Identification of bovine liver mitochondrial NAD⁺ glycohydrolase as ADP-ribosyl cyclase

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The present investigation identifies bovine liver mitochondrial NADase (NAD⁺ glycohydrolase) as a member of the class of bifunctional ADP-ribosyl cyclases/cyclic ADP-ribose hydrolases, known to be potential second messenger enzymes. These enzymes catalyse the synthesis and degradation of cyclic ADP-ribose, a potent intracellular calcium-mobilizing agent. The mitochondrial enzyme utilized the NAD⁺ analogues nicotinamide guanine dinucleotide (NGD⁺) and nicotinamide hypoxanthine dinucleotide (NHD⁺) to form fluorescent cyclic purine nucleoside diphosphoriboses. ADP-ribosyl cyclase activity was also demonstrated using ³²P-labelled NAD⁺ as substrate. The identity of NADase and ADP-ribosyl cyclase was supported by their comigration in SDS/polyacrylamide gels. Cyclase activity was

visualized directly within the gel by detecting the formation of fluorescent cyclic IDP-ribose from NHD⁺. The enzyme catalysed the hydrolysis of cyclic ADP-ribose to ADP-ribose. Moreover, in the presence of nicotinamide and cyclic ADP-ribose the enzyme synthesized NAD⁺. Both the ADP-ribosyl cyclase and NADase activities of the enzyme were strongly inhibited by reducing agents. Treatment of the NADase with dithiothreitol caused the apparent inactivation of the enzyme. Subsequent removal of the reducing agent and addition of oxidized glutathione led to a partial recovery of enzymic activity. The results support a model for pro-oxidant-induced calcium release from mitochondria involving cyclic ADP-ribose as a specific messenger, rather than the non-enzymic modification of proteins by ADP-ribose.

INTRODUCTION

In recent years, evidence has accumulated that NAD⁺, besides its function as a hydrogen acceptor, serves as a precursor of metabolites that participate in a number of regulatory processes. Several enzymes have been identified that transfer the ADP-ribose moiety of NAD⁺ to either protein or water. While the role of the liberated nicotinamide remains uncertain, monoADP-ribosylation of several proteins has been documented to cause significant alterations in function. The discovery of cyclic ADP-ribose (cADP-ribose) [1] and its role as a potent Ca²⁺-mobilizing agent has led to the identification of the ADP-ribosyl cyclases. These enzymes, e.g. the surface antigen of blood cells, CD38 [2–4], catalyse the conversion of NAD⁺ into ADP-ribose or cADP-ribose. In addition, some of these enzymes are capable of hydrolysing cADP-ribose to ADP-ribose [2–5].

A number of reports have described mitochondrial NADase (NAD⁺ glycohydrolase) activity and ADP-ribosylation [6–11]. Evidence has been presented suggesting that treatment of isolated mitochondria with pro-oxidants induces the oxidation and subsequent degradation of mitochondrial pyridine nucleotides (reviewed in [12]). Concomitantly, ADP-ribosylation and Ca²⁺ efflux have been observed. These processes were proposed to be causally linked [12]. It has been proposed that ‘oxidative stress’ may activate a mitochondrial NAD⁺ glycohydrolase. The ADP-ribose formed would then non-enzymically, but specifically, modify mitochondrial proteins. The specific ADP-ribosylation in turn would cause the activation of a mitochondrial Ca²⁺-efflux pathway [12]. Although this is an attractive model, conclusive evidence for such a sequence of events is lacking. The physiological significance of non-enzymic mitochondrial ADP-

ribosylation has been called into question, e.g. by the finding that, in the presence of nicotinamide, the NADase was almost inactive, whereas the extent of ADP-ribosylation was only slightly diminished [9,10].

