Characterization of Detergent-Solubilized Beef Liver Mitochondrial NAD^+ Glycohydrolase and Its Truncated Hydrosoluble Form†

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ABSTRACT: Membrane-bound beef liver mitochondrial NAD^+ glycohydrolase (NADase) was partially purified after its solubilization by either detergent or crude pancreatic lipase, steapsin. Solubilization by steapsin yielded a homogeneous water-soluble enzyme. A fluorescence assay was developed that allowed visualization of NADase activity directly within the gel after sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The apparent molecular masses of the detergent- and steapsin-solubilized forms were estimated to be about 30 000 and 28 000, respectively. The small part that was cleaved by steapsin represents presumably the membrane anchor of the mitochondrial NADase, as its removal converted the enzyme from a highly hydrophobic to a hydrosoluble protein. The fluorescence staining for activity was also successfully applied to other NADases. Kinetic analyses of the two forms of solubilized mitochondrial NADase revealed that the catalytic properties were unaffected after the steapsin treatment. Neither the binding affinity of the substrate analog 1,N^6-etheno-NAD^+ nor the inhibition by nicotinamide differed significantly between these two forms of the enzyme. Moreover, the dependence of enzyme activity on temperature, pH, or ionic strength was also similar for both preparations. However, activity of the detergent-solubilized but not of the truncated steapsin-solubilized enzyme was strongly dependent on the presence of bivalent metal ions such as Zn^{2+}. These results suggest that the membrane part of the mitochondrial NAD^+ glycohydrolase is not required for catalysis. It appears, however, to be of importance for the regulation of the enzyme.

Mitochondrial uptake and release of Ca^{2+} have gained considerable interest in recent years. Several investigations have provided evidence that these processes play an important role in the tightly regulated cellular Ca^{2+} homeostasis [reviewed in Carafoli (1987), Denton and McCormack (1990), and Clapham (1995)]. Recent studies have shown that intramitochondrial enzyme activities can be regulated by changes in the cytosolic calcium concentration (Denton & McCormack, 1990; Hajnóczky et al., 1995). On the other hand, the occurrence of cytosolic calcium waves depended on the activity of the respiratory chain and potential driven mitochondrial calcium uptake (Jouaville et al., 1995). These observations have reinforced the role of mitochondrial Ca^{2+} fluxes in intracellular calcium signalling.

While the uptake of Ca^{2+} into mitochondria has been well characterized and shown to be accomplished by a ruthenium red sensitive uniporter along the membrane potential, the pathway(s) of calcium release from the organelles remains to be clearly determined (Carafoli, 1987). Evidence has been accumulating supporting the conclusion that calcium efflux from mitochondria can be induced by prooxidants such as *tert*-butyl hydroperoxide (Lötscher et al., 1979, 1980; Richter & Kass, 1991) or *N*-acetyl-*p*-benzoquinoneimine (Weis et al., 1992) causing oxidation and subsequent degradation of pyridine nucleotides and ADP ribosylation of proteins within mitochondria. On the basis of these observations, a hypothesis has been put forward suggesting that prooxidants induce the hydrolysis of mitochondrial NAD^+ by NAD^+ glycohydrolase (EC 3.2.2.5). Formed ADP−ribose would then in a nonenzymatic but specific reaction modify mitochondrial proteins that represent or activate a calcium transporter. As a consequence, Ca^{2+} would be released from the organelles (Lehninger et al., 1978; Lötscher et al., 1979, 1980; Hilz et al., 1984; Richter & Kass, 1991). Such a mechanism would suggest hydrolysis of NAD^+ by NAD^+ glycohydrolase (NADase)[1] to be a key step in prooxidant-induced calcium release.

