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Nonenzymic ADP-ribosylation of specific mitochondrial polypeptides

(NAD glycohydrolase/ADP-ribosyl transferase/plasma membranes/Ca^{2+} efflux/nonenzymic glycosylation)

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ABSTRACT **The apparent NAD:protein ADP-ribosyl transferase activity of mitochondria and submitochondrial particles from beef heart and rat liver is simulated by a reaction sequence that consists of an enzymic hydrolysis of NAD to ADP-ribose (ADP-Rib) by NAD glycohydrolase(s) and a nonenzymic ADP-ribosylation of acceptor proteins by the free ADP-Rib formed. The nonenzymic ADP-ribosylation of mitochondrial proteins showed two pH optima and exhibited the same remarkable selectivity as the reaction with NAD. The predominant acceptor in beef heart mitochondria was a 30-kDa protein, whereas in mitochondrial extracts of rat liver a 50–55 kDa polypeptide served as an acceptor. No authentic ADP-Rib transferase activity could be detected even when free ADP-Rib was trapped by NH_2OH. Once formed, the mitochondrial ADP-Rib conjugates were resistant to hydroxylamine. NH_2OH-resistant mono(ADP-Rib)-protein conjugates as found in most cells may also be products of nonenzymic ADP-ribosylation. In mouse tissues, their amounts relate to protein and NAD contents, and they increase specifically and reversibly in the hypothyroid status. Furthermore, intact rat liver mitochondria contain a mono(ADP-Rib)-polypeptide (50–55 kDa) that appeared to be identical with the polypeptide reacting with ADP-Rib *in vitro*.**

Various phage enzymes and bacterial toxins catalyze the transfer of single ADP-ribose (ADP-Rib) residues from NAD to one or several protein acceptors (for reviews, see refs. 1–4). Eukaryotic cells also contain endogenous proteins that are modified by single ADP-Rib residues (cf. ref. 5). In rat liver, most of these mono(ADP-Rib)-protein conjugates are located in the cytoplasm, whereas poly(ADP-Rib)-protein conjugates appear to be confined to the nucleus (6). Two types of endogenous mono(ADP-Rib)-conjugates can be distinguished by their sensitivity towards neutral hydroxylamine. They show independent changes and they are distributed unevenly in various organs of the mouse (5, 7).

Interest in cytoplasmic ADP-ribosylation was greatly stimulated when diphtheria toxin- and cholera toxin-catalyzed ADP-ribosylation was detected (7–12) and when membrane-bound (12, 13), cytosolic (14), and mitochondrial (15, 16) ADP-ribosyl transferase activities were described. It was also reported that free ADP-Rib can form Schiff bases with amino groups of proteins, especially of the histones, when incubated at slightly alkaline conditions (17), while true ADP-Rib transferase activity in mitochondria was said to be operating at pH values <7 (18). However, the use of various inhibitors in mitochondrial preparations indicated to us that nonenzymic ADP-ribosylation was occurring at lower pH values as well.

This paper describes a reaction sequence found in mitochondria (and plasma membranes) that involves the hydrolysis of NAD by NAD glycohydrolases and the nonezymic ADP-ribosylation of *specific* acceptors to form acid-stable conjugates. This reaction sequence simulated ADP-Rib transferase activity, and the similarity of acceptors *in vivo* and *in vitro* provides suggestive evidence that hydroxylamine-resistant mono(ADP-Rib)-protein conjugates of intact tissues may be formed in part by nonenzymic ADP-ribosylation as a result of NAD glycohydrolase and other ADP-Rib-producing enzyme activities *in vivo*.

MATERIAL AND METHODS

Enzymes, coenzymes, and substrates were obtained from Merck or Boehringer Mannheim. [*adenine*-^{3}H]NAD was prepared from [^{3}H]ATP as described (19).

