Modification of Plasma Membrane Protein Cysteine Residues by ADP-Ribose in Vivo*

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Myron K. Jacobson, Paul T. Lofinț, Naaszam About-Elat, Mingkwan Mingmuang, Joel Moss, and Elaine L. Jobson

From the Departments of Biochemistry and Medicine, Texas College of Osteopathic Medicine, University of North Texas, Fort Worth, Texas 76107 and the Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Proteins can be post-translationally modified by ADP-ribose. Previously, two classes of ADP-riboseyl protein linkages have been detected in vivo which have chemical properties indistinguishable from ADP-riboseyl arginine and ADP-ribosyl glutamate or aspartate. Here we report the detection of a third class of endogenous ADP-riboseyl protein linkage. This class is chemically indistinguishable from ADP-ribose linked to cysteine residues by a thiolglycosidic bond. The distribution of ADP-riboseyl cysteine residues was studied in subcellular fractions of rat liver. Proteins modified on cysteine were detected only in the plasma membrane fraction. Pertussis toxin is known to disrupt signal transduction of ADP-riboylation of cysteine residues of plasma membrane GTP binding proteins. The results described here raise the interesting possibility that the endogenous modification of plasma membrane protein cysteine residues may be involved in signal transduction.

NAD+ is a substrate for many dehydrogenases that catalyze hydride transfer reactions central to energy metabolism. It is also the substrate for enzymes that catalyze the cleavage of the linkage between nicotinamide and ribose and the transfer of ADP-ribose to a nucleophilic acceptor. Such ADP-ribose transfer reactions represent a versatile mechanism for the post-translational modification of proteins. For example, poly(ADP-ribose) polymerase catalyzes transfer of ADP-ribose to protein carboxylate groups and to ribosyl hydroxyls of ADP-ribose resulting in the modification of proteins with ADP-ribose polymers (1, 2). While all other known ADP-riboseyltransferases catalyze the transfer of only single ADP-ribose groups, they show a wide range of specificity for acceptors. The best understood mono-ADP-riboseyltransferases are the bacterial toxins which modify a specific amino acid residue in a specific target protein. Diptheria toxin and Pseudomonas exotoxin A transfer ADP-ribose to an imidazole nitrogen of a hydropommodified histidine residue (diphthamide) of elongation factor 2 (3). Cholera toxin and Escherichia coli heat labile enterotoxin modify a guanidino nitrogen of an arginine residue of Gs, a stimulatory GTP binding protein involved in the regulation of adenylate cyclase (4, 5), and pertussis toxin modifies the thiol group of a cysteine residue of Gi, an analogously inhibitory protein of the cyclase system (6-8). Clostridium botulinum C2 toxin modifies an arginine residue of nonmuscle actin (9, 10), and C. botulinum C3 toxin appears to modify an asparagine residue of a 21-kDa molecular mass membrane protein (11).

The synthesis of ADP-ribose polymeric is known to occur at rapid rates in animal cells following DNA damage and is required for cellular recovery from DNA damage (1). However, evidence is accumulating that mono-ADP-ribose transfer reactions are also a versatile component of metabolism although the function is unknown. Endogenous mono-ADP-riboseyltransferases include enzymes that catalyze the hydrolysis of NAD+ to nicotinamide and free ADP-ribose (12) and enzymes which can modify proteins (13-16). Several apparently distinct transferases that can modify free arginine, other guanido compounds, and arginine residues in proteins have been characterized (13), and an activity that modifies the diphthamide residue in elongation factor 2 has been reported (14, 15). Most recently, an activity that modifies free cysteine and cysteine residues in proteins has been described (16).

Evidence that the endogenous arginine-specific mono-ADP-riboseyltransferases actually modify protein in vivo comes from the detection of proteins that are covalently modified with ADP-ribose by linkages indistinguishable from ADP-riboseyl arginine (17). Endogenous protein linkages with properties indistinguishable from ADP-riboseyl carboxylate ester have also been detected (17). Here we report the discovery of a third class of protein ADP-ribose linkages in rat liver in vivo. These linkages are characteristic of modification at cysteine residues. Further, we report that the proteins modified by ADP-ribose on cysteine are located exclusively in the plasma membrane fraction of rat liver.

EXPERIMENTAL PROCEDURES

Materials

Guanidine hydrochloride and hydroxyamine hydrochloride were obtained from Sigma. Chloroacetidihydride (45% in water) was purchased from ICN Pharmaceuticals, Inc. (Plainview, NY). Mercucic acetate was obtained from Aldrich. Transducin was purified as described by West et al. (7). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). NAD+-arginine ADP-riboseyltransferase A was purified as described by Moss et al. (18), and diphtheria toxin was obtained from Connaught Laboratories Ltd. (Toronto, Canada). Eucaryotic elongation factor 2 was kindly provided by Dr. D. Michael Gill (Tufts University). [adenine-14C]NAD+ and [adenosine-32P]NAD+ were from ICN (Irvine, CA).