This apparent contradiction, as well as the identification of ADP-ribosyl cyclase activity in a number of enzymes previously known as NAD⁺ glycohydrolases, prompted us to test for the presence of cyclase activity in the mitochondrial NADase. In recent reports we have described the isolation and characterization of NAD⁺ glycohydrolase from bovine liver mitochondria [13,14]. The enzyme activity was assigned to a single polypeptide with an *M_r* of about 30000. It has been characterized as a hydrophobic integral membrane protein [13,14]. In the present study it is demonstrated that the mitochondrial NADase possesses ADP-ribosyl cyclase as well as cADP-ribose hydrolase activity. This enzyme is, therefore, identified as a member of the class of bifunctional cADP-ribose synthases/hydrolases. Consequently, it may play a role in the regulation of mitochondrial Ca²⁺ fluxes via the formation of a Ca²⁺-mobilizing metabolite rather than by providing the substrate for non-enzymic ADP-ribosylation.

MATERIALS AND METHODS

Materials

Analogues of NAD⁺ [nicotinamide hypoxanthine dinucleotide (NHD⁺) and nicotinamide guanine dinucleotide (NGD⁺)], ADP-ribose and ADP-ribosyl cyclase from *Aplysia californica* were purchased from Sigma. ³²P-labelled NAD⁺ was obtained from ICN. 1,*N*⁶-Etheno-NAD⁺ (ε-NAD⁺) was synthesized in this

Abbreviations used: cADP-ribose (etc.), cyclic ADP-ribose (etc.); ε-ADP-ribose, 1,*N*⁶-etheno-ADP-ribose; ε-NAD⁺, 1,*N*⁶-etheno-NAD⁺; NHD⁺, nicotinamide hypoxanthine dinucleotide; NGD⁺, nicotinamide guanine dinucleotide; LDAO, lauryl dimethylamine *N*-oxide.

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laboratory as described in [15]. All other reagents were of analytical grade.

Isolation of mitochondria and mitochondrial NADase

Bovine liver mitochondria were isolated as described [13], resuspended in 10 mM Tris/HCl, pH 7.5, 250 mM sucrose and 0.5 mM EDTA and stored frozen (-70°C). Washed mitochondria were solubilized with 6% Triton X-100. The NADase was then partially purified by four chromatographic steps, as reported previously [14]. The enzyme was enriched about 300-fold and contained no NAD⁺-metabolizing activity other than NADase [14].

Analysis of nucleotides by TLC

Nucleotides were separated on poly(ethyleneimine)-cellulose plates (Schleicher & Schuell) coated with fluorescence indicator. The solvent consisted of 1 M acetic acid and 0.3 M LiCl. The positions of radiolabelled nucleotides were revealed by autoradiography and compared with those of standard compounds detected by fluorescence quenching.

Determination of NAD⁺ glycohydrolase and purine nucleoside diphosphoribose cyclase activities

NADase activity was determined as described [14], using ϵ -NAD⁺ as substrate.

The production of (fluorescent) cGDP-ribose or cIDP-ribose was followed using 1 mM (non-fluorescent) NGD⁺ or NHD⁺ as substrate respectively at room temperature in a solution consisting of 50 mM Tris/HCl, pH 7.5, and 0.03% (w/v) lauryl dimethylamine *N*-oxide (LDAO). The final volume was 400 μl . The reaction was started by adding an appropriate amount of enzyme. The excitation wavelength was set to 300 nm. Emission was monitored at a wavelength of 410 nm.

Protein separation by SDS/PAGE

Partially purified NADase was separated in Minigels (Hoefer Scientific) containing 12% (w/v) acrylamide as described in [16]. The sample buffer did not contain β -mercaptoethanol and the samples were not boiled. These conditions were required, since the gels were to be stained for NADase activity after electrophoresis [14].

