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ADP-ribosylation in inner membrane of rat liver mitochondria

(protein modification/pyridine nucleotides/NAD^+ glycohydrolase/ADP-ribosyltransferase)

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ABSTRACT **NAD^+ glycohydrolase activity is found at high levels in submitochondrial particles. It leads to the reaction products ADP-ribose, nicotinamide, and small amounts of 5′-AMP. Furthermore, submitochondrial particles catalyze the exchange reaction: [*adenosine*-^{14}C]ADP-ribose + $NAD^+ \rightleftharpoons$ [*adenosine*-^{14}C]-NAD^+ + ADP-ribose. When submitochondrial particles are incubated with NAD^+, mono(ADP-ribosyl)ation of protein molecules migrating with an apparent molecular weight of 30,000 in sodium dodecyl sulfate/polyacrylamide gel electrophoresis is demonstrable. Inhibitor studies suggest attachment of ADP-ribose to arginine residues. ADP-ribose bound to submitochondrial particles is rapidly turning over. The release of ADP-ribose from the protein is probably enzyme catalyzed. The rapid turnover, the specificity of the modification, and the inhibition of ADP-ribosylation by ATP and nicotinamide suggest a regulatory role of mono(ADP-ribosyl)ation of a protein in the inner mitochondrial membrane.**

ADP-ribosylation is a posttranslational protein modification receiving growing interest. Several reviews on both the biological and methodological aspects have been published (1–3). ADP-ribose is attached to acceptor proteins as a monomer, oligomer, or polymer. Mono(ADP-ribosyl)ation has so far been found to be catalyzed mainly by prokaryotic enzymes—e.g., diphtheria toxin (4), *Pseudomonas* exotoxin A (5), choleragen (6), and *Escherichia coli* heat-labile enterotoxin (7). Catalysis of mono-ADP-ribosylation by enzymes from rat liver (8) and erythrocytes from turkey and man (9, 10) has also been reported. Poly(ADP-ribosyl)ation, on the other hand, is found mainly in the nuclei of eukaryotic cells. The accumulating evidence indicates a close relationship between poly(ADP-ribosyl)ation and chromatin activities (11, 12).

During our studies on the mechanism of hydroperoxide-induced release of calcium from rat liver mitochondria we observed the hydrolysis of intramitochondrial pyridine nucleotides at the β-*N*-glycosidic bond between nicotinamide and ADP-ribose in intact mitochondria (13, 14). The hydrolysis of NAD^+ is accompanied by a covalent protein modification at the inner side of the inner mitochondrial membrane by a NAD^+-derived compound. Pyridine nucleotide hydrolysis, protein modification, and release of calcium from rat liver mitochondria are inhibited by ATP (15). On the basis of these findings we put forward the hypothesis that this covalent modification is part of a mechanism controlling calcium release.

Kun *et al.* (16, 17) have described ADP-ribosylation of a soluble protein derived from sonicated mitochondria. In recent papers Hilz and co-workers (18, 19) analyzed the distribution of mono- and poly(ADP-ribose) in liver cells. We describe here a covalent modification by mono(ADP-ribose) of a protein in the inner mitochondrial membrane. The modification is found almost exclusively in protein molecules with a molecular weight of 30,000 in $NaDodSO_4$/polyacrylamide gel electrophoresis. The modifying group has a high turnover. The fast removal of ADP-ribose from the protein is possibly also catalyzed by a membrane-bound protein.