Bovine Heart Mitochondria. Preparation of bovine heart mitochondria was performed according to ref. 20, including treatment with purified digitonin. Submitochondrial particles (SMP) were prepared by sonication of 9.4 mg of mitochondria in 70 ml of 10 mM Hepes (pH 7.5). After centrifugation (rotor Ti 60, 60 min, 50,000 rpm, 0°C), the pellet was resuspended in 25 ml of 10 mM Hepes (pH 7.5).

[*adenine*-^{3}H]ADP-Rib. [^{3}H]NAD (2 μmol) was incubated in pyrophosphate buffer (pH 7.3) with 6 mg of NAD nucleosidase (calf thymus; Boehringer Mannheim) for 60 min at 37°C. The yield was usually 95% as revealed by thin-layer chromatography [polygram GL 300 PEI/UV_{254} (Machery & Nagel; 1 M LiCl)]. Since enzymically prepared ADP-Rib contained contaminants, in some cases we used [^{3}H]ADP-Rib prepared by mild alkaline hydrolysis of [^{3}H]NAD and purified by boronate and paper chromatography (M. Jacobson, personal communication).

[^{3}H]NAD or [^{3}H]ADP-Rib. Incorporation of [^{3}H]NAD or [^{3}H]ADP-Rib into acid-insoluble material was analyzed by incubation (37°C, 60 min) of mitochondrial (1 mg of protein) or membrane (2 mg of protein) fractions in 1 ml containing 50 mM buffer (usually Hepes at pH 6.5) and 50 or 250 μM [^{3}H]NAD or [^{3}H]ADP-Rib (3×10^5 cpm, each). The reaction was terminated by addition of 2 ml of cold 10% trichloroacetic acid, and the precipitate was washed three times with trichloroacetic acid, hydrolyzed in 1 ml 5% trichloroacetic acid (60 min, 100°C), and assayed for radioactivity. When rat liver extracts were analyzed, the reaction was terminated by transfering the samples onto filter paper discs followed by immersion and washing in 10% trichloroacetic acid, ethanol, and ether.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed according to Lämmli (21). Samples were dissolved in 0.06 M Tris phosphate, pH 6.0/0.1% lithium dodecyl sulfate/6 M urea/1 mM dithiothreitol, dialyzed for 2 hr against the same buffer, and subjected to electrophoresis in a 9% polyacrylamide gel or in gradient gels.

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Abbreviations: ADP-Rib, ADP-ribose; SMP, submitochondrial particle(s); INH, Isonicotinic acid hydrazide.

RESULTS

Apparent ADP-Ribosyl Transferase Activity in Mitochondrial Preparations. When SMP of heart mitochondria or extracts from rat liver mitochondria were incubated at pH 6.5 with [*adenine*-^{3}H]NAD, a time- and dose-dependent incorporation of the adenine moiety into the trichloroacetic acid-insoluble fraction could be observed, confirming previous reports with rat liver mitochondria (15, 16). Treatment of the acid-insoluble reaction products with phosphodiesterase or alkali released [^{3}H]AMP, as would be expected for protein-bound mono(ADP-Rib)-residues.

The transfer of [^{3}H]adenine equivalents from labeled NAD to the acid-insoluble acceptors occurred over a wide range of the pH scale, and it appeared to be enzymic in nature as it was eliminated by heating the mitochondrial preparation (Table 1). Unlabeled NAD suppressed incorporation of ^{3}H by isotope dilution. The comparable effect of NADP could be explained by competitive inhibition of the apparent transferase reaction or by rapid conversion of NADP to NAD by phosphatase action. Surprisingly, ADP-Rib was as inhibitory as NAD at pH 4, but much less inhibitory at slightly alkaline conditions. The still greater inhibitory effect of 2′-phospho-ADP-Rib compared to ADP-Rib was unexpected also. Noteworthy also was the marked inhibition by isonicotinic acid hydrazide (INH), which is a rather specific inhibitor of many NAD glycohydrolases (cf. refs. 22 and 23).