Identification of NADase and ADP-ribosyl cyclase activities in gels after SDS/PAGE

After separation of partially purified mitochondrial NADase by SDS/PAGE, the gel was washed for 30 min in a solution containing 50 mM Tris/HCl, pH 7.5, and 0.5% LDAO. To visualize NADase activity, the (respective lanes of the) gels were then incubated for 15 min in a buffer consisting of 50 mM Tris/HCl, pH 7.5, 0.03% LDAO and 150 μM ϵ -NAD⁺ [14]. Staining for IDP-ribose cyclase activity was achieved by using 3 mM NHD⁺ instead of ϵ -NAD⁺. After incubation with the respective pyridine nucleotide, the gel was placed on a UV-transilluminator. Photographs were taken using a 550 nm interference filter. Fluorescent bands were marked and their positions compared with those of standard compounds after subsequent staining with Coomassie Blue.

Analysis of nucleotides by HPLC

Separation of ADP-ribose, cADP-ribose, AMP, nicotinamide and NAD⁺ was carried out on a BioLogic System (Bio-Rad)

using a 4.6 mm $\times$ 250 mm C₁₈ reverse-phase column at a flow rate of 0.5 ml/min. The mobile phase consisted of 50 mM NaCl and 3 mM trifluoroacetic acid (buffer A). Elution was performed as follows. Following 15 min of isocratic elution with buffer A, a linear gradient from 100% (v/v) buffer A to 100% (v/v) buffer B [buffer A supplemented with 5% (v/v) acetonitrile] was run for 6 min. Subsequent washing with 100% (v/v) buffer B for 10 min completed the elution of the nucleotides. Prior to the application of the next sample, the column was equilibrated for 10 min with buffer A.

Protein determination

Protein was determined by using the biuret procedure or the BCA assay (Pierce). BSA was used as a standard.

Data presentation

All data presented are representative of at least three experiments.

RESULTS

Lee and co-workers [17] have developed an elegant test to detect ADP-ribosyl cyclase activity utilizing the ability of these enzymes to form cGDP-ribose from NGD⁺. As opposed to NGD⁺ and GDP-ribose, cGDP-ribose is fluorescent. Therefore its formation can easily be followed fluorimetrically. Other non-fluorescent analogues of NAD⁺, such as NHD⁺, served as precursors of fluorescent cyclic purine nucleoside diphosphoriboses as well [18]. To test the ADP-ribosyl cyclase activity of the mitochondrial NADase, we used a partially purified preparation of the enzyme that has been characterized previously [14]. It is important to note that no other NAD⁺-converting activities were detected in this fraction [14]. Moreover, when the enzyme was purified to apparent homogeneity [13], its mobility on SDS/PAGE coincided with that found for the partially purified form. The partially purified NADase was preferred for enzymic studies, because no denaturation step was required during the purification [14]. Incubation of the enzyme in the presence of 1 mM NGD⁺ or NHD⁺ led to a substantial increase in fluorescence (Figure 1), indicating ADP-ribosyl cyclase activity. Preincubation of the

Figure 1 Synthesis of cyclic purine nucleoside diphosphoriboses from NAD⁺ analogues by mitochondrial NADase

The formation of fluorescent cGDP-ribose from NGD⁺ or of cIDP-ribose from NHD⁺ was followed. The reaction mixture consisted of 50 mM Tris/HCl, pH 7.5, 0.03% (w/v) LDAO and 1 mM of the indicated substrate. The arrow indicates the addition of 5 μg of partially purified bovine liver mitochondrial NADase. Fluorescence was measured at 410 nm. The excitation wavelength was set to 300 nm.

Figure 2 Synthesis of [^{32}P]cADP-ribose (cADPR) by mitochondrial NADase

Partially purified mitochondrial NADase was incubated at 30 °C for 5 min in the presence of 25 μM [^{32}P]NAD $^{+}$ and the additions indicated below in a medium containing 50 mM Tris/HCl, pH 7.5, and 0.03% LDAO. The reaction was stopped by acetone precipitation. After drying, the precipitated nucleotides were dissolved by adding 25 μl of water and mixing. Undissolved protein was sedimented and the supernatants treated with snake venom phosphodiesterase for 20 min at 30 °C. Aliquots were then immediately subjected to TLC (see the Materials and methods section). The autoradiograph of the chromatogram is presented. Lanes: 1, no additions; 2, +1 mM unlabelled ADP-ribose; 3, +20 μM ZnCl $_2$; 4, mitochondrial NADase was preincubated for 20 min with 10 mM dithiothreitol prior to its addition to the reaction mixture, which also contained 10 mM dithiothreitol.

enzyme in the presence of dithiothreitol prevented the fluorescence enhancement (results not shown), confirming the previously detected sensitivity of the mitochondrial NADase to reducing agents [14].