In a recent report, a purification procedure for a detergent-solubilized form of the NADase from beef liver mitochondria was presented. The enzyme was purified 1660-fold, and an apparent molecular mass of 32 000 was estimated (Zhang et al., 1995). As the mitochondrial NAD^+ glycohydrolase was highly hydrophobic, it could only be maintained in detergent solution, but it still tended to aggregate. It was, therefore, highly desirable to obtain a readily soluble, yet functional, preparation of the enzyme. Here we report the

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[1] Abbreviations: ϵ-NAD^+, 1,N^6-etheno-NAD^+; NADase, NAD^+ glycohydrolase; SDS−PAG(E), sodium dodecyl sulfate−polyacrylamide gel (electrophoresis); LDAO, lauryl dimethylamine *N*-oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane sulfonate; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

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preparation of a hydrosoluble form of the enzyme using a crude pancreatic lipase (steapsin) for solubilization. According to earlier reports (Green & Bodansky, 1965; Swislocki et al., 1967; Bock et al., 1971; Schuber & Travo, 1976), this approach yielded water-soluble NAD^+ glycohydrolase activity from different origins. A fluorescence assay is described which permitted detection of NADase activity directly within the gel after SDS−PAGE. The steapsin-solubilized enzyme appeared as a single band containing NADase activity and exhibited only a slightly smaller molecular mass as compared to the intact, membrane-bound form. Characterization of this preparation revealed that it was indistinguishable from the detergent-solubilized enzyme with respect to kinetic properties. An important difference concerning the sensitivity toward bivalent metal ions was, however, noted. The absence of such a sensitivity in the truncated hydrosoluble enzyme suggests a role of the detached membrane part in the regulation of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. ϵ-NAD^+, steapsin (crude pancreatic lipase), and NADases from porcine brain and from *Neurospora crassa* were purchased from Sigma. LDAO was obtained from Fluka. Hydroxyapatite was from BioRad, and all other chromatographic supports were from Serva. [*nicotinamide*-^{14}C]NAD^+ was provided by Amersham. All other reagents were of analytical grade. ϵ-NAD^+ used for activity staining of SDS−PAGEs (see below) was synthesized in our laboratory according to a procedure described by Barrio et al. (1972).

Solubilization of Mitochondrial NADase by Steapsin. Washed beef liver mitochondria (Zhang et al., 1995), 20 mg/mL, were incubated with 20 mg/mL steapsin in a solution containing 50 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$ for up to 60 min at 37 °C. Since the activity of the steapsin varied with different lots, the incubation time was adjusted accordingly. The incubation was stopped by addition of EDTA and PMSF to 20 and 0.5 mM, respectively. After the pH had been adjusted to pH 5.0 with 1 M acetic acid, the solution was centrifuged at 215000*g* for 60 min at 4 °C. The supernatant containing water-soluble NADase was readjusted to pH 7.5 by addition of 1 M Tris base. The efficiency of solubilization of NADase was somewhat variable. In most experiments, about 30−50% of the total activity was recovered in the supernatant.

Isolation of Steapsin-Solubilized Mitochondrial NADase. The solubilized fraction was chromatographed on a hydroxyapatite column equilibrated with a buffer consisting of 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl. The majority of NADase activity bound to the column. The column was washed with the same buffer. Elution was accomplished by running a gradient from 50 mM potassium phosphate buffer (pH 7.0) to 500 mM potassium phosphate (pH 7.0). Fractions containing NADase activity were pooled and dialyzed against 20 mM sodium acetate (pH 5.0). The protein solution was applied to a CM-Trisacryl column equilibrated with the same buffer. The column was washed with the same buffer and then eluted by running a linear gradient from this buffer to 0.25 M potassium phosphate (pH 7.0). The eluted fractions containing NADase activity were pooled and dialyzed against 20 mM Tris-HCl (pH 8.5), followed by chromatography on a DEAE-52 cellulose column equilibrated with the same buffer. The enzyme activity eluted in the void volume. The active pool was then directly applied to a Cibacron Blue F3GA (Affi-Blue) column equilibrated with 50 mM Tris-HCl (pH 7.5). The column was washed with the same buffer. The enzyme was eluted in a linear gradient to this buffer supplemented with 200 mM NaCl and 0.5% CHAPS. CHAPS was used because it greatly facilitated elution of the enzyme. Elution with higher salt concentrations instead was inapplicable, since this led to substantial inhibition of the enzyme. When desired, CHAPS could be removed by dialysis.