MATERIALS AND METHODS

Isolation of Mitochondria and Preparation of Submitochondrial Particles. Liver mitochondria from overnight fasted female Wistar rats (Pathophysiologisches Institut, Bern, Switzerland) were isolated according to the centrifugation method described by Bustamante *et al.* (20), using 210 mM mannitol, 70 mM sucrose, 5 mM Hepes at pH 7.4 (mannitol/sucrose/Hepes buffer), 1 mM EDTA, and 0.5 mg of bovine serum albumin per ml as isolation medium. Mitochondria (80–100 mg of protein per ml) were then treated with purified digitonin (0.65 mg/10 mg of protein) as described by Kun *et al.* (21). They were subsequently washed twice in mannitol/sucrose/Hepes buffer by centrifugation for 10 min at 9,000 × *g*. The resulting "mitoplast" pellet was suspended in 50 mM KCl/5 mM $MgCl_2$/3.3 mM KF/1 mM mercaptoethanol/20 mM Hepes, pH 6.5, at a protein concentration of 20 mg/ml and exposed to sonic oscillation for 1.5 min (Branson B 30 sonifier). Larger particles were removed by 10-min centrifugation at 9,000 × *g*. Submitochondrial particles were collected as a pellet after centrifugation for 35 min at 100,000 × *g*. The pellet was taken up in the buffer used for sonication, centrifuged for 35 min at 100,000 × *g*, suspended in this buffer to a final protein concentration of 20–30 mg/ml, and stored in liquid nitrogen. The protein content was determined by the biuret method with bovine serum albumin as a standard.

Standard Incubation Procedure. Submitochondrial particles (10 mg of protein per ml) were incubated in a medium containing 50 mM KCl, 5 mM $MgCl_2$, 3.3 mM KF, 1 mM mercaptoethanol, 20 mM Hepes at pH 6.5, and 1 mM NAD^+ at 25°C. At the required times, 20-μl aliquots were withdrawn and proteins were precipitated with 500 μl of 12% (wt/vol) $HClO_4$. The precipitated proteins were collected on Whatman GF/A filters and rinsed with 10 ml of 12% $HClO_4$. The radioactivity retained on the filters was determined by liquid scintillation counting.

Preparation of [*adenine*-2,8-^{3}H]ADP-ribose. [*adenine*-2,8-^{3}H]NAD^+ (8 nmol, 4.4×10^7 dpm) was incubated with 71 μg of NAD^+ glycohydrolase (0.04 unit) in 0.1 M potassium phosphate buffer, pH 7.5, at 37°C for 20 min. The reaction volume was 200 μl. The reaction was terminated by the addition of 25 μl of 12% $HClO_4$. After neutralization with 15 μl of 3 M K_2CO_3 in 0.5 M triethanolamine the sample was applied to an AG 1-X4 column (13 × 1.2 cm) and eluted with two formic acid gra-

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Abbreviation: kDa, kilodalton(s).

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dients as shown in Fig. 3. The fractions corresponding to ADP-ribose were collected, applied to a Sephadex G-10 column (28 × 2 cm), and eluted with H_2O. The eluted [*adenine*-2,8-^{3}H]ADP-ribose, free of formic acid, was lyophilized and taken up in 1 ml of water/ethanol, 1:1 (vol/vol). The recovery of [*adenine*-2,8-^{3}H]ADP-ribose was 60–70% of the starting [*adenine*-2,8-^{3}H]NAD^+.

Materials. [*adenine*-2,8-^{3}H]NAD^+ and [*adenosine*-U-^{14}C]-NAD^+ were obtained from New England Nuclear; [*adenylate*-^{32}P]NAD^+ was from The Radiochemical Centre, Amersham; oligomycin, ATP, 5′-AMP, ADP-ribose, arginine methyl ester, and NAD^+ glycohydrolase isolated from *Neurospora crassa* were purchased from Sigma; NAD^+, ADP, and snake venom phosphodiesterase I were obtained from Boehringer Mannheim. The phosphodiesterase was purified according to ref. 22. Digitonin, phenylglyoxal, and butanedione were obtained from Fluka (Buchs, Switzerland). The ion-exchange resin, AG 1-X4, 200–400 mesh, formate form, was purchased from Bio-Rad.

RESULTS

Intact mitochondria catalyze the hydrolysis of endogenous NAD^+ and $NADP^+$ in the presence of Ca^{2+} (13, 14). Studies with submitochondrial particles have localized a NAD^+ glycohydrolase† at the inner side of the inner mitochondrial membrane. NADH, NADPH, or $NADP^+$ do not serve as substrates for this enzyme (14). In the present investigation we observed that the activity of the enzyme is linearly dependent on substrate concentration up to 1 mM NAD^+ and is probably still submaximal at the highest substrate concentration tested here (2.5 mM). Table 1 shows that the enzyme is inhibited by several agents. Of special interest is the inhibition by the physiological compounds ATP [indicated previously (15)] and particularly nicotinamide. Calcium, EDTA, and arginine methyl ester have very little influence on the glycohydrolase activity. In the case of butanedione the observed inhibition is explained by the action of the sodium borate buffer alone. The second α-diketonic reagent, phenylglyoxal, causes only a minor inhibition. Both observations suggest that arginine residues play no important role in the enzyme-catalyzed hydrolysis of NAD^+. In separate experiments (not shown here) it was found that the main products of the hydrolysis under standard conditions are nicotinamide, ADP-ribose, and a small amount of 5′-AMP.