Methods

Preparation of Radiolabeled ADP-Ribosylated Protein Standards—Diphthamide-linked [32P]mono-ADP-ribose elongation factor 2 was prepared essentially as described by Gill (19). Briefly, an incubation volume of 100 μl contained 6 pmol of purified elongation factor 2, 6 pmol of [32P]NAD+ (33 Ci/mmol), 20 mM Tris-Cl, pH 8.0, 10 mM β-mercaptoethanol, and 1 μg/ml preactivated diphtheria toxin. The diphtheria toxin was preactivated by dilution to toxin to 50 μg/ml in 250 mM Tris-Cl, pH 8.0, 50 mM dithiothreitol. The reaction mixture was incubated at 37 °C for 30 min followed by the addition of 100 μg of bovine serum albumin and 25 μl of 100% (v/v) trichloroacetic acid. After standing on ice for 10 min, the sample was diluted to 500 μl.

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with ice-cold 20% (w/v) trichloroacetic acid and collected by centrifugation. The pellet was washed twice with 500 ml of 20% (w/v) trichloroacetic acid, dissolved in 200 ml of ice-cold 98% formic acid, and 5 volumes of ice cold water were added. The solution was adjusted to a final concentration of 20% in trichloroacetic acid, and the precipitate was collected by centrifugation. The final pellet was dissolved in ice-cold 98% formic acid and stored at -20 °C.

Cysteine-linked [3H]mono-ADP-ribosyl transducin was prepared by incubating 160 µg of transducin with 40 µg of activated pertussis toxin in 1.5 ml of 100 mM Tris-Cl, pH 7.5, 20 mM thymidine, 10 mM [32P]NAD, 0.1 mM ATP, 0.1 mM Gpp(NH)p, 1 mM EDTA, 9.5 mM MgCl2, for 60 min at 30 °C. Pertussis toxin activation was accomplished by preincubation in 375 µl of 50 mM glycine, pH 8.0, 20 mM dithiothreitol and 1 mg/ml bovine serum albumin for 10 min at 30 °C. Following the incubation, the sample was adjusted to 20% (w/v) in trichloroacetic acid. After standing on ice for 30 min, the solution was subjected to centrifugation and the pellet was dissolved in 98% ice-cold formic acid and precipitated as described above. The labeled protein was stored in 98% formic acid at -20 °C. Arginine-linked [3H]mono ADP-ribosyl histone was prepared as described by Payne et al. (17).

Release of Cysteine-linked Mono-ADP-Ribose from Protein Standards-The acid-insoluble fraction of rat liver (20) was dissolved in 98% ice-cold formic acid, and radiolabeled mono-ADP-ribosyl protein was added. The solution was diluted with 5 volumes of ice-cold H2O and precipitated by the addition of 100% (w/v) ice-cold trichloroacetic acid to a final concentration of 20% (w/v). The sample was then dissolved in ice-cold 98% formic acid and stored in -20 °C for subsequent use. To release cysteine-linked ADP-ribosyl, the sample in ice-cold 98% formic acid was dissolved with an equal volume of a freshly prepared solution of 20 mM mercure acetate, and the resulting solution was incubated at 37 °C for 10 min. The samples were then placed on ice, and 5 volumes of ice-cold H2O were added followed by 100% (w/v) trichloroacetic acid to a final concentration of 20%. After 10 min on ice, the samples were collected by centrifugation and the supernatant was removed. A sample was taken to determine released radiolabel. The pellet containing the remaining protein-bound mono-ADP-ribosyl was dissolved in 250 mM ammonium acetate, pH 6.0, 10 mM EDTA, and 6 mM guanidine before sampling for radioactivity.

Identification of Material Released by Mercuric Ion—After incubation in the presence or absence of 10 mM mercure acetate, the supernatant containing released material was adjusted to pH 9.0 ± 0.2 and diluted to 10 ml with 250 mM ammonium acetate, pH 9.0. The sample was applied to a 0.5-ml DHB-Sepharose column (20) previously equilibrated with 250 mM ammonium acetate, pH 9.0. The column was washed with 10 ml of application buffer and 0.5 ml of 10 mM phosphoric acid, 50 mM potassium chloride. Bound material was eluted with 2.0 ml of 10 mM phosphoric acid, 50 mM potassium chloride. The eluate was applied to a Whatman Partisil-10 SAX column preceded by a silica guard column. Separations were accomplished isocratically at a flow rate of 1.0 ml/min using 100 mM potassium phosphate buffer, pH 4.7. Unlabeled ADP-ribose and AMP were added as UV standards, and fractions were collected and assayed for radioactivity.