Although these data were not in direct contradiction to the proposed existence of NAD:protein ADP-ribosyl transferase activity in mitochondrial preparations (15, 16), they could as well be explained by a sequence of two reactions composed of an enzymic conversion of NAD to ADP-Rib and a nonenzymic ADP-ribosylation of acceptor proteins by free ADP-Rib.

NAD Glycohydrolase-Catalyzed Formation of Free ADP-Rib and Nonenzymic ADP-Ribosylation Simulate Mitochondrial ADP-Rib Transferase Activity. When [^{3}H]NAD was incubated with beef heart SMP at pH 8.0, two-thirds of the NAD was degraded within 15 min, yielding nearly exclusively ADP-Rib. NAD glycohydrolase activity was significantly slower at pH 4.0, with 50% conversion of NAD to ADP-Rib being reached only after about 50 min. Similar data were obtained with rat liver mitochondrial extracts, in which a Mg^{2+}-dependent, INH-insensitive NAD glycohydrolase activity (pH optimum = 8) and Mg^{2+}-independent, INH-sensitive activity (pH optimum = 6) were found. The data show that mitochondrial NAD glycohydrolase activity is high enough to rapidly produce significant amounts of free [^{3}H]ADP-Rib to react with acceptor proteins.

A comparative study with [^{3}H]NAD and [^{3}H]ADP-Rib as "substrates" was then performed. When beef heart SMP were incubated at pH 6.0 with equimolar concentrations of either precursor, radiolabel from ADP-Rib was incorporated to a significantly higher degree than from NAD (Table 2). Furthermore, the incorporation from NAD could be suppressed by >90% by INH, a potent inhibitor of NAD glycohydrolases, whereas incorporation of ADP-Rib was affected only slightly.*

The greater amount of incorporation from ADP-Rib is seen over a wide range of pH values (Fig. 1). Two pH optima can be distinguished—the alkaline one being identical for both precursors at pH 9, whereas the acid region incorporation from [^{3}H]NAD fell off at pH values <5, compared to the optimal value of [^{3}H]ADP-Rib incorporation at pH 4. This difference at low pH is the consequence of an unfavorable pH dependency of the NAD glycohydrolase releasing [^{3}H]ADP-Rib from [^{3}H]NAD during incubation. Similar characteristics with two pH optima for both precursors were also seen when extracts from rat liver mitochondria were analyzed.

Involvement of NAD glycohydrolase as a producer of free ADP-Rib was also demonstrated in an experiment in which the effects of endogenous and added NAD glycohydrolase on the incorporation of [^{3}H]NAD versus [^{3}H]ADP-Rib into SMP proteins were analyzed (Table 3). Heating of SMP eliminated most incorporation from NAD but not from ADP-Rib. Furthermore, NAD glycohydrolase from pig brain catalyzed an apparent ADP-ribosylation of endogenous protein with

Table 1. Inhibitors of ADP-Rib transfer in SMP

Addition	[^{3}H]ADP-Rib incorporation from [^{3}H]NAD			
	pH 8		pH 4	
	pmol incorporated	%	pmol incorporated	%
None	76.5	100	109.6	100
None, enzyme heated	7.5	10	—	—
NAD	0.2	1	11.6	11
NADP	7.7	11	5.9	5
ADP-Rib	34.9	51	13.6	12
P-ADP-Rib	8.3	12	3.7	3
Nicotinamide	27.0	31	46.1	42
INH	23.2	34	9.7	9

Values are means of triplicate determinations, with SD in the range of 5–10%. Standard incubation conditions were with 250 μM [^{3}H]NAD; all additions were at a concentration of 10 mM.

Table 2. Inhibition of ADP-Rib transfer from NAD by INH

Substrate	[^{3}H]Adenine equivalents incorporated		
	Control, pmol	INH	
		pmol	% of control
[^{3}H]NAD	28.5 ± 0.6	2.3 ± 0.2	8
[^{3}H]ADP-Rib	41.2 ± 0.2	33.2 ± 0.2	80

SMP from beef heart were incubated in buffer (pH 6.0) for 15 min with 0.25 mM [^{3}H]NAD or [^{3}H]ADP-Rib (5×10^5 cpm, each). INH was added at a concentration of 20 mM. Values are presented as mean ± SD of triplicate determinations.