The ability of the mitochondrial NADase to utilize the physiological substrate, NAD $^{+}$, to form cADP-ribose was also tested (Figure 2). Partially purified NADase was incubated in the presence of 25 μM [^{32}P]NAD $^{+}$. After incubation for 5 min the reaction was stopped by acetone precipitation. The redissolved nucleotides were then treated with snake venom phosphodiesterase. This enzyme produces [^{32}P]AMP from NAD $^{+}$ or ADP-ribose. However, cADP-ribose is not cleaved. This property was utilized for the analysis, because, in the chromatographic system used, the separation of ADP-ribose from cADP-ribose was insufficient. As shown in Figure 2, lane 1, a detectable amount of product co-migrating with (c)ADP-ribose was not cleaved by the phosphodiesterase. This fraction was greater when the incubation was performed in the presence of Zn $^{2+}$ (Figure 2, lane 3). This observation is of interest, since the ADP-ribosyl cyclase activity of CD38 is also stimulated by copper [4] or zinc [19] ions. Furthermore, activation of the mitochondrial NADase by Zn $^{2+}$ has been observed previously [14]. Addition of 1 mM unlabelled ADP-ribose inhibited both the cyclase activity (Figure 2, lane 2) and the NADase activity (results not shown). The results with this sample (Figure 2, lane 2), which contained a 40-fold higher substrate concentration for the phosphodiesterase (1 mM ADP-ribose) compared with the other samples, demonstrated that the phosphodiesterase reaction was indeed performed to completion. This was further demonstrated by the fact that preincubation of the NADase with inhibitory dithiothreitol led to no detectable conversion of NAD $^{+}$; consequently, the label was completely recovered as [^{32}P]AMP after phosphodiesterase treatment (Figure 2, lane 4). Under the conditions used, ADP-ribose was cleaved by the phosphodiesterase approximately three times faster than was NAD $^{+}$ (results not shown).

Figure 3 demonstrates that the mobilities on SDS/PAGE of the two mitochondrial activities (lanes 1) catalysing the NADase reaction (using ϵ -NAD $^{+}$ as substrate; right-hand side) and the cyclase reaction (using NHD $^{+}$, left-hand side) coincided. The enzyme activities were visualized after SDS/PAGE directly

Figure 3 Identity of mitochondrial NADase and ADP-ribosyl cyclase

Partially purified mitochondrial NADase (lanes 1) and ADP-ribosyl cyclase from *Aplysia californica* (lanes 2) were run in duplicate along with marker proteins on SDS/12%-PAGE. After electrophoresis, the gel was washed in 50 mM Tris/HCl, pH 7.5, and 0.5% LDAO. Thereafter the gel was cut in half. One half was then incubated for 15 min in 50 mM Tris/HCl, pH 7.5, 0.03% LDAO and 3 mM NHD $^{+}$ (left-hand side). The other half was incubated with 50 mM Tris/HCl, pH 7.5, 0.03% LDAO and 150 μM ϵ -NAD $^{+}$ (right-hand side). The fluorographs were taken using a UV-transilluminator. Numbers on the right indicate the positions of marker proteins of the indicated M_r s revealed after subsequent staining of the gel with Coomassie Blue.

