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ORIGINAL ARTICLES

CD38 in Bovine Lung: A Multicatalytic NADase

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Abstract We report the kinetics and molecular properties of CD38 purified from bovine lung microsomal membranes after its solubilization with Triton X-100. The enzyme was found to be a novel member of a multicatalytic NAD^+-glycohydrolase (NADase, EC 3.2.2.6). It was able to utilize NAD^+ in different ways, producing nicotinamide (Nam) and either adenosine diphosphoribose (ADPR, NADase activity) or cyclic ADPR (cADPR, cyclase activity); it also catalyzed the hydrolysis of cADPR to ADPR (cADPR, hydrolase activity). In addition, the enzyme catalyzed the pyridine base exchange reaction with conversion of NAD^+ into NAD analogues. These data are evidence that CD38 is involved in the regulation of both NAD^+ and calcium-mobilizing agents, the concentration resulting in an essential enzyme that plays a key role in cellular energy and signal-transduction systems.

Keywords CD38 · NAD^+-glycohydrolase · Multicatalytic enzyme · cADPR · NAD homeostasis

Introduction

Nicotinamide adenine dinucleotide (NAD^+) plays a central role in many cellular functions. In addition to its well-known role as a coenzyme involved in energy metabolism, NAD^+ plays a key role in signal transduction (Berger et al. 2004). In particular, NAD^+ is the substrate for the synthesis of the second messengers cyclic ADP ribose (cADPR), ADP ribose (ADPR) and nicotinic acid adenine dinucleotide phosphate (NaADP) by way of ADPR cyclase and NAD-glycohydrolase (NADase) (Dousa et al. 1996; Lee et al. 1999).

cADPR, ADPR and NaADP act as calcium-mobilizing agents in many cell types and tissues; they are involved in physiological processes including egg fertilization, gene expression, cell proliferation, insulin secretion and regulation of vascular function (Lee 2002).

Moreover, a wide variety of processes are regulated by changes in intracellular Ca^{2+} concentration, so these biologically active molecules are the key molecules in many calcium-signaling pathways in plants, invertebrates and vertebrates.

Clearly, not all stimuli that increase intracellular calcium concentration initiate a common cell response; in addition, not all stimuli that initiate a given cell response do so by eliciting Ca^{2+} signals with the same spatiotemporal pattern (Berridge et al. 2000). The specificity of the mechanism is determined, in part, by the release of Ca^{2+} from intracellular stores in dependence of the second messengers and their associated Ca^{2+}-release channels.

The homeostasis of the pyridine nucleotides depends both on CD38 enzymatic activities and on NAD^+ concentration (Magni et al. 2004).

NAD^+ is also the substrate for mono-ADP-ribosyltransferases (ARTs) and poly(ADP-ribose) polymerases (PARPs). The biological functions of these enzymes could be related to regulation of chromatin expression, cell differentiation, immune response, apoptosis, DNA repair, physiological cleavage of NAD^+ and pathological conditions such as cellular death (Burkle 2005; Ziegler 2000).

Furthermore, new evidence suggests that NAD^+ is the substrate and regulator of NAD^+-dependent deacetylases

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such as SIRT1, which modulates aging, cell protection and energy metabolism in mammalian cells (Aksoy et al. 2006a).

Acquiring knowledge about the catalytic properties of CD38 is important in order to elucidate the mechanisms that regulate NAD homeostasis and its maintenance, vital factors for cell life.

We report on the purification and characterization of CD38 from bovine lung. In particular, this membrane-bound protein was a novel member of the multifunctional NADases, showing the transglycosylation reaction in addition to NADase, ADPR cyclase and cADPR hydrolase activities.

Materials and Methods

Materials

Fresh lung was obtained from a bovine immediately after its death.

Polybuffer 74 and Mono P HR 5/5 were from Pharmacia (Uppsala, Sweden). Matrex gel Red-A was from Amicon (Beverly, MA). IDA-sepharose 6B and CM-sepharose CL 6B were from Sigma (St. Louis, MO). DEAE Cellulose A52 was from Whatman (Clifton, NJ). Imidazole, Triton X-100, potassium and sodium phosphate and chloride, Tris and sodium acetate were obtained from Baker (Phillipsburg, NJ). NAD^+ and phenylmethylsulfonyl fluoride (PMSF) were from Jansen (Piscataway, NJ). M_r markers were from Bio-Rad (Richmond, CA). All other chemical reagents were purchased from Sigma.

