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SUBCELLULAR DISTRIBUTION OF MONO(ADP-RIBOSE) PROTEIN CONJUGATES IN RAT LIVER *

P. Adamietz, K. Wielckens, R. Bredehorst, H. Lengyel, and H. Hilz

Institut für Physiologische Chemie der Universität, Hamburg 20, Germany

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SUMMARY

Rat liver nuclei isolated by neutral sucrose or citric acid procedures contain < 5 % of the total mono(ADP-ribosyl) proteins found in the corresponding homogenates. The extra-nuclear location of these conjugates is not a consequence of artifacts like leakage or selective degradation during isolation of nuclei. Comparably low levels of mono(ADP-ribosyl) proteins were also found in the nuclei of mouse kidney, and of Ehrlich ascites tumor cells although the latter also had low conjugate levels in non-nuclear compartments. Fractionation of liver tissue revealed that most of the NH_2OH-resistant mono(ADP-ribosyl) protein conjugates were associated with the mitochondrial fraction, and, to a small degree, with the plasma membranes. The NH_2OH-sensitive conjugates were primarily found in the fractions containing the endoplasmic reticulum. This distribution is in accordance with multiple and independent functions of mono ADP-ribosylation and poly ADP-ribosylation reactions.

INTRODUCTION

In 1966, Mandel and coworkers described a nuclear activity able to form poly (ADPR) from NAD (1). Similar data were presented by Hayaishi's and Sugimura's groups (2, 3). Subsequently, covalent linkage of $(ADPR)_n$ residues to proteins was demonstrated (4, 5). The nuclear ADPR transferase preferentially elongates preexisting mono(ADPR) protein conjugates (6). Mono(ADPR) groups were therefore thought to serve as starting points for chain elongation. However, when separate quantitation of protein-bound monomeric and polymeric (ADPR) residues was performed in intact tissues (7, 8), many more acceptor sites were found to be occupied by single ADPR residues than by poly(ADPR). This was true for all conditions so far studied, including stimulation of poly(ADPR) formation by an alkylating agent (unpublished results). These data suggested that mono ADP-ribosylation may serve functions independent from the formation of poly(ADPR) residues. In order to obtain support for this idea, the subcellular distribution

* Dedicated to Helmut Holzer at the occasion of his 60th birthday.

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of mono(ADPR) protein conjugates was analyzed. The study includes the quantitation of those conjugate subfractions that can be discriminated by their sensitivities towards NH_2OH (9).

MATERIALS AND METHODS

(^{3}H)NAD was synthesized enzymically from adenine (2.8-^{3}H)ATP (19 Ci/mmol; Amersham-Buchler, Braunschweig) and nicotinamide mononucleotide according to the method of Römer et al. (10). Ehrlich ascites tumor cells were harvested from Balb/c mice 6 days after inoculation.

Preparation of nuclei: Nuclei from rat liver (♂ Wistar; 220 g) and from mouse kidney (♀ Balb/c) were isolated under neutral and acidic conditions by similar procedures: Both tissues were homogenized in a glass-teflon homogenizer (Braun, Melsungen) with three volumes of 0.5 M sucrose containing either 60 mM KCl - 15 mM NaCl - 0.15 mM spermine - 0.5 mM spermidine - 15 mM 2-mercaptoethanol - 15 mM Tris/HCl, pH 7.4 (11), or 25 mM citric acid (12). EAT cells were disrupted in 25 mM citric acid by use of a Dounce homogenizer (13).
All homogenates were made 1.6 M with respect to sucrose by the addition of 2-3 volumes of a concentrated sucrose solution. The nuclei were isolated by sedimentation through a 2.2 M sucrose layer during 30 min at 40 000 g (SW 27 rotor, Beckman Instruments, München).

