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ADP-ribosyl Transferase and NAD Glycohydrolase Activities in Rat Liver Mitochondria†

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ABSTRACT: ADP-ribosyl transferase and NAD glycohydrolase activities have been estimated in mitochondria in mitoplasts as well as in other submitochondrial fractions. A high activity of these two enzymes was present in mitoplasts as compared to the outer membrane preparation or intermembrane compartment. Inhibitor studies provide strong evidence for the involvement of ADP-ribosyl transferase in the process of ADP-ribosylation of mitochondrial proteins. When NAD glycohydrolase was blocked by nicotinamide or 3-aminobenzamide, the incorporation of ADP-ribose into mitochondrial proteins still occurs. ADP-ribosyl transferase activity could also be detected when NAD glycohydrolase was separated by hydroxylapatite chromatography. The protein-linked ADP-ribose moiety appears to be an oligomer in mitochondria.

ADP-ribosylation is a covalent posttranslational modification of proteins catalyzed by specific enzymes (Mandel et al., 1982; Ueda & Hayaishi, 1985). The ADP-ribose moiety of NAD is transferred to acceptor proteins producing an acceptor-linked monomer or polymer. Nuclear ADP-ribosyl transferase discovered by Chambon et al. (1966) is established to be a chromatin-linked enzyme synthesizing a protein-bound homopolymer. Histones (Okazaki et al., 1980a; Poirier et al., 1982) and various non-histone proteins serve as acceptor proteins for the elongation of poly(ADP-ribose) chains. Apparently, the predominant acceptor is the enzyme itself, i.e., ADP-ribosyl transferase (Okazaki et al., 1980b; Kawaishi et al., 1981).

Enzymatic transfer of ADP-ribose from NAD to acceptor proteins has also been reported in free messenger ribonucleoprotein particles (Thomassin et al., 1985) in mitochondria of rat liver and testis (Kun et al., 1975; Burzio et

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Table I: ADP-ribosyl Transferase and NAD Glycohydrolase Activities in Different Mitochondrial Fractions[a]

	mitochondria		mitoplasts		outer membrane		soluble intermembrane proteins	
	ADPRT	NADase	ADPRT	NADase	ADPRT	NADase	ADPRT	NADase
sp act.	23.75 ± 1.76	7.01 ± 0.26	24 ± 3.4	5.87 ± 0.3	62.3 ± 10.7	6.97 ± 0.37	8.77 ± 2.2	0.095 ± 0.001
total act.	4870 ± 791	1437 ± 5.3	2880 ± 814	842 ± 4.3	374 ± 86	41.8 ± 2.2	255 ± 47	2.76 ± 0.29
recovery	100	100	60	58	7	3	5.2	0.2

[a] The specific activity of ADPRT was expressed as picomoles of ADP-ribose incorporated for 10 min at 37 °C. The specific activity of NADase was expressed as nanomoles of ethano-NAD hydrolysed per minute at 37 °C. Mean values ± SD from five independent experiments are given. Mitochondria or mitoplasts were used at 500 μg of protein per assay medium. Outer membrane or soluble intermembrane proteins were used at 100 μg of protein per assay.

al., 1981) as well as in *Xenopus oocytes* mitochondria (Burzio et al., 1979).

There seems a major difference between nuclear DNA dependent ADP-ribosyl transferase and mitochondrial ADP-ribosyl transferase activity. The latter produces mainly mono-ADP-ribosylation in a rather small degree (Kun & Kirsten, 1982). It has been suggested that in submitochondrial particles—derived from mitochondria treated with digitonin and subsequent sonication—NAD glycohydrolase generates ADP-ribose from NAD and this ADP-ribose is transferred to proteins by a nonenzymatic mechanism (Hilz et al., 1984).

It has however been shown that a high amount of NAD glycohydrolase activity is present in intact mitochondria as well as in submitochondrial particles (Kun & Kirsten, 1982) while the highest incorporation of labeled ADP-ribose has been observed in the soluble extract of rat liver mitochondria (Hilz et al., 1984).