Determination of Enzymatic Activities

Fluorimetric Assay

Identification of NADase activity was followed by a continuous fluorimetric assay, utilizing nicotinamide 1,N^6-ethenoadenine dinucleotide (εNAD^+) as substrate and detecting the fluorescence emission at 410 nm, upon excitation at 300 nm. The reaction mixture, containing 25 μM εNAD^+, 100 mM potassium phosphate buffer (pH 7.0) and an aliquot of the enzyme sample in a final volume of 500 μl, was incubated at 38°C.

HPLC Assay

The enzymatic activity of NADase, ADPR cyclase and cADPR hydrolase was assessed as already published (Orsomando et al. 2000). The reaction mixtures were incubated at 38°C and contained 100 mM potassium phosphate buffer (pH 7.0), an aliquot of the enzyme sample and 1 mM NAD^+ or 0.1 mM cADPR as substrate, in a final volume of 250 μl. In the presence of NAD^+ we calculated at the same time NADase and ADPR cyclase activities, following the increase of ADPR and cADPR, respectively; instead, in the presence of cADPR, hydrolase activity was calculated following the increase of ADPR concentrations. One unit is defined as the amount of the enzyme catalyzing the synthesis of 1 μmol ADPR (NADase) or cADPR (cyclase) from NAD^+ or 1 μmol ADPR (hydrolase) from cADPR, per minute, at 38°C.

The samples for HPLC analysis were prepared as follows: 50-μl aliquots of each reaction mixture were stopped at different times by adding 25 μl of 1.2 M $HClO_4$; after 10 min on ice and centrifugation for 5 min at 16,000 × *g*, 75 μl of each supernatant were neutralized by adding 17.5 μl of K_2CO_3 0.79 M, kept on ice and centrifuged again. Aliquots of 50 μl of the supernatant obtained were directly injected onto RP Supelcosil LC-18 columns (5 μm, 250 × 46 mm) equilibrated with buffer A (100 mM potassium phosphate [pH 6.0], 8 mM tetrabutylammonium hydrogen sulfate). The flow rate was 1.3 ml/min and the elution conditions were as follows: 2.5 min at 100% buffer A, 9.5 min at up to 15% buffer B (buffer A containing 30% methanol), 6 min at up to 40% buffer B, 2 min at up to 100% buffer B and held at 100% buffer B for 5 min. Finally, the gradient returned to 100% buffer A in 1 min. The eluate was followed at 254 nm.

Purification Procedure

All purification steps were carried out at 4°C.

Microsome Extraction

Bovine lung (200 g), separated from membranes and cut into small pieces, was extensively washed in physiological saline solution and homogenized three times (30 s each, at 10-s intervals) with an Ultra Turrax T-25 (IKA Works, Staufen, Germany), at 10,000 rpm, in one volume of ice-cold homogenization buffer (100 mM Tris-HCl [pH 8.0], 150 mM KCl, 1 mM EDTA, 1 mM PMSF). The homogenate was then centrifuged at 10,000 × *g* for 30 min. A crude microsomal fraction was prepared by centrifugation of the supernatant of the previous step at 110,000 × *g* for 90 min. The microsomal pellet was resuspended in homogenization buffer and washed twice in the same buffer.

Solubilization

CD38 was solubilized from the pelletted microsomes with homogenization buffer containing 3% Triton X-100. After gentle stirring overnight, the insoluble material was removed by centrifugation at 110,000 × *g* for 90 min.

Acid Treatment (pH 5)

The pH of the solubilized enzyme was decreased by adding 1 M CH_3COOH until pH 5.0. After 30 min on ice, under gentle stirring, the sample was centrifuged for 20 min at 20,000 × *g*.

CM-Sepharose CL 6B Column Chromatograph

The supernatant obtained after the acidic treatment was directly loaded onto the CM-sepharose CL 6B column, previously equilibrated with 20 mM CH_3COONa (pH 5.0) containing 1% Triton X-100. The NADase-enriched fractions were eluted by a linear NaCl gradient, from 0 to 0.5 M in the same buffer. The active fractions were pooled and dialyzed against 10 mM Tris-HCl (pH 8.0) containing 0.5% Triton X-100.