within the gel by utilizing the formation of the fluorescent products (see the Materials and methods section). Since 1, N^6 -etheno-ADP-ribose (ϵ -ADP-ribose) (Figure 3, right-hand side) exhibits much stronger fluorescence than cADP-ribose (Figure 3, left-hand side), the background in the latter case is higher. The ADP-ribosyl cyclase from *Aplysia californica*, which apparently acts exclusively as an ADP-ribosyl cyclase and not as an NAD $^{+}$ glycohydrolase [20], was electrophoresed on the same gel (Figure 3, lanes 2). Its activity was also detected using either substrate, and the mobility corresponded to an apparent M_r of approx. 28000. This estimation is in agreement with previous observations [20]. The fluorescence enhancement after cleavage of ϵ -NAD $^{+}$ is based upon the separation of the modified adenine ring from the nicotinamide moiety, which also occurs during the formation of cyclic ϵ -ADP-ribose. However, the fluorescence of cyclic ϵ -ADP-ribose is less strong than that of ϵ -ADP-ribose [18]. The NADase from *Neurospora crassa* was readily 'activity-stained' with ϵ -NAD $^{+}$ [14], but no activity was revealed using NHD $^{+}$ (results not shown). This agrees with previous reports demonstrating that this enzyme does not exhibit ADP-ribosyl cyclase activity [17]. Therefore ϵ -NAD $^{+}$ represents a valuable tool for the detection of NADase or ADP-ribosyl cyclase activity in SDS/polyacrylamide gels, owing to the bright fluorescence of the products. On the other hand, NHD $^{+}$ may be used to verify the presence of cyclase activity.

Of the known ADP-ribosyl cyclases, CD38 [4] and the canine spleen enzyme [5] have been shown to catalyse the hydrolysis of cADP-ribose. The ability of bovine liver mitochondrial NADase to hydrolyse cADP-ribose to ADP-ribose is demonstrated in Figure 4(A). Comparison of these results with those from similar experiments using NAD $^{+}$ as substrate indicated that the rates of hydrolysis differed by a factor of about 7–9 in favour of NAD $^{+}$ (results not shown). The yield of cADP-ribose in the latter experiments was less than 2%, ADP-ribose being the predominant product. These values are comparable with those reported for CD38 [4]. However, as also indicated in Figure 2, in the presence of Zn $^{2+}$ the yield of cADP-ribose increased more than 3-fold.

The proposed mechanism of the bifunctional ADP-ribosyl cyclases/cADP-ribose hydrolases suggested that these enzymes have the ability to support the formation of NAD $^{+}$ from cADP-ribose and nicotinamide [5]. This activity is indeed present in the

Figure 4 Hydrolysis of cADP-ribose and synthesis of NAD^+ by mitochondrial NADase

Partially purified mitochondrial NADase was incubated in the presence of 100 μM cADP-ribose (cADPR) (A) or 100 μM cADP-ribose plus 1 mM nicotinamide (Nam) (B) for 30 min at room temperature in a buffer consisting of 50 mM Tris/HCl, pH 7.5, and 0.03% LDAO. Samples were then applied immediately to HPLC. Typical chromatograms are shown. The cADP-ribose used did not contain any detectable amount of ADP-ribose (ADPR), and was stable throughout the incubation period in the absence of enzyme (broken line in A). The ordinate units are absorbance units full scale.

mitochondrial NADase (Figure 4B), similar to that for the NAD^+ glycohydrolase from canine spleen [5].

Regarding the inactivation of the mitochondrial NADase by reducing agents, the possibility of the reversal of this effect is of interest, since such a mechanism could account for the pro-oxidant-induced calcium release from mitochondria. The partially purified enzyme was inactivated by the addition of 100 mM dithiothreitol. Thereafter, dialysis was carried out against 1000 vol. of buffer containing 20 mM Tris/HCl, pH 7.5, 0.03% LDAO and 1 mM oxidized glutathione, 400 μM t-butyl hydroperoxide, 400 μM H_2O_2 or no additions. The buffer was changed once after 1 h. Recovery of enzyme activity was determined by the fluorimetric test using $\epsilon\text{-NAD}^+$ [14]. After 2 h, some activity was detected in all samples. Approx. 20% of activity was regained in the sample dialysed against oxidized glutathione, whereas t-butyl hydroperoxide and H_2O_2 led to only 3–5% recovery of activity. The control sample (dialysis against buffer without additions) exhibited activity corresponding to less than 1% of the original activity measured using enzyme that was not treated with dithiothreitol. The efficiency of oxidized glutathione supports the conclusion that a disulphide bridge within the enzyme is required for activity.