This paper deals with the distribution of NAD glycohydrolase and ADP-ribosyl transferase activities in submitochondrial fractions. It is observed here that NAD glycohydrolase and ADP-ribosyl transferase activities are present in mitochondria. ADP-ribosyl transferase activity appeared to be lower than NAD glycohydrolase activity. However, the occurrence of ADP-ribosylation when NAD glycohydrolase was inhibited and the existence of two or more ADP-ribose moieties bound to protein favor the notion that the transfer of ADP-ribose to acceptor proteins proceeds enzymatically and is catalyzed by ADP-ribosyl transferase.

Materials and Methods

Isolation of Mitochondria. All the steps were carried out at 4 °C. Adult rats of the Wistar strain were used. Six rats were fasted overnight and killed by decapitation. Mitochondria were isolated from 40–50 g of rat liver as described previously (Schnaitman & Greenawalt, 1968) except that the medium contained 0.25 M sucrose, 10 mM Tris-HCl,[1] pH 7.4, 1 mM EDTA, and 0.5 mM PMSF. The nuclear contamination was excluded by successive centrifugations at a low speed, followed by five washings each time sedimenting at 12000*g* for 10 min. The final pellet was resuspended in the same medium at a protein concentration of 100 mg/mL.

Isolation of Submitochondrial Fractions. The separation of mitochondrial inner membrane from the outer membrane was carried out as described previously (Greenawalt, 1974). A total of 0.12 mg of digitonin/mg of mitochondrial protein was used to remove the outer mitochondrial membrane. The resulting intact mitoplasts were centrifuged at 12000*g* for 10 min, and the pellet was resuspended in isolation medium. The supernatant was centrifuged at 145000*g* for 60 min. The pellet thus obtained was suspended in a minimal volume and served as the outer membrane preparation. The resulting supernatant was removed with a Pasteur pipet, which formed the soluble intermembrane fraction.

Assay of ADP-ribosyl Transferase Activity. The standard reaction mixture contained 100 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 0.4 mM dithiothreitol, and 100 μM [^{32}P]NAD (100 cpm/pmol) in a total volume of 125 μL. The reaction was carried out for 10 min at 37 °C and terminated by the addition of 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate. The precipitate was collected and washed by filtration on glass-fiber GFB Whatman filters, and radioactivity was quantified by liquid scintillation. One unit of ADP-ribosyl transferase activity was defined as the amount catalyzing the incorporation of 1 pmol of ADP-ribose into acid-insoluble material per 10 min at 37 °C.

Fluorometric Assay of NAD Glycohydrolase Activity. NAD glycohydrolase activity was determined as previously described (Muller et al., 1983). The reaction mixture contained 50 μM 1,N^6-etheno-NAD in 10 mM potassium phosphate, pH 7.4, in a total volume of 2 mL. The excitation and emission wavelengths were 310 and 410 nm, respectively. One unit of NAD glycohydrolase activity was defined as the amount of enzyme that hydrolyzes 1 nmol of 1,N^6-etheno-NAD/min under the saturating conditions. Activity was determined by the procedure of Wilkinson (1961) with the use of a BASIC program for a microcomputer.

Lithium Dodecyl Sulfate Gel Electrophoresis. The electrophoretic separation of [^{32}P]NAD-labeled mitochondrial proteins by LDS gel electrophoresis was performed according to the procedure of Jones et al. (1981) as modified by Huletsky et al. (1985). Mitochondrial proteins were ADP-ribosylated at 37 °C for 1 h with 100 μM [^{32}P]NAD in the same buffer as described under Assay of ADP-ribosyl Transferase Activity. The ADP-ribosylated proteins were precipitated with trichloroacetic acid for 2 h at 0 °C. Pellets were rinsed twice with cold ether and resuspended in LDS solubilization buffer for 2 h at room temperature (Huletsky et al., 1985). Electrophoresis was performed at 15 mA for 16 h with a slab gel thickness of 1.5 mm. Gels were stained with 0.1% Coomassie Brillian Blue R-250. After being destained, gels were dried and submitted to autoradiography with Kodak XAR-5 films.