The enzymatic hydrolysis of NAD^+ catalyzed by submitochondrial particles is accompanied by a covalent modification of these membranes (Fig. 1). Considerable binding is, however, also observed during incubation of submitochondrial particles with ADP-ribose instead of NAD^+. The activity of the modification reaction was found to be linearly dependent on the concentration of NAD^+ up to the highest level tested (1 mM).

Modified submitochondrial particles were subjected to Na$DodSO_4$/polyacrylamide gel electrophoresis (Fig. 2) and analyzed for the distribution of protein and radioactivity. Most of the bound radioactivity was found in a band migrating at about M_r 30,000. After electrophoresis the labeled protein migrating at M_r 30,000 was cut out and quantitatively eluted from the gel. Treatment with snake venom phosphodiesterase (for details see legend to Fig. 3) released virtually all radioactivity. Analysis on an AG 1-X4 column (Fig. 3) showed that 78% of this radioactivity migrated with 5′-AMP. The minor component migrating close to ADP was not further analyzed. It was, however, noticed in separate experiments that formic acid causes some degradation of ADP-ribose. The fact that most of the radioactivity liberated from the modified protein by phosphodiesterase treatment is 5′-AMP suggests that the protein is modified by mono(ADP-ribose). The absence of poly(ADP-ribose)

Table 1. NAD^+ glycohydrolase activity in submitochondrial particles

Conditions	Enzyme activity: nmol NAD^+ hydrolyzed in 50 min per mg protein	Enzyme activity: % of control
Control	163 ± 12 (7)	
ATP, 10 mM + oligomycin, 4 μg/mg of protein	82 ± 38 (6)	50
ADP, 10 mM + oligomycin, 4 μg/mg of protein	132 ± 10 (5)	81
AMP, 10 mM	140 ± 10 (2)	86
Nicotinamide, 10 mM	32 ± 3 (2)	20
Ca^{2+}, 20 mM	154 ± 8 (2)	94
EDTA, 20 mM	146 ± 4 (2)	90
Phenylglyoxal, 10 mM	123 ± 4 (3)	75
Butanedione, 50 mM, in 50 mM borate buffer, pH 8.0	80 ± 3 (3)	49
Borate buffer, pH 8.0	74 ± 6 (3)	45
Arginine methyl ester, 10 mM	161 ± 4 (2)	99

Submitochondrial particles (5 mg of protein per ml) were incubated under standard conditions in the presence of the effectors indicated. After 50 min the amount of NAD^+ remaining was determined with the alcohol dehydrogenase assay (24). Values are given as mean ± SD. Figures in parentheses show the number of experiments.