Isolation of Detergent-Solubilized Mitochondrial NADase. The purification of detergent-solubilized mitochondrial NADase has recently been reported (Zhang et al., 1995). However, to compare the steapsin-solubilized form to the detergent-solubilized one, it seemed reasonable to prepare the latter using a similar preparation scheme. Since the reported purification procedure for the detergent-solubilized NADase included, for example, a denaturation/renaturation step in the presence of SDS and Triton X-100, it was inapplicable to the water-soluble form of the enzyme. Thus, the mitochondrial protein was solubilized by 6% Triton X-100 in a buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl. Unsolubilized matter was sedimented by centrifugation at 215000*g* for 45 min at 4 °C. The subsequent chromatographic steps were carried out under the same conditions as for the steapsin-solubilized enzyme. However, the detergent, LDAO, was included into all solutions at a concentration of 0.05%, except for the elution buffer from the Affi-Blue column (which contained CHAPS). Moreover, the chromatography on DEAE-52 cellulose was carried out before using the CM-Trisacryl column. While Triton X-100 was most efficient in solubilizing the enzyme, LDAO was preferred as detergent in the chromatographic steps, because it exhibits only little absorbance at 280 nm. This allows one to monitor the protein separation during column chromatography.

Both steapsin- and detergent-solubilized enzyme preparations were quite stable, when maintained at 4 °C (about 10% loss of activity within 4 weeks). However, some preparations of steapsin-solubilized enzyme appeared to contain significant protease contaminations, which led to drastic loss of activity. It is not clear which preparation step was crucial for the removal of proteases. It was, however, obvious that different lots of steapsin varied considerably in their protease/lipase contents which, at least in part, accounted for the variations in enzyme preparations.

Compared to washed mitochondria, the detergent- and steapsin-solubilized forms were enriched about 280- and 510-fold, respectively.

Identification of NADase in SDS−PAGE by Fluorescence Staining. The protein solution of interest was separated on SDS−PAGE according to Laemmli (1970) using the acrylamide concentration indicated. Unless stated otherwise, the sample buffer did not contain reducing agents such as β-mercaptoethanol. After electrophoresis, the gel was washed in a solution containing 50 mM Tris-HCl (pH 7.5) and 0.5% LDAO for 30 min. Thereafter, the gel was incubated for 15 min in 50 mM Tris-HCl (pH 7.5) supplemented with 0.05% LDAO and 150 μM ϵ-NAD^+. NADase was then visualized as a fluorescent band by placing the gel on a UV transilluminator. Photographs were taken using a 550 nm interference filter. The apparent molecular masses

of the proteins corresponding to a fluorescent band were estimated by marking the band using a small piece of wire and comparing its position to that of marker proteins after staining of the gel with Coomassie Brilliant Blue.

Fluorimetric Assay of NADase Activity. Use was made of the fluorescence enhancement that follows cleavage of the substrate analog ϵ-NAD^+ (Barrio et al., 1972). The assay was carried out in a final volume of 700 μL in a solution consisting of 50 mM Tris-HCl (pH 8.0), 0.03% LDAO, and 10 μM ϵ-NAD^+ at room temperature, unless indicated otherwise. Data acquisition was started concomitantly with the addition of an appropriate amount of enzyme. Activity was estimated according to the initial slope of the reaction. Fluorescence measurements were performed using a Perkin-Elmer LS50B spectrofluorimeter. The excitation wavelength was set to 310 nm. Fluorescence emission was followed at 410 nm.

The concentration of ϵ-NAD^+ was determined by conversion of ϵ-NAD^+ to ϵ-NADH using the alcohol dehydrogenase reaction and assuming a molar extinction coefficient for ϵ-NADH of 6.2×10^6 cm^2/mol at 340 nm.

Identification of Reaction Products of Isolated Mitochondrial NADase. Each form of isolated mitochondrial NADase (10 μg) was incubated with either 25 μM [*nicotinamide*-^{14}C]NAD^+ (0.1 μCi) or 25 μM ϵ-NAD^+ in 50 mM Tris-HCl (pH 7.5) for 15 min at 37 °C. The final volume was 50 μL. The products were separated by thin layer chromatography on cellulose plates according to Lötscher et al. (1980). The running buffer consisted of isobutyric acid, H_2O, and 25% NH_4OH (96/19/4, v/v/v). Standard compounds were identified by fluorescence quenching, whereas the radioactive products of the reaction were identified by autoradiography of the plates. Etheno derivatives of adenine nucleotides were identified by their blue fluorescence.

Protein Determination. Protein was determined by either a biuret procedure or the micro BCA procedure from BioRad using bovine serum albumin as standard.