DEAE Cellulose A 52

The dialyzed CM-sepharose CL 6B pool was then applied to a DEAE Cellulose A 52 column, previously equilibrated with 10 mM Tris-HCl (pH 8.0) containing 0.5% Triton X-100. In these conditions the enzymatic activity was recovered in the unbound fraction.

Matrex Gel Red-A Column Chromatography

The active DEAE Cellulose A 52 pool was applied to a red-A column previously equilibrated in 10 mM Tris-HCl (pH 7.2) containing 0.1% Triton X-100. The column was first washed in 20 mM Tris-HCl (pH 7.2), 0.2 M NaCl and 0.1% Triton X-100, then eluted with a linear NaCl gradient, from 0.4 to 1.5 M in the same buffer. The active fractions were pooled and dialyzed against 50 mM TFNa (pH 7.2) containing 0.1% Triton X-100.

IDA-Sepharose 6B Column

The active red-A pool was finally loaded onto an IDA-sepharose 6B column, previously saturated with an excess of Cu^{2+} and equilibrated with 50 mM TFNa (pH 7.2) containing 0.1% Triton X-100. After washing the column in the same buffer containing 0.5 M NaCl, NADase activity was eluted with a linear imidazole gradient, from 0 to 0.2 M. The active fractions were pooled and dialyzed against 10 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100.

Throughout the purification procedure, NADase activity was detected by fluorimetric assay and the eluate absorbance was monitored at 245 nm to minimize the interference caused by Triton X-100. The final enzyme preparation, containing pure bovine lung CD38, was lyophilized and stored at −20°C.

Electrophoresis

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous electrophoresis was performed under denaturing conditions (Laemmli 1970) in a 12% polyacrylamide gel. The final enzyme preparation (10 μg) was treated with a standard denaturing mixture both in the presence and in the absence of 5% 2-mercaptoethanol. Samples and M_r markers were run after boiling for 5 min.

NADase Renaturation in SDS-PAGE

The enzyme sample in a standard mixture without 2-mercaptoethanol and not boiled was loaded onto SDS polyacrylamide gels cooled at 4°C. After the run, gels were either stained for protein with Coomassie Brilliant Blue R-250 or treated to allow renaturation of the enzyme activity. The gel was washed twice with 200 mM Tris-HCl (pH 7.5) and 6% Triton X-100 and incubated for 90 min at 38°C with the same buffer containing 1 mM εNAD. NADase activity resulted as a fluorescent band evidenced by UV light.

Chromatofocusing

The isoelectric point of NADase was calculated by FPLC using a Mono P HR 5/5 column, previously equilibrated with 25 mM Bis-Tris buffer (pH 7.1) and 0.1% Triton X-100 at a flow rate of 1 ml/min. After loading the sample, NADase was eluted with Polybuffer 74 (diluted 1:10 in distilled water and adjusted to pH 4.0 with 1 M HCl) containing 0.1% Triton X-100. The fractions were tested for the pH value and immediately neutralized by adding 200 μl of 0.5 M Tris-HCl (pH 7.0) before the enzymatic assay.

pH Optimum

Enzyme assays were carried out by spectrofluorimeter using εNAD^+ as substrate. In the pH studies, a 0.028 M universal buffer (citric acid, KH_2PO_4, barbital and boric acid adjusted to the appropriate pH with 0.2 M NaOH) was used.

Protein Determination

Quantitative measurement of proteins was carried out by the Bradford (1976) method, using BSA as standard. Because of the interference caused by Triton X-100, determinations were performed in the presence of detergent concentration not exceeding 0.1%, both in the standard and in the sample.

Results

Enzyme Purification

CD38 was purified from bovine lung after its solubilization from the microsomal fraction utilizing Triton X-100. The purification procedure involved an acidic treatment and four different chromatographic steps; the data are summarized in Table 1.

SDS-PAGE conducted on the final enzyme preparation showed the presence of a 30-kDa band under reducing conditions, while under nonreducing conditions two bands corresponding to 30 and 60 kDa were detected. Both the bands could be renatured directly on the gel as described in "Materials and Methods," and the NADase activity was evidenced as two fluorescent bands at 30 and 60 kDa.