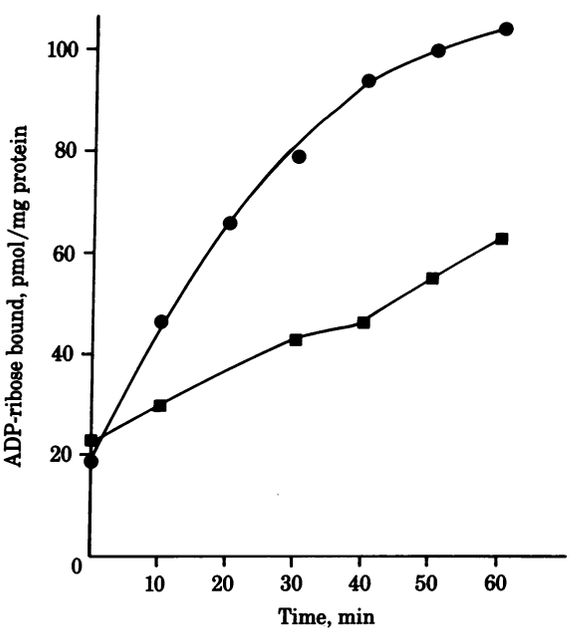

FIG. 1. Covalent modification of submitochondrial particles in the presence of [*adenine*-2,8-^{3}H]NAD^+ and [*adenine*-2,8-^{3}H]ADP-ribose. Submitochondrial particles were incubated under standard conditions in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD^+ at 2.5 × 10^4 dpm/nmol (●) or 1 mM [*adenine*-2,8^{3}H]ADP-ribose at 2.4 × 10^4 dpm/nmol (■). The radioactivity bound to submitochondrial particles was determined after precipitation of the proteins with $HClO_4$.

† Enzymes that cleave the nicotinamide-glycosyl bond of NAD^+ (EC 3.2.2.5) have been categorized as either NAD^+ glycohydrolase (NADase) or ADP-ribosyltransferase. The distinction, however, is not entirely clear, because transferases—e.g., diphtheria toxin—can hydrolyze NAD^+ in the absence of the normal acceptor. Also, the membrane-bound NADases from pig brain or calf spleen, besides hydrolyzing NAD^+, also catalyze the exchange of the nicotinamide moiety with exogenous pyridines and imidazoles (23).

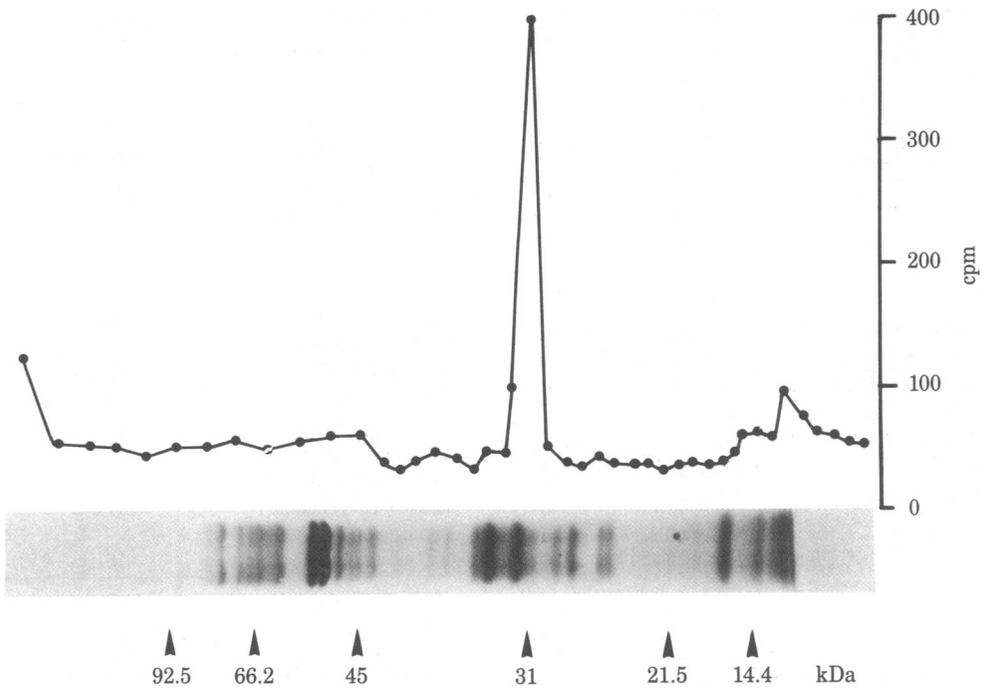

FIG. 2. $NaDodSO_4$ polyacrylamide gel electrophoresis of submitochondrial particles after incubation with [*adenine*-2,8-^{3}H]NAD^+. Submitochondrial particles were incubated as described in the legend of Fig. 1 in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD^+ (0.53×10^6 dpm/nmol). After 60 min of incubation the membranes were diluted with cold incubation buffer and centrifuged for 35 min at 100,000 × *g* to remove unbound radioactive material. The pellet was dissolved in $NaDodSO_4$ and subjected to polyacrylamide gel electrophoresis according to Laemmli (25). One part of the gel was stained with Coomassie blue. The other part was sliced at 1.5-mm intervals, and the amount of protein-bound radioactivity was determined. Lower figures indicate the apparent molecular masses (in kilodaltons) determined with protein standards (Bio-Rad).

was further evidenced by the finding that the radioactive compound released at pH 10 (see below) could not be precipitated by acid. The possibility of a modification by poly(ADP-ribose) residues longer than 5 monomers (1) is therefore excluded.