Chain Length Determination. The average chain length of the polymer was determined as previously described by Okazaki et al. (1980b). After thin-layer chromatography on PEI–cellulose, the radioactivity was determined directly by the use of Berthold linear analyzer L.B. 2820.

Protein Determination. Proteins were assayed by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results and Discussion

The ADP-ribosyl transferase activity was determined in

[1] Abbreviations: LDS, lithium dodecyl sulfate; INH, isonicotinic acid hydrazide; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; ADPRT, ADP-ribosyl transferase; NADase, NAD glycohydrolase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PEI, poly(ethylenimine).

Table II: Effect of Nicotinamide or 3-Aminobenzamide on ADPRT and NAD Glycohydrolase Activities into Mitochondrial Fractions[a]

	nicotinamide (mM)	% of residual ADPRT activity	% of residual NADase activity	3-aminobenzamide (mM)	% of residual ADPRT activity	% of residual NADase activity
mitochondria	1	82	20	1	92	ND
	10	55	ND	10	78	ND
mitoplasts	1	90	ND	1		ND
	10	66		10	82	ND
outer membrane	1	100	5	1	100	ND
	10	90		10	88	
soluble intermembrane proteins	1	90	48	1	90	ND
	10	86	ND	10	88	ND

[a] Experimental conditions were the same as in Table I. Inhibitors at the indicated concentrations were added in the assay medium prior to the substrate. Values are the means of three separate experiments. ND = nondetected.

intact mitochondria and in different submitochondrial fractions (Table I). The highest incorporation of ADP-ribose from NAD into mitochondrial proteins was observed in mitoplasts. In terms of specific activity, ADP-ribosyl transferase was highest in the outer membrane as prepared here. Digitonin treatment (0.12 mg/mg of protein) seems to solubilize ADP-ribose acceptors present in the membrane and thus favors the transfer of ADP-ribose into proteins. These data do indicate that in the outer mitochondrial membrane preparation there is a striking increase of ADPRT specific activity. This may be attributed to the separation of mitoplasts which contain a rather high amount of proteins as compared to the outer membrane.

The hydrolysis of NAD by NAD glycohydrolase was evaluated in the different mitochondrial fractions analyzed (Table I) by use of a sensitive assay of 1,N^6-etheno-NAD and the release of 1,N^6-etheno-ADP-ribose measured by fluorometric emission. The NAD glycohydrolase activity appeared to be much higher than ADP-ribosyl transferase activity. When 7 nmol of NAD was cleaved per milligram of mitochondrial protein per minute by NAD glycohydrolase, the extent of ADP-ribosylation was only 2.3 pmol min^{-1} (mg of protein)$^{-1}$.

These results are in agreement with Richter and co-workers (Richter et al., 1983), reporting that the ADP-ribosyl transferase activity appears to be lower than the potential NAD glycohydrolase activity. The bulk of NAD glycohydrolase activity was found in mitoplasts as compared to other mitochondrial fractions (Table I). The presence of this high activity in the outer membrane may be due partially to lysosomal or microsomal contamination, which contains endogenous NAD glycohydrolase activities (Bock et al., 1971; Mellors et al., 1975).

The existence of ADP-ribosyl transferase activity in mitochondria was further supported by the experiments in Table II. While NAD glycohydrolase activity was strongly inhibited by nicotinamide or 3-aminobenzamide, ADP-ribose from labeled NAD was still incorporated into mitochondrial proteins. The incorporation is lower than in the controls without nicotinamide or 3-aminobenzamide since these compounds also inhibit mitochondrial ADP-ribosyl transferase, although at a much lower extent than the NAD glycohydrolase. It is known that nuclear ADP-ribosyl transferase activity is strongly inhibited by nicotinamide or 3-aminobenzamide.