Statistical Treatment of Kinetic Data. Kinetic parameters of the enzyme preparations were obtained by fitting the experimental data to the Michaelis−Menten equation by means of nonlinear regression using the software package SPSS.

RESULTS

NAD^+ glycohydrolase appears to represent only a minor fraction of the total protein in beef liver mitochondria (Zhang et al., 1995). As a consequence, at least during initial steps of the purification, enriched fractions of NADase were not supposed to yield a protein band after SDS−PAGE at the expected position (approximately 30 000) that could be stained with Coomassie Blue. Moreover, it is essential to correlate the occurrence of a specific protein band with enzyme activity. These problems could be overcome by application of a procedure used previously which included slicing the gel after electrophoresis followed by a renaturation protocol. Eventually, the gel slices were incubated with ϵ-NAD^+ and the ability to enhance fluorescence was interpreted as the presence of NADase in a particular gel slice (Zhang et al., 1995). A more efficient and faster procedure has been developed in this study (Figure 1). After SDS−PAGE, the gel was first washed in 0.5% LDAO and thereafter incubated for 15 min in 0.03% LDAO containing

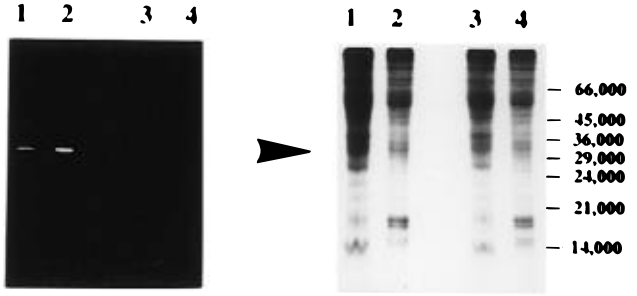

FIGURE 1: Direct detection of mitochondrial NADase activity after SDS−PAGE. Beef liver homogenate (80 μg) and mitochondria (50 μg) were separated in a 12% SDS−PAGE. (Left panel) After electrophoresis, the gel was activity stained for NADase using the fluorescent analog ϵ-NAD^+ as described in Experimental Procedures. (Right panel) The gel was then stained with Coomassie Brilliant Blue: lane 1, homogenate; lane 2, mitochondria; lane 3, homogenate prepared in sample buffer containing β-mercaptoethanol; and lane 4, mitochondria prepared in sample buffer containing β-mercaptoethanol. The arrow indicates the position of the fluorescent bands after activity staining. The numbers on the right indicate the positions of molecular weight standards.

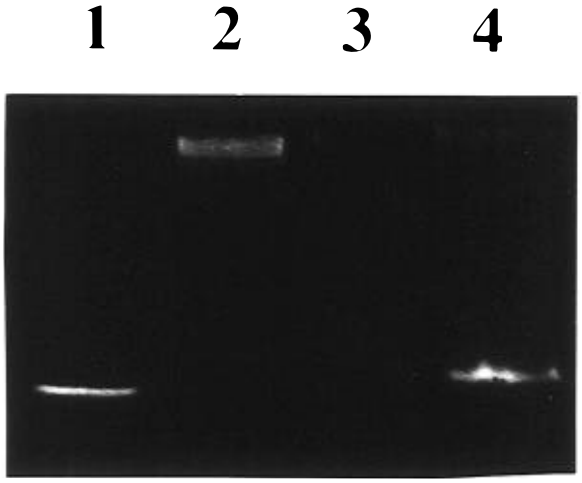

FIGURE 2: Activity staining of different NADases after SDS−PAGE. Mitochondria (lane 1), crude NADase from *N. crassa* (lane 2), phosphodiesterase I from snake venom (lane 3), and crude NADase from porcine brain (lane 4) were separated in a 10% SDS−PAGE followed by fluorescence staining for NADase activity using ϵ-NAD^+ as described in Experimental Procedures.

150 μM ϵ-NAD^+. The band containing NADase activity was then visualized by its fluorescence under UV light (Figure 1, left panel). Conveniently, the gel could then be stained with Coomassie Blue (Figure 1, right panel). Figure 1 shows the electrophoretic separation of beef liver homogenate (lanes 1 and 3) and mitochondria (lanes 2 and 4) followed by incubation of the gel with ϵ-NAD^+. Using the fluorescence staining for activity, only a single band could be identified in the homogenate (Figure 1, left panel, lane 1) as well as in mitochondria (left panel, lane 2) with an apparent molecular mass of about 30 000. Figure 1 further demonstrates that the activity staining failed completely, if the sample buffer contained β-mercaptoethanol (Figure 1, lanes 3 and 4).