In contrast to the NAD^+ glycohydrolase activity (see above), ADP-ribosylation is prevented (Fig. 4) by the arginine blocking reagent phenylglyoxal and largely by butanedione and arginine methyl ester. In other experiments, after incubation under standard conditions for 50 min, the membranes were collected by centrifugation and resuspended in the presence of various effectors. It was found that at pH 6.0 the label was not removed by 400 mM hydroxylamine during an incubation period of 2 hr. In the presence of 100 mM picrylsulfonic acid, pH 8.5, 75% of the label was lost from the protein within 30 min. At pH 10,

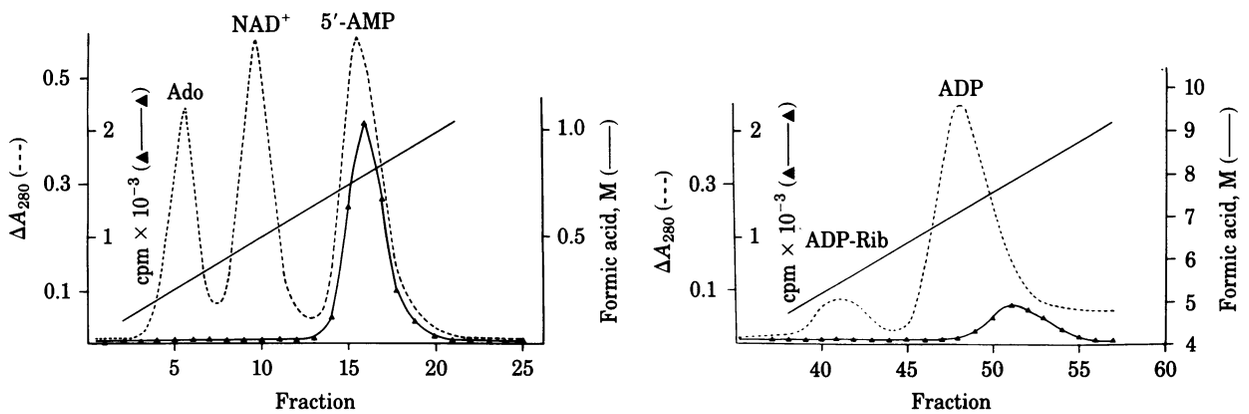

FIG. 3. Analysis of the radioactive material bound to the 30-kDa protein of submitochondrial particles. Submitochondrial particles were incubated with [*adenylate*-^{32}P]NAD^+ (1.63×10^5 cpm/nmol) and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as shown in Fig. 2. Gel pieces containing the bulk of the radioactivity (≈30 kDa) were cut out, homogenized, and suspended in 0.1 M Tris·HCl, pH 7.4/0.01% $NaDodSO_4$. The labeled protein (13,500 cpm) was extracted from the gel by vigorous shaking for 2 hr at 20°C. Subsequently the suspension was centrifuged for 10 min at 12,000 × *g*. The supernatant, after filtration through quartz sand, was centrifuged for 30 min at 100,000 × *g*. The resulting supernatant (8.2 ml, 12,500 cpm) was treated with 6 μg of purified snake venom phosphodiesterase I in a medium containing 0.1 M Tris·HCl at pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, and 0.01% $NaDodSO_4$ for 60 min at 20°C. The reaction was terminated by heating (95°C, 10 min) and the denatured proteins were removed by centrifugation. The supernatant was lyophilized, taken up in 2 ml of 0.1 M Tris·HCl at pH 7.4, applied to a Sephadex G-10 column (12 × 0.7 cm), and eluted with the same buffer. The fractions containing radioactivity (9,300 cpm total) were collected, lyophilized, and taken up in 1 ml of H_2O. Subsequently, the sample was applied to an AG 1-X4 column (9 × 1.3 cm) together with adenosine (Ado), NAD^+, 5'-AMP, ADP, and ADP-ribose (ADP-Rib) as markers (2 μmol each). The column was eluted with two linear formic acid gradients, 0–1 M and 4–10 M. The volume of each fraction was 4.75 ml.