Fractionation of rat liver homogenate: Livers were homogenized as described above in ten volumes of 0.25 M sucrose - 10 mM Hepes, pH 7.5 - 5 mM dithiothreitol - 1 mM $MgCl_2$ and filtered through gauze. Centrifugation for 10 min at 12 000 g (HB4 rotor, Sorvall, DuPont) yielded a post-mitochondrial supernatant. The pellet was resuspended in the same buffer and put on top of a discontinuous gradient consisting of three sucrose layers of increasing density: 0.25 M sucrose - 9 % Ficoll (Pharmacia, Uppsala); 0.25 M sucrose - 15 % Ficoll; and 2.1 M sucrose. When spun for 30 min at 95 000 g (SW 27 rotor) nuclei were found at the bottom of the tubes, mitochondria at the 15 % Ficoll - 2.1 M sucrose boundary, and plasma membranes distributed over the 9 % Ficoll layer (cf. 14). These three fractions were collected with a syringe and either precipitated with 20 % trichloroacetic acid (determination of mono(ADPR) protein conjugates), or used for enzymic analysis.

Mitochondrial extract: Liver homogenates (see above) were centrifuged for 15 min at 1 000 g (HB 4 rotor, Sorvall DuPont) which removed most nuclei and plasma membranes. Mitochondria were then sedimented at 12 000 g. Contaminating lysosomes were removed by digitonin (Merck, Darmstadt) treatment according to Kun (15). The purified mitochondria were suspended in 10 mM sodium phosphate pH 6.5 and disrupted by sonication (three 20 sec bursts with maximal output setting, Branson Model S125, Danbury), and spun for 60 min at 100 000 g (Ti60 rotor, Beckmann, München). The supernatant was concentrated by ultrafiltration (UM10, Amicon, Lexington) to 20 mg protein/ml.

Synthesis of mitochondrial (^{3}H)(ADPR) protein conjugates: 150 µl of the mitochondrial extract was incubated for 10 min at 37° C with 0.3 mM (^{3}H)NAD (0.16 Ci/mmol) in the presence of 100 mM sodium pyrophosphate pH 8.5 - 50 mM KCl - 5 mM $MgCl_2$ - 10 mM thymidine in a total volume of 500 µl. The reaction was terminated by heat inactivation (5 min at 95° C) after lowering the pH to 6.5 with HCl.

Table 1

Preparation	mono(ADPR) Residues (pmol / mg DNA)	(% of total)
Rat Liver		
homogenate	7 830 ± 800	100
nuclei (neutral sucrose)	70 ± 15	1
nuclei (citric acid)	220 ± 25	3
Mouse Kidney		
homogenate	1 960 ± 110	100
nuclei (citric acid)	90 ± 15	4
EAT cells		
homogenate	730 ± 35	100
nuclei (citric acid)	310 ± 30	42

Table 1 Total protein-bound mono(ADPR) residues in homogenates and in nuclei from different tissues. - For details see Methods.

Mono(ADPR) protein conjugates were determined by radioimmunoassay (9). Two conjugate subfractions can be distinguished on the basis of NH_2OH-induced release of the ADPR groups. They were quantitated as described previously (9).

Poly(ADPR) residues were quantitated by chromatography on boronate beads, phosphodiesterase I digestion and radioimmunoassay as described previously (8).

RESULTS

Amounts of mono(ADPR) protein conjugates in nuclei versus total tissue.

In adult rat liver protein-bound mono(ADPR) residue levels are more than 100 times higher than those of polymeric ADPR (8). When nuclei were prepared at neutral pH in the presence of sucrose, small amounts of mono(ADPR) residues were detected (table 1). To exclude possible degradation of the ADPR residues at pH 7.4 nuclei prepared by the citric acid procedure were also analyzed. Again only a small fraction of the total mono(ADPR) residues was found associated with the nuclei. The same was true for mouse kidney. The absolute amounts of the nuclear mono(ADPR) residues in Ehrlich ascites tumor cells were also in a comparable range. However, the tumor cells had very low levels of extra-nuclear conjugates leading in this case to a relatively high proportion of the monomeric ADPR residues in the nucleus.