*It seems likely that INH in resembling semicarbazide reacts with the aldehyde form of the reducing ribose in ADP-Rib, thus competing with protein acceptor sites (see Table 4).

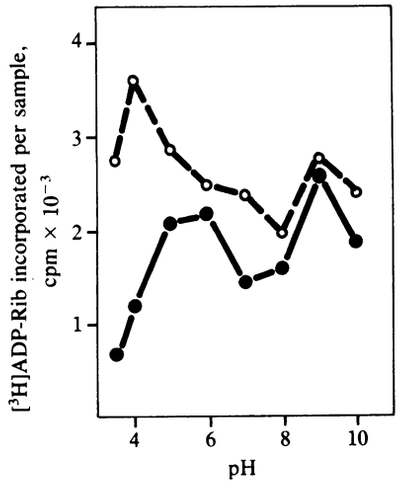

FIG. 1. pH-dependent formation of acid-insoluble products in SMP with [^{3}H]NAD (●) versus [^{3}H]ADP-Rib (○). SMP from bovine heart (0.9 mg of protein) were incubated for 60 min at 37°C in the presence of 0.25 mM [^{3}H]NAD or [^{3}H]ADP-Rib (2×10^4 cpm/nmol, each). The buffers (0.1 M) used were acetate (pH 3.5); Hepes (pH 7 and 8); *N*,*N*-bis(2-hydroxyethyl)glycine and glycine (pH 9 and 10).

Table 3. Effect of NAD glycohydrolase on the formation of acid-insoluble products from [^{3}H]NAD versus [^{3}H]ADP-Rib in SMP

Addition	[^{3}H]Adenine equivalents incorporated	
	pmol	Difference
[^{3}H]NAD		
SMP	76.8 ± 0.1	69
SMP, 5 min at 95°C	7.5 ± 0.2	—
NAD glycohydrolase	25.4 ± 0.6	23
NAD glycohydrolase, 5 min at 95°C	2.4 ± 0.4	—
SMP + NAD glycohydrolase	142.5 ± 1.9	133
[^{3}H]ADP-Rib		
SMP	140.0 ± 1.6	1
SMP, 5 min at 95°C	139.1 ± 0.8	—
NAD glycohydrolase	40.8 ± 5.0	−14
NAD glycohydrolase, 5 min at 95°C	65.0 ± 2.5	—
SMP + NAD glycohydrolase	147.5 ± 3.3	−57

SMP were incubated at pH 4 for 30 min at 37°C. NAD glycohydrolase (1 mg) was a partially purified insoluble preparation from pig brain (Boehringer Mannheim). Values are presented as mean ± SD of triplicate determinations. Difference: non-heated versus heated sample.

NAD as a substrate, which was also eliminated by heating the enzyme. Again, [^{3}H]ADP-Rib was incorporated into the native as well as the denatured NAD glycohydrolase preparation, the degree of incorporation being higher than with intact [^{3}H]NAD.† Finally, when brain NAD glycohydrolase was combined with SMP, a synergistic action was seen with [^{3}H]NAD as a precursor but not with free [^{3}H]ADP-Rib, as would be expected if NAD glycohydrolase activity of mitochondrial preparations was the rate-limiting step in the overall reaction with [^{3}H]NAD. Similar data were obtained when the reactions were carried out at pH 8.

Additional support for nonenzymic ADP-ribosylation came from experiments with rat liver mitochondrial extracts from which NAD glycohydrolases had been separated by chromatography on blue Sepharose. This extract still incorporated [^{3}H]ADP-Rib to the same extent as untreated extract, but it was unable to transfer ADP-Rib residues from [^{3}H]NAD. No significant incorporation into mitochondrial protein could be detected when [^{3}H]AMP was used instead of [^{3}H]ADP-Rib.