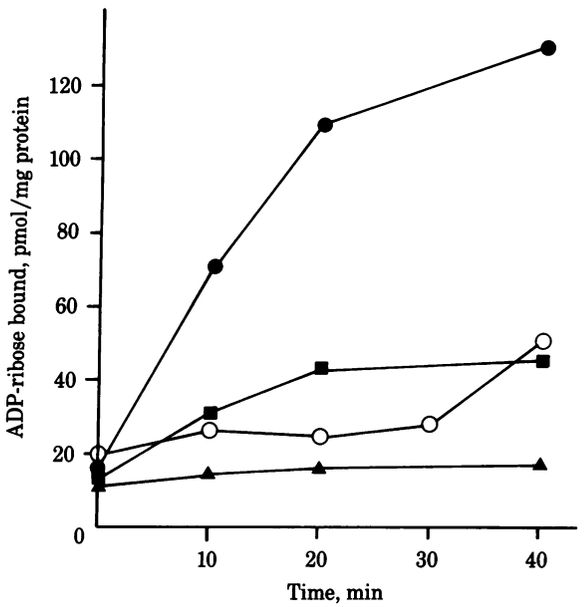

FIG. 4. Inhibition of ADP-ribosylation in submitochondrial particles by arginine blocking reagents and arginine methyl ester. Submitochondrial particles were incubated under standard conditions in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ (3.3×10^4 dpm/nmol). The protein-bound radioactivity was measured as described for Fig. 1. ●, No inhibitor present; ▲, in the presence of 10 mM phenylglyoxal; ■, in the presence of 50 mM butanedione and 50 mM borate buffer, pH 8.0; ○, in the presence of 10 mM arginine methyl ester.

80% of the label was lost within 30 min.

While about 160 nmol of NAD$^+$ is hydrolyzed, there is a net ADP-ribosylation of only about 100–150 pmol/mg of protein during incubation for 60 min under standard conditions (see above). This seemingly inefficient ADP-ribosylation can be explained in at least two ways: (*i*) The apparent high NAD$^+$ glycohydrolase activity represents a high ADP-ribosyltransferase activity ("on reaction"), which is followed by a somewhat slower, possibly enzymatic, removal of ADP-ribose from the protein ("off reaction"). (*ii*) ADP-ribosyltransferase activity is a minor pathway in comparison to the NAD$^+$ glycohydrolase activity. If the first interpretation were correct, blocking of the on reaction should result in a rapid loss of ADP-ribose from submitochondrial particles. NAD$^+$ glycohydrolase is inhibited to 50% of control activity by 10 mM ATP and to 20% of control activity by 10 mM nicotinamide (Table 1). The same amounts of inhibitors lead to a complete rapid loss of ADP-ribose with ATP, whereas the more effective glycohydrolase inhibitor nicotinamide causes only a partial loss (Fig. 5). Obviously, inhibition of the glycohydrolase cannot be the only reason for the decreased net binding of ADP-ribose to submitochondrial particles. On the contrary, there might exist an ATP-stimulated enzyme responsible for the removal of ADP-ribose.

The experiment shown in Fig. 6 investigates turnover of ADP-ribose directly. A slight modification (200 μM NAD$^+$, 17°C) of the standard incubation conditions was necessary because in some membrane preparations a loss of the ability to remove ADP-ribose had been observed when they were assayed under standard conditions. Submitochondrial particles were allowed to ADP-ribosylate with [^{3}H]NAD$^+$ as substrate until the net ADP-ribosylation had almost ceased. The incubation mixture was then divided and one half was supplemented with [^{14}C]-NAD$^+$ of a specific radioactivity equal to that of [^{3}H]NAD$^+$. It can be seen that during the incorporation of [^{14}C]ADP-ribose there is a loss of about 40% of the [^{3}H]ADP-ribose from submitochondrial particles, providing direct proof of a rapid turnover of ADP-ribose bound to submitochondrial particles.