Analysis of Rat Liver Extracts—A trichloroacetic acid-insoluble fraction of rat liver was prepared as described previously (20). Fifty µg of trichloroacetic acid powder was dissolved in 1 ml of ice-cold 98% formic acid and diluted 10-fold with ice-cold H2O and precipitated using 100% (w/v) ice-cold trichloroacetic acid to a final concentration of 20% (w/v). The precipitate was collected and dissolved in ice-cold 98% formic acid. Release of cysteine-linked mono-ADP-ribosyl residues from protein was performed by incubating the sample in the presence of 10 mM mercure acetate as described above. After incubation, protein was precipitated by adjusting the sample to 20% (w/v) in trichloroacetic acid, and the pellet was collected by centrifugation at 12,000 × g for 5 min. The pellet was used for determination of ADP-ribosyl arginine-like and carboxylate ester-like linkages as described in detail elsewhere (17, 20), with the following modifications. The loosely packed upper layer of the pellet from the first centrifugation was collected and washed as described by Emmelot et al. (22). The resulting pellet was layered on a two-step sucrose gradient consisting of 22.5% and 8% sucrose, and plasma membranes were isolated as described by Quist et al. (23). The total protein content of each subcellular fraction was determined by the method of Bradford (24), and a trichloroacetic acid-insoluble fraction was prepared and analyzed for ADP-ribosyl cysteine linkages as described above. Each fraction was also assayed for Na+, K+-ATPase activity as described by Quist et al. (23).

RESULTS AND DISCUSSION

The use of neutral hydroxylamine to release ADP-ribose from carboxylate ester linkages on glutamate or aspartate and glycosyl linkage of the guanidinium group of arginine has been reported previously by this laboratory (17, 20). Cysteine and diphthamide linkages to ADP-ribose are stable to hydroxylamine (17). It has been reported that thioglycolic linkages can be cleaved in the presence of mercure ion (25), and Meyer et al. (26) have recently reported that ADP-ribosyl cysteine linkages in transducin formed in vitro by the action of pertussis toxin can also be cleaved using this reagent. In order to search for proteins modified in vivo by ADP-ribose

![Image of Fig. 1. Release of cysteine-linked ADP-ribose by mercure ion. Cysteine-linked [3H]ADP-ribose transducin was added to liver extracts and subjected to the conditions for release as described under "Experimental Procedures" in the presence (O) or absence (C) of mercure ion. Panel A shows a time course at 10 mM mercure ion, and panel B shows a 10-min incubation at the indicated concentrations of mercure ion.](http://www.jbc.org/)

![Image of Fig. 2. Strong anion exchange HPLC analysis of radiolabel released in the absence (panel A) or presence (panel B) of mercure ion. Chromatography was performed as described under "Experimental Procedures." The solid lines represent the absorbance at 254 nm of AMP and ADP-ribose standards which had retention times of 7.4 and 9.7 min, respectively. The closed circles represent radioactivity.](http://www.jbc.org/)
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TABLE I

Comparison of the stability of arginine-linked, cysteine-linked, and diphthamide-linked ADP-ribose in the presence of mercuric ion

The indicated proteins radiolabeled with ADP-ribose were added to crude extracts of rat liver protein and analyzed for release by mercuric ion as described under "Experimental Procedures." The values shown are the means ± S.D. of triplicate determinations.

| Protein     | Linkage  | Control                | Plus mercuric ion                  |
|-------------|----------|------------------------|-----------------------------------|
|             |          | % Released | cpm       | % Released | cpm       |
| Histones    | Arginine | 3800       | 88        | 2.0        | 4100      | 60        | 1.5      |
| Transducin  | Cysteine | ±248       | ±10        | ±211       | ±25       |
| EF-2        | Diphthamide | ±90       | ±4        | ±29        | ±22       | 88.2      |

The selectivity of release of ADP-ribose by mercuric ion is shown in Table I. The presence of mercuric ion did not result in release of ADP-ribose from cysteine. The effect of mercuric ion on ADP-ribose carboxylates was also examined by analyzing cell extracts (data not shown). The presence of mercuric ion did not release carboxylate ester linkages to ADP-ribose. Further, Aktories et al. (27) have shown that the putative ADP-ribose asparagine linkage is also stable in the presence of mercuric ion. Taken together, these results show that, for the known protein ADP-ribose linkages, mercuric ion catalyzes the selective release of ADP-ribose from cysteine.