Catalytic Properties and Kinetic Parameters

Bovine lung CD38 was demonstrated to be a multicatalytic enzyme able to synthesize nicotinamide (Nam), ADPR and cADPR from NAD^+. In fact, the same protein possesses NADase, ADPR cyclase and cADPR hydrolase activities (Fig. 1). Furthermore, the protein is able to catalyze the base exchange reaction as demonstrated by the production of 3-acetylpyridine adenine dinucleotide (3-AcPyr AD^+) after its incubation in the presence of both 1 mM NAD^+ and 10 mM 3-acetylpyridine (Fig. 2).

ADPR cyclase, cADPR hydrolase and NADase showed a ratio of about 1/20/100.

In order to determine the K_m values for different substrates, the enzyme activity was determined in the presence of the following:

(a) NAD^+ (0.05–3 mM)
(b) εNAD^+ (0.0025–0.1 mM)
(c) $NADP^+$ (0.05–1 mM)
(d) cADPR (0.02–1 mM)

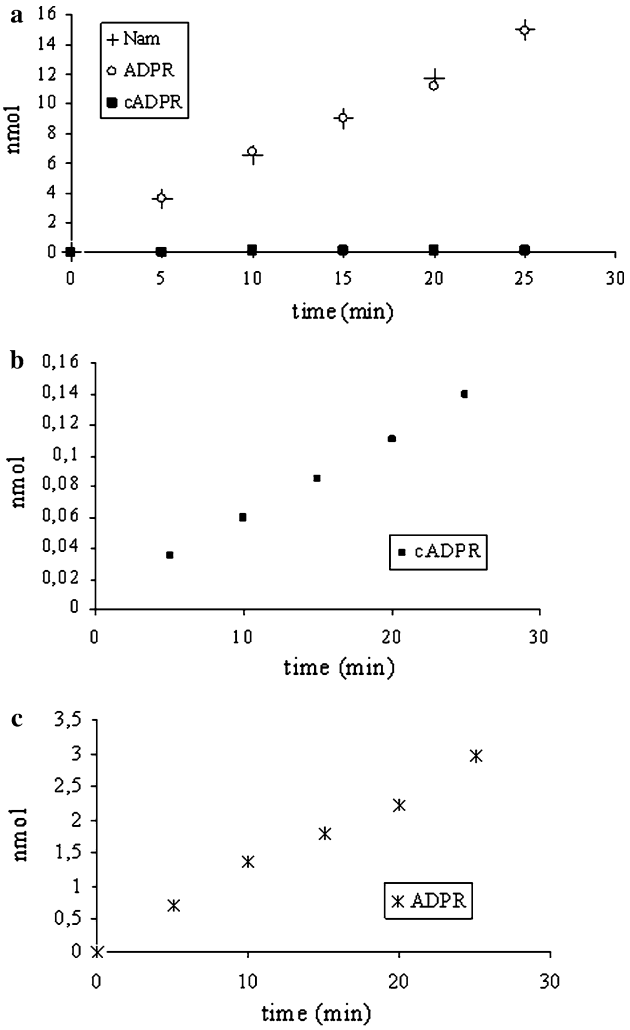

Fig. 1 Bovine lung NADase, ADPR cyclase and cADPR hydrolase activities. The pure enzyme was incubated with 1 mM NAD^+: NADase activity was evidenced by the production of both Nam (+) and ADPR (o) (**a**), and ADPR cyclase was calculated by measuring cADPR (■) formation (**b**), corresponding to about 1% of ADPR. Incubation with 1 mM cADPR showed cADPR hydrolase activity, resulting in ADPR (*) production (**c**)

Table 1 Purification of NADase from bovine lung

Step	Protein content (mg)	Total activity (U total)[a]	Specific activity (U/mg)[a]	Yield (%)[b]	Purification (fold)[b]
Solubilized Triton X-100	4,248	84.96	0.02	100	
pH 5.0	1,100	66	0.06	77.7	3.3
CM-CL6B	117	23.4	0.20	27.5	10
DEAE A52	16.25	4.87	0.30	5.7	15
Matrex Gel Red-A	2	1.18	0.59	1.4	29.5
Cu^{2+} IDA	0.37	0.44	1.18	0.52	59

[a] One unit is defined as the amount of the enzyme able to synthesize 1 μmol ADPR from NAD^+, per minute, at 38°C