Table II shows that 3-aminobenzamide inhibited the NAD glycohydrolase activity in mitochondria, mitoplasts, the outer membrane preparation, and the intermembrane space. In different mitochondrial fractions, NAD glycohydrolase activity was blocked with 1 mM 3-aminobenzamide whereas 10 mM nicotinamide (Table II) was required to produce a similar effect; the mitochondrial ADP-ribosyl transferase was slightly reduced at these concentrations, but at least 80% of this enzymatic activity still remained. This was further supported by LDS–polyacrylamide gel electrophoresis followed by autoradiography (Figure 1); despite the inhibition of NAD glycohydrolase, the transfer of ADP-ribose to the major protein of 50–55 kDa as well as to a minor one of 30 kDa still occurred.

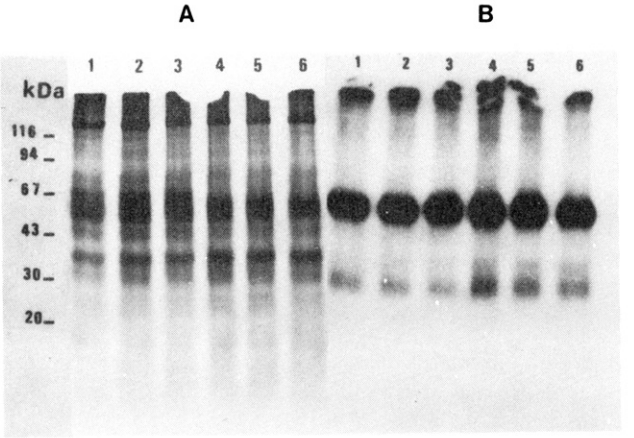

FIGURE 1: LDS–polyacrylamide gel electrophoresis (10%) of mitochondria or mitoplasts showing the pattern of protein ADP-ribosylation. (A) Gel stained with Coomassie Blue: (Lane 1) mitochondria (100 μg); (lane 2) mitochondria + 10 mM nicotinamide; (lane 3) mitochondria + 10 mM 3-aminobenzamide; (lane 4) mitoplasts (100 μg); (lane 5) mitoplasts + 10 mM nicotinamide; (lane 6) mitoplasts + 10 mM 3-aminobenzamide. (B) Autoradiogram of the same gel exposed to Kodak XAR-5 film.

It has been reported that isonicotinic acid hydrazide (INH) is a potent inhibitor of mitochondrial NAD glycohydrolase (Hilz et al., 1984). The effect of INH on ADP-ribosylation in submitochondrial fractions as well as on nuclear ADP-ribosyl transferase activity was studied. We have shown that ADP-ribosylation of proteins in mitochondria or in mitoplasts was partially inhibited by 10 mM INH (53%), while nuclear ADP-ribosyl transferase activity (Mandel et al., 1977) was inhibited by 48%. Under similar conditions, mitochondrial NAD glycohydrolase activity was entirely inhibited. Thus, it may be argued that the inhibition of NAD glycohydrolase activity by INH is not a specific one. The partial inhibition of the transfer of ADP-ribose to the proteins from labeled NAD by the nuclear enzyme supports such a view. Moreover, in view of the observation that ADP-ribosylation in mitochondria may occur through a ADP-ribosyl transferase reaction without involvement of NAD glycohydrolase, it may be safe to emphasize that this reaction is enzymatic. To our knowledge nonenzymatic oligo(ADPR) transfer to proteins was never reported.

Further support for the presence of ADP-ribosyl transferase activity in mitochondria came from experiments in which NAD glycohydrolase was separated from solubilized submi-