To validate these results, commercially available crude preparations of NADases, namely from *N. crassa* and porcine brain, were also run on SDS−PAGE and then stained with ϵ-NAD^+. Figure 2 shows that, in addition to the mitochondrial NADase (lane 1), these two NADases could also be activity stained (lanes 2 and 4). The apparent molecular

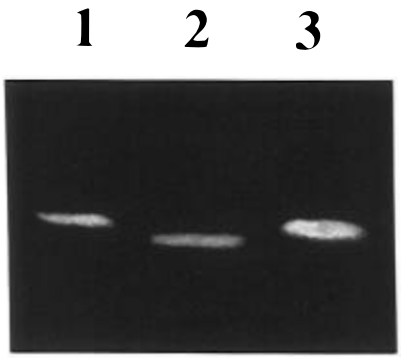

FIGURE 3: Detection of detergent-solubilized and hydrosoluble mitochondrial NADase by activity staining after SDS−PAGE. Detergent-solubilized NADase (1 μg) (lane 1), steapsin-solubilized NADase (0.5 μg) (lane 2), and mitochondria (90 μg) (lane 3) were electrophoresed in a 12% SDS-PAG. The positions of the NADases were visualized fluorimetrically as described in Experimental Procedures. The molecular masses were estimated after subsequent staining of the gel with Coomassie Brilliant Blue by comparison of the position of the fluorescent bands with those of marker proteins (see text).

masses of the NADases were estimated to be about 205 000 and 36 000 for the enzymes from *N. crassa* and porcine brain, respectively. The enzyme from *N. crassa* was rerun on an 8% SDS−PAGE for this estimation (not shown). Phosphodiesterase I from snake venom (Figure 2, lane 3) did not yield a fluorescent band. This is remarkable because, when tested in an activity assay in the cuvette (see Experimental Procedures), the velocity by which it cleaved ϵ-NAD^+ was 10 times higher than that of the mitochondrial NADase, respective to the amount used for the gel analysis.

Previous reports have shown that NADase from various origins could be solubilized by steapsin, a crude pancreatic lipase also containing proteolytic activities (Green & Bodansky, 1965; Swislocki et al., 1967; Bock et al., 1971; Schuber & Travo, 1976). In the present study, it was found that incubation of washed beef liver mitochondria with steapsin also substantially solubilized NADase activity. This solubilized NADase (see Experimental Procedures) could then be treated as a "normal" water-soluble protein. A disadvantage of this method is, however, that addition of steapsin itself increases tremendously the amount of contaminating proteins. For the characterization and comparison of the detergent- or steapsin-solubilized forms, partially purified enzyme preparations were obtained (see Experimental Procedures). The purification schemes included several chromatographic steps and were similar for both preparations with the exception that the detergent-solubilized fraction was always maintained in the presence of LDAO. As shown in lanes 1 and 3 of Figure 3, the apparent molecular mass of detergent-solubilized NADase coincided with that found for mitochondria (about 30 000). The steapsin-solubilized form appeared as a single band with a slightly reduced apparent molecular mass of about 28 000 (Figure 3, lane 2). Thus, the removal of only a small part of the enzyme converted it from a highly hydrophobic to a water-soluble protein. The nature of the cleaved part (lipid or peptide or both) was not further investigated.

The products of the enzymatic reaction were analyzed by thin layer chromatography. It was found for both solubilized enzyme preparations that they cleaved ϵ-NAD^+ ($R_f = 0.32$) to a compound whose mobility coincided with that of ϵ-ADP−ribose ($R_f = 0.18$). No formation of ϵ-AMP ($R_f = 0.43$) was observed, ruling out the presence of phosphodiesterase activity. In addition, the only detectable radioactive product formed after cleavage of [*nicotinamide*-^{14}C]NAD^+ ($R_f = 0.35$) had the mobility of nicotinamide ($R_f = 0.88$). Thus, formation of nicotinamide mononucleotide ($R_f = 0.44$), an expected product of a phosphodiesterase reaction, did not occur. It was, therefore, concluded that the enriched fractions contained a single activity utilizing NAD^+, NAD^+ glycohydrolase. Consequently, these preparations could be used for the kinetic characterization of detergent- and steapsin-solubilized NADase.