DISCUSSION

This study establishes that bovine liver mitochondrial NADase catalyses the formation of cADP-ribose from NAD^+ , as well as that of cyclic nucleoside diphosphoriboses from NAD^+

Figure 5 Hypothetical mechanism of pro-oxidant-induced calcium release from mitochondria

Pro-oxidants such as oxygen radicals or t-butyl hydroperoxide cause the oxidation of intramitochondrial glutathione and pyridine nucleotides [12]. When activated by oxidized glutathione (GSSG), presumably by formation of a disulphide bridge within the enzyme via disulphide interchange, the NADase forms cADP-ribose (cADPR) from NAD^+ . Binding of cADP-ribose to its receptor may then trigger the opening of a putative specific calcium-release channel within the inner membrane. The mitochondrial outer membrane has been omitted.

analogues. Since it also catalysed the hydrolysis of cADP-ribose to ADP-ribose, this NADase would appear to belong to the class of bifunctional ADP-ribosyl cyclases [2–5]. The ability of the enzyme to synthesize NAD^+ from cADP-ribose and nicotinamide may serve as supportive evidence for the proposed catalytic mechanism of these enzymes, involving a stabilized, non-covalent, enzyme–ADP-ribose intermediate [5].

The presence of ADP-ribosyl cyclase activity in mitochondria raises the possibility that this enzyme participates in the regulation of mitochondrial Ca^{2+} fluxes via synthesis of cADP-ribose rather than by liberating ADP-ribose for non-enzymic ADP-ribosylation of a putative Ca^{2+} transporter. Such a model appears plausible, since cADP-ribose has been demonstrated to be a potent calcium-mobilizing agent in a variety of experimental systems [21].

The influence of thiol reagents on the activity of NADases and ADP-ribose cyclases has been noted previously [22–24]. Studies using site-directed mutagenesis have revealed that the presence or absence of specific cysteine residues in the NAD^+ glycohydrolase from *Aplysia* or in human CD38 determines the ability of these enzymes to function as ADP-ribose cyclases or cADP-ribose hydrolases [25]. Addition of reducing agents led to complete loss of activity of the bovine mitochondrial enzyme. This observation suggests a requirement for a disulphide bridge for activity, which is further implied by the partial reversal of inactivation in the presence of oxidized glutathione. Therefore the results presented here demonstrate that the mitochondrial ADP-ribosyl cyclase may be reversibly switched from the active to the inactive state depending on its thiol status. Interestingly, it has been reported that mitochondrial degradation of pyridine nucleotides requires the oxidation of vicinal thiols in these organelles [26]. Taking into consideration previous observations on pro-oxidant-induced calcium efflux from mitochondria (reviewed in [12]), a hypothesis may be derived that links the oxidation of intramitochondrial glutathione and pyridine nucleotides to the efflux of calcium via activation of the NADase and the production of cADP-ribose (Figure 5).

It should be pointed out that calcium efflux from mitochondria can be triggered by several agents, including oxidants. A proposed mechanism for the efflux includes the cyclosporin A-sensitive,

reversible, formation of a non-specific pore (mitochondrial transition pore) within the inner membrane [27]. Owing to the specificity of cADP-ribose-mediated calcium release in other systems, it would appear unlikely that the mechanism proposed here would proceed via formation of the mitochondrial transition pore, permitting the exchange of other solutes besides calcium. Nevertheless, it has been observed that degradation of mitochondrial pyridine nucleotides, which is catalysed by the NADase, was inhibited by cyclosporin [28]. Further studies will be aimed at testing the potential involvement of cADP-ribose in mitochondrial calcium release.

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