Interestingly, in the above experiment not all [^{3}H]ADP-ribose is lost from submitochondrial particles after supplementation with [^{14}C]NAD$^+$. When, in a similar experiment, [^{3}H]-ADP-ribosylated membranes were supplemented with unlabeled NAD$^+$ an incomplete loss of the total radioactivity followed by further binding of radioactive ADP-ribose was ob-

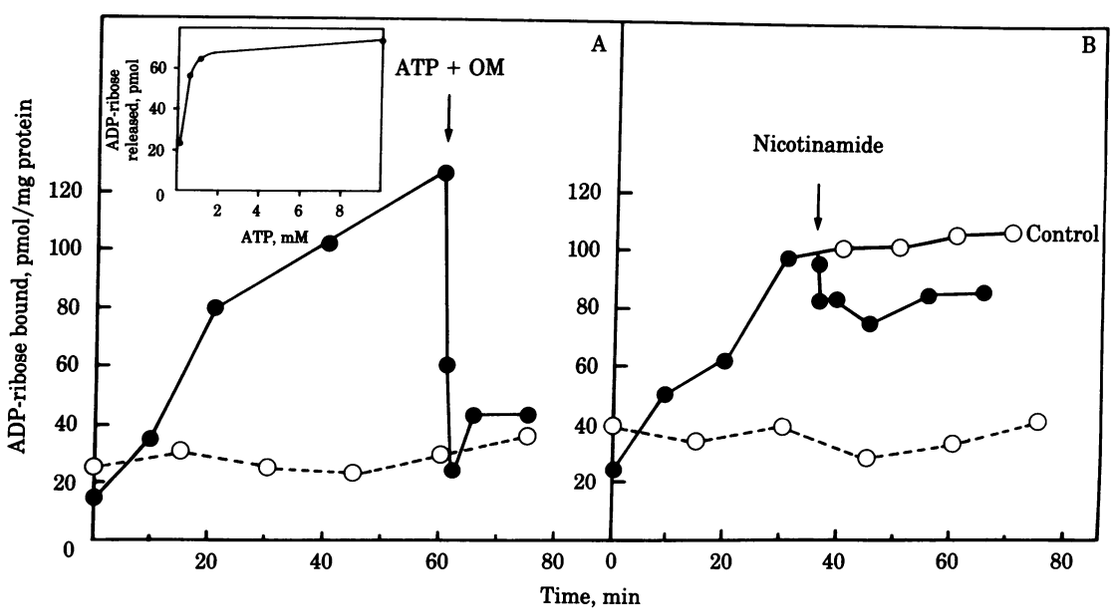

FIG. 5. Release of ADP-ribose bound to submitochondrial particles in the presence of the NAD$^+$ glycohydrolase inhibitors ATP and nicotinamide. Submitochondrial particles were incubated under standard conditions in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ (3.1×10^4 dpm/nmol). At the time indicated by the arrow 10 mM ATP together with 2 μg of oligomycin (OM) per mg of protein (*A*) or 20 mM nicotinamide (*B*) was added. The protein-bound radioactivity was determined as described for Fig. 1. Broken lines indicate activity in the presence of 10 mM ATP + 2 μg of oligomycin per mg of protein (*A*) or 20 mM nicotinamide (*B*) over the total incubation period. (*Inset*) Quantity of ADP-ribose released per mg of protein 1 min after addition of various amounts of ATP together with oligomycin.