Absence of significant ADP-ribosyl transferase activity was further indicated by a comparative study on the influence of different inhibitors (Table 4). Typical carbonyl reagents such as NH_2OH and semicarbazide nearly eliminated incorporation from both precursors even at very low concentrations. This was apparently caused by the trapping of ADP-Rib and not by a secondary splitting of the ADP-Rib-protein bond since the conjugates when formed, proved to be quite resistant to NH_2OH (see below). Basic amino acids also inhibited the incorporation of ADP-Rib equivalents from both precursors. Furthermore, AMP and especially ATP markedly inhibited incorporation of free and NAD-derived ADP-Rib (cf. ref. 16). Formation of insoluble complexes with masking of the acceptor sites may be the reason for this type of inhibition, as "coagulation" of dispersed SMP was seen in the presence of ATP.

Once formed, the conjugates obtained with NAD or free ADP-Rib and bovine heart mitochondrial preparations were not split by treatment with 0.3 M neutral hydroxylamine (Table 5). They also remained intact when exposed to dilute hydrochloric acid. Only treatment with alkali or with picrylsulfonate at pH values >7 (cf. ref. 15) resulted in release of most of the adenine equivalents.

†The fact that native NAD glycohydrolase in the presence of [^{3}H]ADP-Rib yielded lower levels of ADP-Rib conjugates than the denatured enzyme may be due to contaminating phosphodiesterase activity.

Table 4. Inhibition of ADP-Rib incorporation into SMP proteins by various compounds

Addition	Relative incorporation of [^{3}H]Adenine equivalents	
	[^{3}H]NAD	[^{3}H]ADP-Rib
None	100	100
NH_2OH		
10 mM	15	10
100 mM	5	5
Semicarbazide		
10 mM	84	74
100 mM	16	8
Arginine methylester, 100 mM	33	34
Lysine, 100 mM	68	52
Glycylglycine, 100 mM	103	96
Glutathione, 100 mM	75	81
AMP, 10 mM	57	48
ATP, 10 mM	17	14

SMP of bovine heart were incubated at pH 6.5 under standard conditions in the presence of the compounds listed.

Chemical stability of submitochondrial ADP-Rib conjugates differed significantly from that of acid-stable adducts of ADP-Rib formed with poly(lysine) and poly(arginine): submitochondrial conjugates were stable towards 3 M NH_2OH for at least 6 hr at 37°C. Under the same conditions, the ADP-Rib polypeptides released about 50% of their bound ADP-Rib equivalents. Picrylsulfonate, which decomposed submitochondrial ADP-Rib conjugates (Table 5), had no effect on poly(lysine) or poly(arginine) adducts exceeding that slow release seen under the slightly alkaline conditions. Finally, the pH dependency of the ADP-Rib reaction with the basic poly amino acids showed only a single maximum at about pH 9, whereas the reaction of ADP-Rib with mitochondrial extracts and SMP exhibited the additional optimum at acidic pH values as shown in a preceding section (Fig. 1).

Reaction of Free [^{3}H]ADP-Rib with Mitochondrial Proteins Exhibits High Specificity. When homogenates of beef heart mitochondria were fractionated into extracts and SMP, highest incorporation (90%) of labeled NAD or ADP-Rib into the acid-insoluble fractions occurred in the SMP, whereas the bulk of incorporation in rat liver mitochondria was associated with the mitochondrial extract. [^{3}H]Adenine equivalents

Table 5. Stability of ADP-ribosyl conjugates formed with SMP from bovine heart mitochondria

Treatment	[^{3}H]Adenine equivalents released, % of total	
	pH 4 conjugates	pH 9 conjugates
NH_2OH, 0.3 M	4	2
HCl, 0.1 M	2	<1
Picryl sulfonate, 0.1 M		
pH 6.5	13	12
pH 8.2	ND	61
NaOH, 0.1 M	90	81