In order to demonstrate that these results were not due to an artifactual release of mono(ADPR) protein conjugates from nuclei into the soluble fraction during the isolation procedure with citric acid, the following set of experiments was performed: Rat liver was homogenized with citric acid and incu-

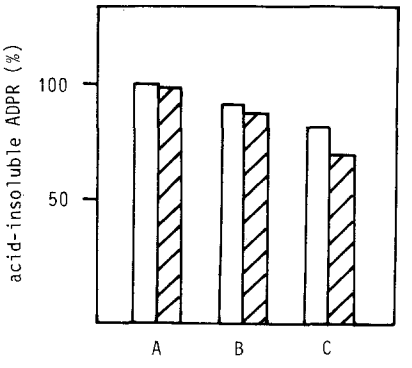

Figure 1 Limited solubilization of mono(ADPR) protein conjugates in rat liver homogenates by citric acid treatment. - Rat liver was homogenized with 20 % TCA (A) or 25 mM citric acid (B and C). In B, 20 % TCA was added after incubation of the citric acid homogenate. Aliquots were centrifuged for 10 min at 40 000 g immediately after homogenization (open column) or after 50 min at 4° (hatched column). Mono(ADPR) residues were determined in the pellets as described in Methods. The 100 % - value corresponds to 15.6 nmol ADPR / g liver.
A=TCA precipitable ADPR residues kept in TCA (4°). B=TCA precipitable ADPR residues of citric acid homogenate kept at (4°). C=ADPR residues insoluble in citric acid.

bated for 60 min at 4° thereby simulating the effect of ionic conditions on the possible solubilization and degradation of ADPR protein conjugates during isolation on nuclei. Under these conditions, 30 % of the total mono(ADPR) residues became soluble in citric acid; 15 % by solubilization of intact conjugates, and another 15 % by degradation of conjugates (fig. 1). The data demonstrate that 70 % of the mono(ADPR) residues remained bound to their original non-nuclear compartments. Therefore it can be safely assumed that the vast majority of the mono(ADPR) protein conjugates in rat liver is located in extra-nuclear organelles.

In contrast to the conjugates carrying mono(ADPR) residues, location of poly-(ADP-ribosyl) proteins poses greater problems. A substantial fraction of these conjugates appears in the soluble fraction when nuclei are prepared by the citric acid procedure, although a major fraction remains associated with the nuclear pellet. Isolation of nuclei by non-aqueous methods will be required to clarify the intracellular distribution of poly ADP-ribosylated proteins.

Distribution of mono(ADPR) protein conjugates in cellular compartments.

In order to analyze the intracellular distribution of the mono(ADPR) conjugates and their subfractions, fractionation of liver homogenates under neutral

Table 2

Cell Fraction	Mono(ADPR) Residues (pmol / g liver ± SEM.)		
	NH_2OH-sensitive conjugates	NH_2OH-resistant conjugates	Ratio resistant / sensitive
Homogenate	6500 ± 1660	9110 ± 1550	1.40
12 000 g supernatant	3640 ± 370	3200 ± 1040	0.88
12 000 g pellet	1780 ± 60	7200 ± 2100	4.04
plasma membrane fraction	40 ± 10	480 ± 350	12.00
mitochondrial fraction	1210 ± 390	5090 ± 970	4.20
nuclear fraction	230	220	0.96

Table 2 Distribution of hydroxylamine-sensitive and hydroxylamine-resistant mono(ADPR) protein conjugates in cellular subfractions.- Rat liver homogenates were fractionated as described in Methods. The protein bound mono (ADPR) residues were freed from acid-soluble contaminations by 20 % TCA precipitation and either released from its protein acceptor by NH_2OH or by alkali. For further details see Methods. Mean values from three independent preparations (except nuclei).

nondenaturing conditions was applied. Surprisingly little degradation occured during separation into a post-mitochondrial supernatant and the pellet fraction as indicated by the good recovery of the protein bound ADPR (Table 2). The data in table 2 also show that about two thirds of the NH_2OH-sensitive conjugates were associated with the post-mitochondrial supernatant, while the majority of the NH_2OH-resistant subfraction was found in the pellet.

When the pellet was further subdivided into a mitochondrial fraction, plasma membranes and nuclei, uneven distribution was again encountered. The most interesting observation is made with respect to the mitochondria. This fraction contained over 80 % of the pelleted NH_2OH-resistant conjugates. At the same time, the mitochondria were nearly devoid of NH_2OH-sensitive conjugates leading to a high ratio of the NH_2OH-resistant to sensitive fraction. The same situation applies to the plasma membrane fraction which is practically free of NH_2OH-sensitive conjugates. In contrast to the mitochondria, however, it contained only about 5 % of the total NH_2OH-resistant conjugates. The nuclei differ from both fractions by the low absolute amounts of mono(ADPR) protein conjugates (as already discussed), and by the nearly equal content of both conjugate subfractions.