Next, total rat liver proteins were examined for the presence of linkages characteristic of ADP-ribose in subcellular fractions of rat liver.

TABLE II

Distribution of linkages characteristic of ADP-ribose in cysteine in subcellular fractions of rat liver

Crude homogenates of rat liver were subjected to fractionation and analyzed as described under "Experimental Procedures." The results of a representative experiment are shown. The values are the means of triplicate determinations which differed from the mean by less than 10%.

| Fraction         | Protein      | ADP-ribose cysteine | Na⁺, K⁺-ATPase |
|------------------|--------------|---------------------|----------------|
|                  |              | pmol/ mg protein    | µmol P/ mg protein/h |
| Cytoplasmic      | 291          | <100                | <0.1           |
| Mitochondrial    | 166          | <160                | <0.3           |
| Nuclear          | 2.1          | <60                 | <0.1           |
| Microsomal       | 5.7          | <90                 | <0.1           |
| Plasma membrane  | 9.2          | 2440                | 10.5           |
| Sum of fractions | 544          | 2440                | 4.5            |
| Unfractionated   | 544          | 2520                | 4.6            |

a For values shown with the symbol, <, ADP-ribose linkages or enzymatic activity were not detected. The numbers shown represent the limit of detection.
fluorescent compounds unrelated to ADP-ribose. Fig. 3D shows that a small amount of authentic etheno-ADP-ribose added to extracts prepared as in Fig. 3A resulted in an enhancement of the peak. Taken together, the results of Fig. 3 demonstrate that rat liver proteins are modified in vivo with ADP-ribose in linkages chemically indistinguishable from ADP-ribose linked to cysteine.

To further examine endogenous ADP-ribosyl cysteine linkages, crude homogenates of rat liver were subjected to subcellular fractionation using methodology that has been described in detail elsewhere (21-23). The isolated subcellular fractions were analyzed for linkages characteristic of ADP-ribosyl cysteine and total protein. These data along with analysis of an equivalent amount of crude homogenate are shown in Table II. ADP-ribosyl cysteine linkages were detected in the plasma membrane fraction only. With regard to recovery, this fraction contained 97% of the linkages detected in the crude homogenate. In contrast to the distribution of ADP-ribosyl cysteine linkages, ADP-ribosyl arginine linkages were detected in each of the subcellular fractions except nuclear, with only 4% of the total ADP-ribosyl arginine present in the plasma membrane fraction (results not shown). Na+,K+-ATPase activity was measured as a criteria of purity for the plasma membrane fraction. A specific activity of 10.5 nmol of Pi/mg of protein/h was obtained for this fraction which is in close agreement with the value of 11.7 previously reported for a highly purified plasma membrane fraction of rat liver (29).

In total, these results indicate that proteins modified on cysteine by ADP-ribose are located primarily, if not exclusively, in the plasma membrane.

The function of endogenous mono-ADP-ribosyltransferases in animal cells is unknown. Since ADP-ribose acceptor proteins have not been identified, these enzymes are presently categorized according to functional group specificity for ADP-ribose transfer. A number of apparently distinct endogenous transferases that are specific for transferring ADP-ribose to the guanidinium group of free arginine and arginine residues in protein have been characterized (13). The detection of proteins covalently modified by ADP-ribose with linkages indistinguishable from ADP-ribosyl arginine has provided evidence that protein molecules can be in vivo substrates for these enzymes (17). The presence of proteins that are modified by ADP-ribose with linkages indistinguishable from carboxyester linkages of ADP-ribose has also been reported (17). Mono-ADP-ribosyltransferases that catalyze the formation of carboxyester linkages to AD-ribose have not been described. However, such a linkage can be formed by poly(ADP-ribose) polymerase or the concerted activity of poly(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase (1, 30). Thus, the mono-ADP-ribosyl carboxyester-like linkages are likely the result of ADP-ribose polymer metabolism.

In the present study, a third class of endogenous ADP-ribosyl protein linkages has been detected. The linkages are chemically indistinguishable from ADP-ribosyl cysteine. Demonstration of the function of endogenous protein modification by ADP-ribose will require identification of the acceptor proteins. The methods for selective release of ADP-ribose from protein described here combined with those described previously (17, 20) should prove valuable in this regard. The exclusive location of proteins modified on cysteine in the plasma membrane fraction has interesting implications since pertussis toxin is known to disrupt signal transduction by the modification of a cysteine residue of the plasma membrane protein G, and related G proteins (6-8). It is of interest that a mono-ADP-ribosyltransferase has been recently detected in human erythrocytes that catalyzes the ADP-ribo-
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