Conjugates were formed under standard conditions with acetate (pH 4) or *N*,*N*-bis(2-hydroxyethyl)glycine (pH 9) buffer at 37°C for 60 min. After precipitation and washing with cold trichloroacetic acid, the pellet was dissolved in 6 M guanidine·HCl/100 mM buffer, pH 7.0, and chromatographed on Sephadex G-25 columns previously equilibrated with the same solution. Aliquots of the high molecular weight peaks were treated for 30 min at 37°C (56°C with NaOH), and the released material was separated by Sephadex G-25 chromatography by using the conditions described above (6 M guanidine·HCl). ND, not determined.

incorporated from labeled NAD as well as from ADP-Rib into heart SMP migrated with the same acceptors whether the reaction was performed at pH 4 or pH 8 (Fig. 2 *A–D*). The principal polypeptide had an apparent molecular size of about 30 kDa. This is the same size as described previously for the product of a supposed enzymic ADP-ribosylation in SMP of rat liver (16). ADP-ribosylation of this acceptor was thought to be connected with peroxide-induced degradation of pyridine nucleotides and an efflux of Ca^{2+} ions (16). A minor ADP-ribosyl polypeptide of 50–55 kDa was also formed with bovine heart SMP. In rat liver, this 50- to 55-kDa polypeptide was the principal acceptor with NAD or ADP-Rib (Fig. 2 *E* and *F*). A polypeptide of similar size has been described as the substrate of an assumed mitochondrial ADP-ribosyl transferase (15, 18).

Specificity of the nonenzymic reaction was also seen with plasma membranes from rat liver. In this case, free ADP-Rib formed conjugates preferentially with a 55-kDa acceptor (cf. ref. 13) as well as with three other polypeptides (unpublished data).