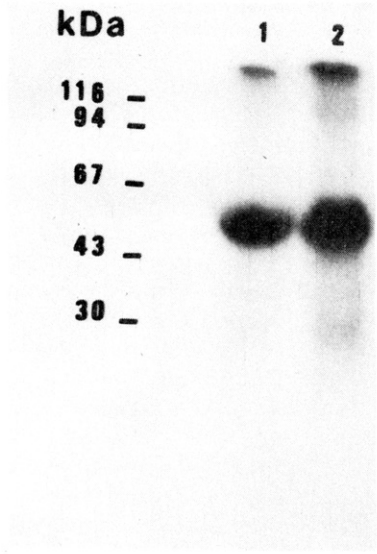

FIGURE 2: LDS–polyacrylamide gel electrophoresis (12%) of the fraction bound to hydroxylapatite chromatography. Gel was run for 16 h at 15 mA. Autoradiography of the gel: (lane 1) ADP-ribose-labeled proteins at 10 μM [^{32}P]NAD; (lane 2) same fraction incubated with 100 μM [^{32}P]NAD. Molecular weights ($\times 10^{-3}$) of markers are indicated with dashes.

tochondrial proteins by hydroxylapatite chromatography. In these conditions, NAD glycohydrolase passes through the column (Moser et al., 1983). The bound material was eluted by employing 0.5 M KCl buffered with 0.5 M potassium phosphate, pH 7.4. The eluted material was still able to transfer ADP-ribose to proteins from labeled NAD. The ADPRT activity recovered in the eluted material ranged between 20 and 25 pmol of ADP-ribose incorporated per milligram of protein under the same conditions as in Table I.

Figure 2 illustrates the LDS–polyacrylamide gel electrophoresis followed by autoradiography of the fraction eluted from the hydroxylapatite column with 0.5 M potassium phosphate and 0.5 M KCl buffer. The incorporation of ADP-ribose from NAD into a major protein band of 50–55 kDa clearly appeared. The extent of ADP-ribosylation was distinctly enhanced when the concentration of NAD was increased in the assay medium (Figure 2, lane 2).

Our data are in contradiction with the observation of Hilz and co-workers (Hilz et al., 1984). These authors have shown that when NAD glycohydrolase activity was separated by Blue Sepharose chromatography, the transfer of ADP-ribose from NAD to the proteins did not occur. It is difficult to ascribe any rational between the observations from two laboratories at the this moment.

According to Kun and Kirsten (1982), ADP-ribosylation proceeds involving either ADP-ribosyl transferase or NAD glycohydrolase, which cleaves selectively NAD and liberates ADP-ribose. The latter has been considered to bind protein covalently by a schiff base type of reaction (Kun et al., 1976). Since blocking the mitochondrial NAD glycohydrolase activity does not impair the ADP-ribosylation, the existence of mitochondrial ADP-ribosyl transferase with its intact enzymatic role can be asserted.

The observation (Hilz et al., 1984) that very little ADPRT activity was present in intact liver mitochondria while sonicated submitochondrial particles catalyzed the reaction of ADP-ribose incorporation into proteins is in sharp contradiction to the study reported here. From the study (Hilz et al., 1984), it is not clear whether the NAD glycohydrolase activity was observed in intact mitochondria and what was the extent of ADPRT activity in sonic and submitochondrial particles. It is well-known that sonication (Malviya et al., 1968) of intact mitochondria or digitonin fragments produces an inside-out orientation of the inner membrane, i.e., inner membrane subunits that lay interior become exterior to the vesicular structures. Thus according to Hilz et al. it is the inside-out membrane configuration of mitochondria that favors the transfer of ADP-ribose to proteins. We observe ADPRT as well as NAD glycohydrolase activity in intact mitochondria and in mitoplasts. Therefore, it may be argued that these activities are expressed in the native membrane configuration of mitochondria.

The average chain length of ADP-ribose units bound to mitochondrial proteins with purified snake venom phosphodiesterase (Oka et al., 1978) appeared to be two to three (2.56) residues. The formation of ADP-ribose oligomers linked to the protein cannot be performed by NAD glycohydrolase, since this enzyme is only able to produce nonenzymatic mono(ADP-ribosylation). Thus, the formation of an ADP-ribose chain length of more than two residues favors the contention that the ADP-ribosylation reaction is catalyzed enzymatically by an ADP-ribosyl transferase. Recently (Tanuma et al., 1986) the presence of (ADP-ribose) glycohydrolase II activity has been observed whereas about 5% of this activity was identified in mitochondria. The physiological significance of this activity is difficult to evaluate at this stage. Nonetheless, the effect of this enzyme on regulation of turnover of ADP-ribose chain length in mitochondria cannot completely be ruled out.