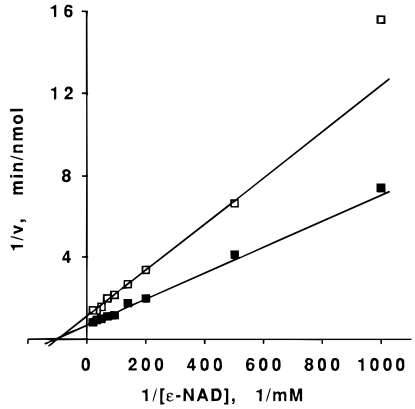

FIGURE 4: Kinetics of mitochondrial NADase solubilized by steapsin or detergent. The activity of steapsin-solubilized NADase (■) and detergent-solubilized NADase (□) was measured fluorimetrically in a buffer containing 50 mM Tris-HCl (pH 8.0) and 0.03% LDAO as described in Experimental Procedures using various substrate concentrations. The assays were carried out using 7 μg of enzyme. Activity was calculated according to the initial slopes of the fluorescence traces. The data were fitted to the Michaelis−Menten equation to achieve adequate weighting of the individual values (Cleland, 1979) for the determination of apparent K_M values (given in the text). The lines reflect the results of the computation. Data points represent mean values of three determinations using at least two different enzyme preparations each.

The enzyme assay was based upon the fluorescence enhancement observed when ϵ-NAD^+ is cleaved. Both forms of the enzyme exhibited similar binding affinities toward ϵ-NAD^+ with apparent K_M values of 9 and 10 μM for the steapsin- and detergent-solubilized forms, respectively (Figure 4).

Nicotinamide, a known inhibitor of NADases, exerted a similar inhibitory effect on both the steapsin- and detergent-solubilized NADases. Virtually complete inhibition was achieved by 2 mM nicotinamide (see, for example, Figure 5B). Inhibitor titrations at several substrate concentrations and subsequent Dixon plot analysis of the data indicated K_I values of 128 and 150 μM for the steapsin- and detergent-solubilized forms of the enzyme, respectively.

The two forms of mitochondrial NADase also behaved similarly with respect to changes in temperature, pH, and ionic strength. The temperature range between 35 and 45 °C was optimal for enzyme activity. The optimal pH was found to be in the range of pH 8−9. Increasing salt concentrations caused significant loss of activity. At 1 M NaCl, the activity of both forms of the enzyme decreased by about 80%. After 10 min, only 40−50% of the original activity could be recovered upon dilution into the assay medium. This observation was also important for the elaboration of an appropriate purification procedure. For example, elution with high salt concentrations or hydrophobic interaction chroma-

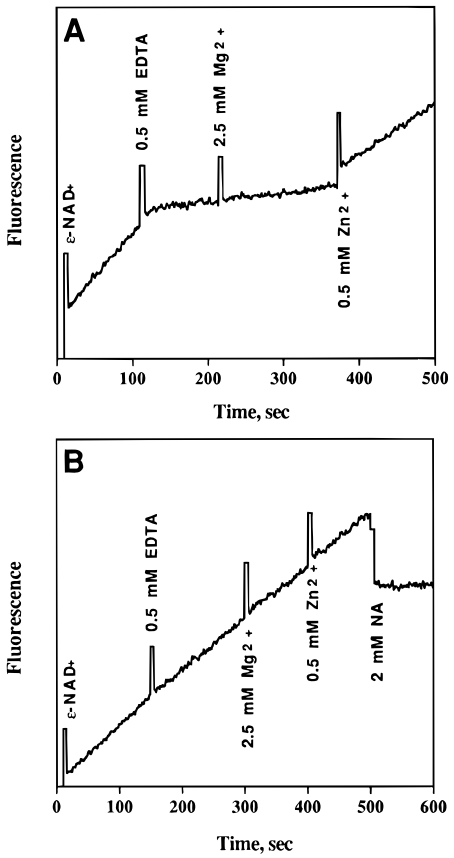

FIGURE 5: Different effects of metal ions and their chelation on the activity of mitochondrial NADase solubilized by steapsin or detergent. The activity of detergent-solubilized NADase (A) and steapsin-solubilized NADase (B) was assayed fluorimetrically as described in Experimental Procedures in a buffer containing 50 mM Tris-HCl (pH 8.0) and 0.03% LDAO. The reaction was started with 10 μM ϵ-NAD$^+$ as shown. Further additions were made as indicated. NA, is nicotinamide.

tography (using a high salt concentration as starting conditions) needed to be avoided.