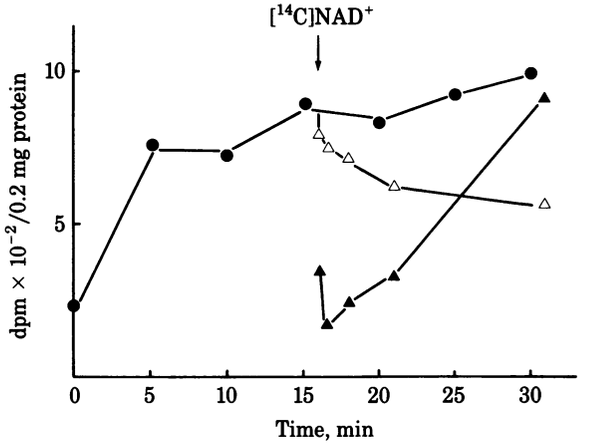

FIG. 6. Turnover of ADP-ribose bound to submitochondrial particles. Submitochondrial particles were incubated in standard medium in the presence of 0.2 mM [*adenine*-2,8-^{3}H]NAD$^+$ (4×10^5 dpm/nmol) at 17°C. At 16 min (arrow) one half of the incubation mixture was supplemented with 0.2 mM [*adenosine*-U-^{14}C]NAD$^+$ (3.5×10^5 dpm/nmol). The protein-bound radioactivity was determined as described for Fig. 1. ●, Protein-bound ^{3}H radioactivity without supplementation (control); △, protein-bound ^{3}H radioactivity after supplementation with [*adenosine*-U-^{14}C]NAD$^+$; ▲, protein-bound ^{14}C radioactivity after supplementation with [*adenosine*-U-^{14}C]NAD$^+$.

served. The finding that the decrease of the amount of radioactive ADP-ribose residues bound to submitochondrial particles is only transient after supplementation with unlabeled NAD$^+$ suggested that the mitochondrial NAD$^+$-splitting enzyme might catalyze an exchange of the ADP-ribose moiety of NAD$^+$ with free ADP-ribose. To test this possibility submitochondrial particles were allowed to hydrolyze radioactive NAD$^+$ almost completely and were then supplied with 1 mM unlabeled NAD$^+$. The incubation mixture was then analyzed for radioactive NAD$^+$ and radioactive ADP-ribose on thin-layer chromatography. After addition of unlabeled NAD$^+$ there was indeed a rapid formation of radioactive NAD$^+$ concomitant with a corresponding decrease in the amount of radioactive ADP-ribose. Within 1 min more than 10% of NAD$^+$ exchanged its ADP-ribose. This was followed by a decrease in the amount of radioactive NAD$^+$ and an increase in the amount of radioactive ADP-ribose.

DISCUSSION

Sensitivity towards phosphodiesterase I and subsequent product analysis allows the conclusion that the radioactive material bound to submitochondrial particles during incubation with NAD$^+$ labeled at the adenine moiety is mono(ADP-ribose), with possibly a very small contribution of oligo(ADP-ribose) chains. The ADP-ribose residues bound to submitochondrial particles are rapidly turning over. Turnover of ADP-ribose in the nuclear system, albeit much slower, has already been indicated (11, 26, 27). The minimal number of enzymes involved in the mitochondrial turnover of ADP-ribose cannot be precisely deduced from the present experiments. Our inhibitor studies could not, for example, reveal whether the enzyme that splits NAD$^+$ is identical with the protein that accepts ADP-ribose. Purification of the splitting enzyme and the ADP-ribose acceptor protein will help to solve this problem. The inhibitor studies do, however, strongly suggest an arginine residue as acceptor of ADP-ribose. Although nicotinamide is a better inhibitor of the glycohydrolase than is ATP, the release of ADP-ribose from submitochondrial particles caused by ATP is much faster and more extensive. In other experiments it was found that some aged particle preparations lost the ability to remove ADP-ribose upon supplementation with ATP and showed a severalfold higher net ADP-ribosylation activity. These findings are compatible with the existence of an ATP-stimulated enzyme that catalyzes the removal of ADP-ribose from submitochondrial particles.

The importance of ADP-ribosylation in the inner mitochondrial membrane for the function of mitochondria is presently not known. The specificity of the modification, the rapid turnover, and its regulation by physiological compounds such as ATP and nicotinamide, however, indicate that the mono-(ADP-ribosyl)ated protein has a regulatory function. The present and our previous studies suggest that the release of calcium from intact rat liver mitochondria is paralleled by ADP-ribosylation in the inner mitochondrial membrane. Both ADP-ribosylation and calcium release are inhibited by ATP. These studies therefore provide experimental support for the hypothesis (15) that ADP-ribosylation in the inner mitochondrial membrane triggers calcium release from rat liver mitochondria.

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