structural states of chromatin are responsible for the observed differences in ADP-ribosylation between H3/H4 and H2A/H2B, since the structural function of the H3/H4 tetramer in the nucleosome is entirely different from that of H2A/H2B dimers.

Our result indicating a preferential ADP-ribosylation of acetylated histone subspecies, as shown for H4, is in line with the data of Smulson and coworkers (Malik & Smulson, 1984; Wong & Smulson, 1984), showing that identical histone molecules may be accessible to both modifications. This finding was substantiated by Boulikas (1988), who reported that, in dimethyl sulphate-treated cells, histone H4 exists in up to tetra(ADP-ribosyl)ated forms, with acetylated subspecies being present mainly as tri- and tetra-(ADP-ribosyl)ated H4; in the absence of DNA strand breaks, H4 is mainly mono(ADP-ribosyl)ated (Boulikas, 1989). The result of the predominant modification by ADP-ribosylation of acetylated core histones is not confined to ADP-ribosylation alone, but has also been reported for the postsynthetic modification by phosphorylation (Sterner & Allfrey, 1983) and methylation (Hendzel & Davie, 1989). This raises the question of whether acetylation of core histones is somehow triggering other postsynthetic modifications. This is suggested by our experiments, especially those using butyrate for hyperacetylation, since we measure ADP-ribosylation in isolated nuclei containing histones with a distinct predisposed steady-state acetylation; therefore ADP-ribosylation could be somehow primed by acetylation, but not vice versa. This possibility is also supported by a model (Loidl & Gröbner, 1987*b*; Loidl, 1988) that explains the biological function of histone acetylation as a modulation of histone–protein as well as histone–DNA interactions. Histone acetylation could therefore act as a signal for nuclear enzymes, such as ADP-ribosyltransferase, to interact with histones, their substrate molecules.

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