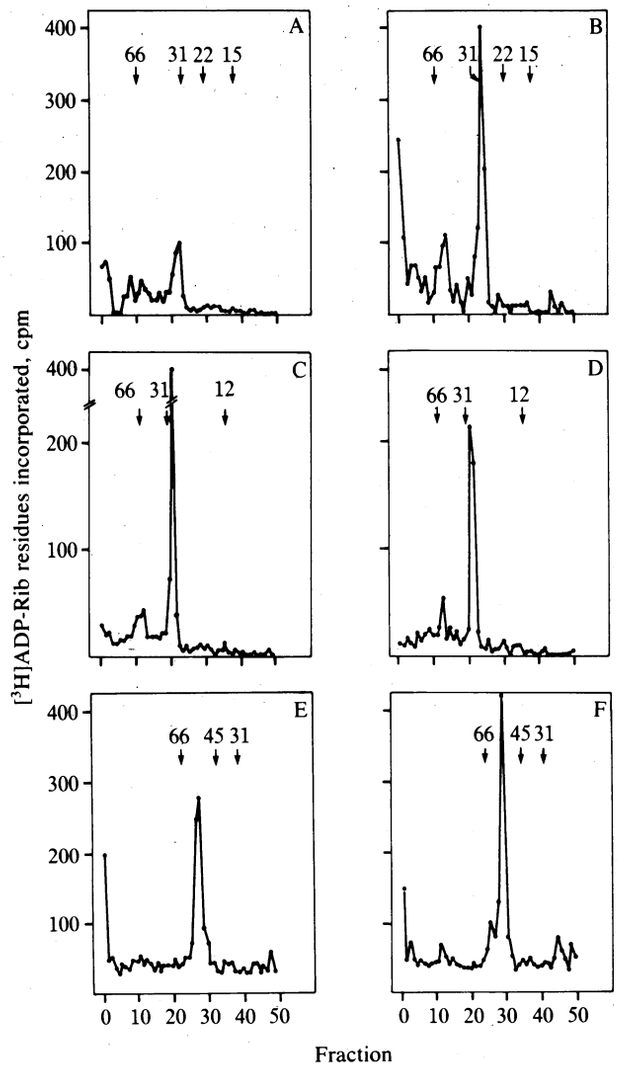

FIG. 2. $NaDodSO_4$ gel electrophoretic analysis of ADP-ribosylated proteins formed with [^{3}H]NAD or [^{3}]ADP-Rib in bovine heart SMP and in extracts of mitochondrial rat liver. Tissue preparations were incubated with 250 μM [^{3}H]NAD (2.8×10^5 cpm/nmol) (*A*, *C*, and *E*) or [^{3}H]ADP-Rib (3.2×10^5 cpm/nmol) (*B*, *D*, and *F*) for 60 min at 37°C. The incubates were then processed. (*A–D*) SMP incubated at pH 4 (*A* and *B*) or pH 8 (*C* and *D*); (*E* and *F*) rat liver extract incubated at pH 6.5. Molecular sizes are given in kDa.

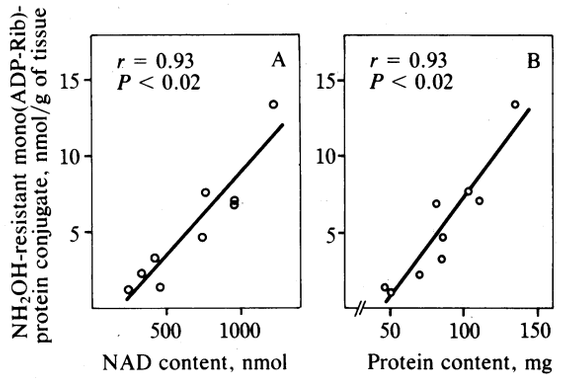

FIG. 3. Correlation of endogenous NH_2OH-resistant mono-(ADP-Rib)-protein conjugates in mouse tissues with levels of NAD(H) (*A*) and protein (*B*). Calculated from ref. 7.

NH_2OH-Resistant Mono(ADP-Rib)-Protein Conjugates of Tissues as Possible Products of Nonenzymic ADP-Ribosylation *in Vivo*. Free ADP-Rib can be formed in intact cells by the action of NAD glycohydrolases. Under conditions of DNA alkylation or cell starvation, free ADP-Rib is also produced by a dramatically stimulated synthesis and degradation of poly(ADP-Rib)- and mono(ADP-Rib)-residues (24–26). Since all eukaryotic cells appear to contain the enzymes catalyzing NAD- and poly(ADP-Rib) hydrolysis, it is to be expected that free ADP-Rib is permanently formed, leading to nonenzymic glycosylation of proteins *in vivo*. The following findings support the existence of such products in tissues. (*i*) All eukaryotic tissues so far studied contain mono(ADP-Rib)-protein conjugates that are hydroxylamine-resistant but cleaved by alkali (5), as found with the conjugates formed nonenzymically (Table 5). Most of the NH_2OH-resistant conjugates in rat liver are associated with the mitochondrial fraction (6). (*ii*) Nonenzymic formation of conjugates should depend on the concentration of the reactants and the turnover of the reaction products. Although procedures for the quantitation of endogenous free ADP-Rib and the individual unmodified acceptor proteins are presently not available, it was found that the amounts of the NH_2OH-resistant ADP-Rib-protein conjugates of various mouse tissues, but not the NH_2OH-sensitive subfraction, were positively correlated ($P < 0.02$) to the NAD(H) content and to the amount of protein (Fig. 3). No correlation was obtained with DNA and RNA contents. Furthermore, induction of a hypothyroid status in mice, which is associated with a reduction of general metabolism, should also increase the concentration of ADP-Rib conjugates because of a presumed increased persistance of the reactants and a reduced elimination of the conjugates. Indeed, in hypothyroid rats, a specific increase in the hydroxylamine-resistant subfraction of the mono(ADP-Rib)-protein conjugates of the liver was observed (7). (*iii*) Freshly prepared rat liver mitochondria contain a mono(ADP-Rib)-protein conjugate that exhibits the same electrophoretic mobility (50–55 kDa) as the principal polypeptides reacting with free [^{3}H]ADP-Rib *in vitro* (unpublished experiments). It is suggested that nonenzymic ADP-ribosylation of specific proteins is operating in intact cells.