Nonenzymatic ADP-ribosylation of poly(ADP-ribose) (Ikejima & Gill, 1985) can occur at high salt concentration or at alkaline pH by the hydrolysis of NAD through a glycosidic bond. However, the velocity of this reaction is very slow as compared to that of the ADP-ribosylation reaction.

In conclusion, we report that the ADPRT and NAD glycohydrolase activities are present in various mitochondrial compartments. The ADP-ribosylation reaction proceeds without the involvement of an NAD glycohydrolase system, and the ADP-ribose chain length consists of more than one residue. Therefore, ADP-ribosylation in mitochondria appears to take place through an enzymatic reaction. The biological significance of ADP-ribosylation in mitochondria remains unknown at this time.

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Registry No. NAD-protein ADP-ribosyl transferase, 58319-92-9; NAD glycohydrolase, 9032-65-9.

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Inhibition of Thiaminase I from *Bacillus thiaminolyticus*. Evidence Supporting a Covalent 1,6-Dihydropyrimidinyl-Enzyme Intermediate†

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ABSTRACT: Thiaminase I from *Bacillus thiaminolyticus* strain Matsukawa et Misawa is completely and irreversibly inhibited by treatment with 4-amino-6-chloro-2-methylpyrimidine. Inhibition is a time-dependent first-order process, exhibiting a half-time of 4 h at an inhibitor concentration of 5 mM. A specific active-site-directed inactivation is supported by protection of the enzymatic activity in the presence of the substrates thiamin and quinoline as well as by the observation that a stoichiometric amount of inorganic chloride is released during inactivation. 4-Amino-5-(anilinomethyl)-6-chloro-2-methylpyrimidine, which resembles the structure of the product of base exchange of thiamin with aniline, inactivates thiaminase approximately 2 orders of magnitude faster. Inactivation is again complete and irreversible and is a time-dependent first-order process, in this case exhibiting saturation at low inhibitor concentrations (K_I = 96 μM). Enzyme inactivation can be explained as the result of displacement of chloride from the chloropyrimidine by a nucleophile at the enzyme active site. The inactivation suggests that the Zoltewicz–Kauffman model of bisulfite-catalyzed thiamin cleavage [Zoltewicz, J. A., & Kauffman, G. M. (1977) *J. Am. Chem. Soc. 99*, 3134–3142], which calls for the reversible nucleophilic addition of catalyst across the 1,6 double bond of thiamin's pyrimidine ring, may be applicable to thiaminase as well.

Thiaminases are enzymes that destroy thiamin by cleaving the vitamin between the pyrimidinylmethyl group and the thiazole (Fujita, 1954; Murata, 1965, 1982). Thiaminase I (EC 2.5.1.2) catalyzes a base exchange reaction in which the (4-amino-2-methyl-5-pyrimidinyl)methyl group of thiamin is transferred to any one of a variety of organic nucleophiles, e.g., aniline, quinoline, pyridine, and cysteine. A related activity, thiaminase II, catalyzes the transfer of the pyrimidinylmethyl specifically to water. Scheme I shows the reaction catalyzed by thiaminase I between thiamin and aniline.

Thiaminase I occurs in such diverse organisms as ferns, fish, mollusks, crustacea, and microorganisms (Fujita, 1954; Murata, 1965, 1982). Although the biological function of the enzyme is unknown, it is of some veterinary importance, as it has been demonstrated that animals fed a diet containing excessive quantities of the enzyme develop the neurological symptoms of thiamin deficiency (Green et al., 1941; Woolley, 1941). Production of thiaminase by ruminal microorganisms has been suggested to be the cause of certain thiamin deficiencies in livestock (Edwin & Jackson, 1970; Edwin et al.,

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