A pronounced difference between the two forms of the enzyme concerning their sensitivity toward bivalent metal ions was detected. While the activity of the steapsin-solubilized NADase was not affected by either EDTA, Mg^{2+}, or Zn^{2+} (Figure 5B), the detergent-solubilized form was strongly inhibited in the presence of 0.5 mM EDTA (Figure 5A). The activity could be largely recovered by the addition of 0.5 mM $ZnCl_2$ but not by 2.5 mM $MgCl_2$ (Figure 5A). It should be pointed out that the additions shown in Figure 5 were also made separately in separate experiments (e.g. addition of Zn^{2+} directly after EDTA) with the same results. Addition of Mg^{2+} or Ca^{2+} to either form of the enzyme had no effect on activity.

It is also shown in Figure 5B for the steapsin-solubilized enzyme that it was virtually completely inhibited by 2 mM nicotinamide.

DISCUSSION

It is an important result of the present investigation that the mitochondrial NAD$^+$ glycohydrolase can be isolated in a water-soluble or detergent-solubilized form with virtually identical kinetic properties. Therefore, further studies, e.g. of the reaction mechanism or of essential parts of the protein involved in catalysis, can be carried out using the hydrosoluble form. The possibility of avoiding the presence of detergents will undoubtedly facilitate such studies. A microsomal NADase from calf spleen has been solubilized by steapsin (Schuber & Travo, 1976). Its molecular mass was estimated by gel filtration to be about 24 000 as compared to about 32 000 for the intact enzyme (Muller-Steffner et al., 1993). However, this microsomal steapsin-solubilized enzyme preparation appeared to be inhomogeneous, as it contained more than one catalytically active peptide (Schuber & Travo, 1976).

The occurrence of a single activity stainable band after SDS−PAGE of the mitochondrial steapsin-solubilized preparation (Figure 3, lane 2) suggests the presence of a specific cleavage site in the membrane-bound enzyme. It is quite surprising that the removal of a component of the protein with a molecular mass as small as about 2000 rendered it water-soluble, because the uncleaved mitochondrial enzyme has been found to be highly hydrophobic (Moser et al., 1983; Zhang et al., 1995). It is likely that this detached part represents the moiety of the enzyme that anchors it within the membrane. Since steapsin contains lipolytic as well as proteolytic activities, the nature of the cleaved part remains unknown (see below).

With respect to the putative membrane anchor, it is an important finding that the activity of the truncated, water-soluble form of mitochondrial NADase is independent of the presence of bivalent metal ions, whereas the membrane-bound enzyme can be almost completely inhibited by addition of EDTA. These observations indicate that the membrane part of the enzyme may play a role in the regulation of enzyme activity but not in catalysis itself. The reversal of EDTA inhibition by Zn^{2+} but not by Mg^{2+} suggests that a metal ion with a significantly higher affinity to EDTA than Mg^{2+}, such as, for example, Mn^{2+}, Co^{2+}, or Zn^{2+}, is required for enzyme activity. That is, the apparent reactivation by Zn^{2+} shown in Figure 5A could also be due to competetive binding of Zn^{2+} to EDTA, thereby displacing another metal ion that had been chelated by EDTA. It is interesting to note that the well-characterized NADase and ADP-ribosyl cyclase CD38 is activated by Zn^{2+} and Cu^{2+} (Zocchi et al., 1993). The absence of a dependence on metal ions of the steapsin-solubilized form could have several reasons. One could speculate that the part of the enzyme that is removed by the steapsin treatment contains (part of) a metal binding site. Alternatively, the mitochondrial NADase could interact with another, inhibitory membrane protein which is not solubilized by steapsin but contains a metal binding site. Binding of the metal ion might cause dissociation from the NADase, leading to activation of the enzyme. In any case, it is important to emphasize that the metal ion serves as a regulator but is not involved in the catalytic step.