DISCUSSION

The present results demonstrate that the apparent ADP-ribosyl transferase activity in mitochondria can be explained by a reaction sequence involving formation of free ADP-Rib by NAD glycohydrolase(s) and a nonenzymic reaction of ADP-Rib with mitochondrial proteins. This reaction exhibits a surprisingly high degree of specificity. Depending on the type of mitochondria or the submitochondrial compartment used (or both), a 30-kDa polypeptide or a 50- to 55-kDa polypeptide

served as the main acceptor. In plasma membranes of rat liver, other polypeptides were found to function as the principal acceptors of free ADP-Rib. The high specificity of the reaction suggests that the acceptor proteins contain defined structural features that are not destroyed at elevated temperatures (see Table 3). To a certain degree, the reaction resembles nonenzymic glycosylation of proteins by glucose (27), galactose (28), or sialic acid (29), which, too, exhibit some specificity. However, nonenzymic ADP-ribosylation appears to proceed at higher rates and to exert higher affinities than protein glycosylation with the free sugars (unpublished experiments).

The nature of the acceptor site(s) is not yet known. Certainly, it is not identical with the NH_2OH-sensitive ester glycoside linkage first described by Nishizuka *et al.* (30). Model reactions with poly(lysine) and poly(arginine) indicated significant differences with the mitochondrial systems, including the lack of the additional acidic pH optimum. Whether a specific amino acid acceptor, as in diphtheria toxin-mediated ADP-ribosylation (cf. ref. 31), or specific influences of the microenvironment determine the selectivity of the reaction remains to be determined.

Our findings may also be of significance with regard to experimental pitfalls when searching for ADP-ribosyl transferase activity in other subcellular compartments and tissues. Incorporation of labeled NAD into polypeptides is apparently not sufficient to show the existence of an ADP-Rib transferase. Also, "control" experiments using nicotinamide or heating may only show that NAD glycohydrolase is required to produce the true reactant ADP-Rib. Control incubations with low concentrations of NH_2OH or semicarbazide to trap free ADP-Rib and substitution of labeled NAD by labeled ADP-Rib should be used when nonenzymic ADP-ribosylation is to be excluded. ADP-Rib transferase activity can also be present as a nuclear contamination of the subcellular preparations. However, this activity has an optimum at pH 8. It is little affected by 10 mM NH_2OH, and the reaction product is nearly exclusively poly(ADP-Rib). Careful purification of liver mitochondria by use of digitonin (15) yielded preparations that showed very little ADP-Rib transferase activity when analyzed as intact particles. Only when sonicated, NAD glycohydrolase-catalyzed hydrolysis of NAD followed by the nonenzymic reaction of ADP-Rib with the protein acceptor(s) could be observed.

Although the reactions of ADP-Rib presented here proceed nonenzymically, they may still be of biological significance. This can be inferred from two findings. (*i*) Organic peroxides induce mitochondrial Ca^{2+} efflux and a depletion of mitochondrial pyridine nucleotides with the concomitant appearance of free ADP-Rib and nicotinamide (32, 33). (*ii*) Intact rat liver mitochondria contain an ADP-Rib conjugate that is similar to or identical with the mitochondrial 50- to 55-kDa polypeptide reacting with ADP-Rib *in vitro*. Thus, it is tempting to speculate that mitochondrial efflux of Ca^{2+} as induced by organic peroxides uses activation of NAD glycohydrolase and nonenzymic ADP-ribosylation of the 30-kDa polypeptide to promote the release or transport of the divalent cation.

Nonenzymic ADP-ribosylation of proteins may also occur in nuclei. Induction of DNA repair by alkylating agents leads to a dramatic stimulation of poly(ADP-Rib) turnover (24–26) and a correspondingly high production of free ADP-Rib by the action of poly(ADP-Rib) glycohydrolase. Under these conditions, accumulation of NH_2OH-resistant histone H1-associated mono(ADP-Rib)-residues has been observed (34).

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