The conjugates of the post-mitochondrial supernatant appear to be mainly associated with the endoplasmic reticulum, the cytosol being largely devoid of ADPR proteins, as deduced from preliminary data.

Table 3

Treatment	ADPR Residues Remaining Acid-insoluble (cpm ± s.d.)	(% of total)
none	7370 ± 680	100
Tris·HCl pH 7.4 (0.4 M; 37°)	7450 ± 480	101
NH_2OH pH 7.4 (0.4 M; 37°)	7440 ± 400	101
Tris·glycine pH 10.5 (0.4 M; 37°)	6020 ± 210	81
NaOH (1 M; 56°)	2150 ± 220	28
HCl (0.1 M; 37°)	7520 ± 270	102

Table 3 Stability of the mono(ADPR) protein conjugates formed from endogenous acceptors by mitochondrial ADPR transferase. - Extracts from purified mitochondria were incubated with (^{3}H)NAD for 10 min under the conditions described in Methods. 50 µl aliquots were treated for 60 min as indicated. After precipitation with trichloroacetic acid (10%) and washing of the precipitate, acid-insoluble (^{3}H)ADPR protein conjugates were hydrolyzed (0.5 M perchloric acid, 60 min, 95°). Mean values from 4 determinations.

Extra-nuclear mono(ADPR) transferases.

Polymerizing ADPR transferase activity appears to be located mainly or exclusively in the cell nucleus (cf. 1-3). This enzyme is dependent on DNA (16) and can be inhibited by thymidine (17). The location of mono(ADPR) protein conjugates in various extra-nuclear compartments of the cells suggested the presence in these compartments of other, non-polymerizing mono(ADPR) transferases. Indeed, ADPR transferase activity has been observed in all compartments listed in table 1 (18, 19 and unpublished results).

A more detailed analysis was performed with the mitochondrial system. The enzyme used in these studies was obtained from extracts of purified mitochondria. It was free of contamination with the nuclear polymerizing ADPR transferase as deduced from the complete insensitivity to thymidine. The enzyme transfers ADPR residues from NAD to endogenous acceptors as described previously by Kun et al. (18). In contrast to this report, however, optimal activity was seen at pH 8.5. It acts as a mono(ADPR) transferase as shown by enzymic and chemical analysis of the groups transferred to the acceptors (not documented).

The enzyme forms ADPR protein conjugates that exhibit the same properties as the endogenous conjugates analyzed from intact mitochondria (Table 3): They are resistant to neutral NH_2OH, to mild acid and mild alkaline conditions, but cleaved by 0.4 - 1 M NaOH at elevated temperature (cf. (9)).

The mitochondrial enzyme is inhibited by arginine methyl ester, similar to the transferase from plasma membranes of avian erythrocytes described by Moss et al. (19). However, the fact that other basic compounds like imidazole, histones and even tris buffer inhibit the transfer to the endogenous acceptor proteins, may point to a different enzyme.

These data support and extend the finding that mitochondria contain the bulk of the NH_2OH-resistant mono(ADPR) protein conjugates in rat liver. Similar analyses are presently being performed with respect to the other subcellular compartments.

DISCUSSION

Previous work on ADP-ribosylation in eukaryotic cells was largely restricted to isolated nuclei or chromatin and to permeabilized cells because of the lack of a suitable precursor for in vivo studies (cf. 20). This led to the tacit assumption that ADP-ribosylation is primarily a nuclear event. The development of procedures for the quantitation of mono(ADPR) protein conjugates and their subfractions in intact tissues (9, 21) allowed for the first time to analyze their intracellular location. Surprisingly, these conjugates carrying single ADPR groups were localized primarily in extranuclear compartments. The uneven distribution of NH_2OH-sensitive and NH_2OH-resistant subfractions supports and extends previous findings on an independent synthesis (22) and independent functions (7). Preliminary experiments on the distribution of poly(ADPR) proteins indicate that these conjugates are mainly confined to the nucleus. Different location and independent changes of mono ADP-ribosylated and poly ADP-ribosylated proteins under various conditions (8) again suggest different functions. Thus a picture emerges which indicates that covalent modification of proteins by ADP-ribosylation serves multiple functions in multiple compartments of the eukaryotic cell.

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