We were unable to achieve measurable solubilization by phosphatidylinositol (PI)-specific phospholipase C.[2] This feature clearly distinguishes the mitochondrial enzyme from CD38, a PI-anchored ectoenzyme found in the plasma membrane of blood cells (Takasawa et al., 1993; Zocchi et al., 1993). In addition, treatment of mitochondria with phospholipase (A_2 or C) or trypsin did not yield any solubilized NADase activity. It was also observed that Ca^{2+} greatly stimulated the solubilization, while PMSF and EDTA

[2] M. Ziegler, D. Jorcke, and M. Schweiger, unpublished observations.

were inhibitory.[2] Therefore, solubilization of NADases by steapsin appears to require a combined action of protease(s) and lipase(s) as has also been observed and discussed by other researchers (Green & Bodansky, 1965; Bock et al., 1971; Schuber & Travo, 1976). It seems likely that the digestion of membrane phospholipids by lipases renders the cleavage site of the NADase susceptible to proteolytic attack, thus removing the hydrophobic membrane part of the enzyme. At least for the mitochondrial NADase, a quite specific cleavage site would be suggested by the fact that a single sharp activity stainable band is obtained after the steapsin treatment.[3]

The successful application of the fluorescent NADase assay to identify NADases directly within the gel after SDS−PAGE has provided some valuable information. The molecular mass of the purified mitochondrial NADase from beef liver could be confirmed to be about 30 000 rather than about 62 000, as has been proposed for the enzyme from rat liver mitochondria (Moser et al., 1983). It is of interest that the mitochondrial NADase could not be visualized in the gel if the sample buffer contained β-mercaptoethanol. This may indicate that the enzyme contains a disulfide bridge which possibly stabilizes the structure during electrophoresis. A disulfide bond is also implicated by the fact that DTT exerted an inhibitory effect on both forms of the enzyme.[2] The apparent molecular mass of the purified detergent-solubilized enzyme was estimated to be about 32 000 after separation in an SDS−PAGE under reducing conditions (Zhang et al., 1995). It has been found for other proteins, e.g. the δ-subunit of the *Escherichia coli* F_1-ATPase, that the presence of an internal disulfide bridge may cause an appreciably higher mobility on SDS−PAGE (Ziegler et al., 1994), suggesting a smaller apparent molecular mass. It seems, therefore, reasonable to assume that the detergent-solubilized NADase run under nonreducing conditions might exhibit a somewhat lower apparent molecular mass as compared to that found for the reduced form.

All NADases tested were able to renature after gel electrophoresis, whereas phosphodiesterase activity could not be visualized. Importantly, at least the three NADases used in this study (from beef liver mitochondria, *N. crassa*, and porcine brain) appear to function as monomers or homo-oligomers, as they exhibited a single band containing NADase activity. The molecular masses of the monomers of the enzymes from porcine brain and *N. crassa* were estimated to be about 36 000 and 205 000, respectively. The unusually large size of the NADase from *N. crassa*, which was confirmed even in the presence of reducing agents in the sample buffer (not shown), may be taken as an indication that this enzyme serves more functions than just the splitting of NAD^+. This is a quite intriguing aspect, as this NADase apparently does not serve as an ADP-ribosyl cyclase (Graeff et al., 1994).

NADase activity has been implicated in the regulation of mitochondrial Ca^{2+} efflux (Richter & Kass, 1991). Under conditions causing peroxidation reactions, e.g. in the presence of *tert*-butyl hydroperoxide, pyridine nucleotide degradation and concomitant Ca^{2+} efflux have been observed. Our results do not provide any indication that the mitochondrial NADase might function directly as a calcium sensor, since Ca^{2+} appeared to have no effect on NADase activity of either form of the enzyme. Further studies will be aimed at testing the influence of prooxidants on NADase activity.

Finally, tight regulation of the activity of NAD^+ glycohydrolase is an obvious physiological necessity, since resynthesis of NAD^+ is quite expensive for the cell. The influence of bivalent metal ions on the mitochondrial enzyme shown here offers a possible mechanism for such a regulation.

[3] Attempts to convert the detergent-solubilized enzyme to the hydrosoluble form by treatment with steapsin led to a rapid decline of activity, presumably due to proteolysis. No formation of a truncated form was detectable.

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