[b] Data represent the mean of three independent determinations

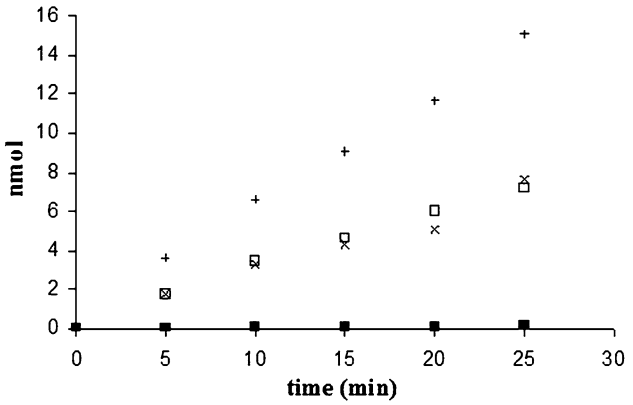

Fig. 2 The multicatalytic bovine lung NADase:tranglycosidation reaction. The ability of the enzyme to catalyze the pyridine base exchange reaction was demonstrated by incubating the pure enzyme in the presence of 1 mM NAD and 10 mM 3-acetylpyridine. The reaction products Nam (+), ADPR (×), cADPR (■) and 3-AcPyr AD^+ (□) were identified by HPLC

The enzymatic activities were calculated following the time-dependent formation of ADPR or its analogues by HPLC. The K_m values, calculated by double-reciprocal plot, were 126, 50, 88 and 290 μM, respectively.

Both Nam and ADPR showed a product inhibition for NADase activity; in particular, Nam proved to be a non-competitive inhibitor, with a $K_i = 0.53$ mM, while ADPR was a competitive one, with a $K_i = 2.35$ mM.

The effect of metal ions on NADase activity was investigated in the presence of several ions at a final concentration of 0.1 mM. As reported in Table 2, the activity was inhibited totally by Hg^{2+} and partially by Cu^{2+}. Further analyses demonstrated that 0.023 mM Hg^{2+} and 0.036 mM Cu^{2+} were the ion concentrations able to determine a 50% inhibition effect. Instead, the enzymatic activity was not affected in the presence of anions.

Table 2 Effect of metal ions on bovine lung NADase activity

Cation 0.1 mM	Residual activity (%)
Control	100
Zn^{2+}	80
Mn^{2+}	87.5
Cd^{2+}	87.5
Cr^{2+}	102
Ca^{2+}	105.5
Mg^{2+}	97.5
Fe^{2+}	93
Ag^{2+}	99
Hg^{2+}	5
Co^{2+}	93.5
Cu^{2+}	25
Sn^{2+}	100
Li^{+}	105
Pb^{+}	85
Na^{+}	109
Cr^{3+}	100
Fe^{3+}	69

In addition, NADase activity was inhibited in the presence of dye molecules such as Red-120 and Cibacron Blue 3FGA; in particular, 50% inactivation was evidenced at 2.15 and 6 μM, respectively.

A 50% NADase residual activity was measured in the presence of 10 mM DTT and 0.42 mM isoniazide, while full inactivation was observed after addition of SDS, even at micromolar concentrations. However, after SDS treatment, about 80% of enzyme activity could be recovered by renaturation with Triton X-100 (20:1 v/w ratio with SDS).

The chromatofocusing experiments, conducted as described in "Materials and Methods," revealed an isoelectric point of 8.3. NADase was active in the pH range 4.0–10.0, with a pH optimum at 7.1.

NADase showed maximal activity at 45.5°C and was rapidly inactivated at temperatures higher than 55°C.

Enzyme Stability

NADase activity was fully stable in 10 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100 for at least 3 months after storage at 4, −20, −80 and −196°C. After lyophilization and storage at −20°C, the enzyme was fully active after 1 year. NADase lost 50% of its enzymatic activity after incubation at 50°C for 20 min.

Discussion

CD38 was purified to homogeneity from bovine lung microsomal membranes. The catalytic properties evidenced led us to identify this membrane-bound protein as a novel member of the multifunctional NADases, showing the transglycosylation reaction in addition to NADase, ADPR cyclase and cADPR hydrolase activities. The kinetic parameters determined for the bovine lung NADase demonstrated that the enzyme is similar to the enzyme from other sources.

In recent decades, an emerging physiological role has been attributed to those ubiquitous enzymes. In particular, cADPR, ADPR and NAADPR, endogenous metabolites of NAD^+ via NADase, have been identified as intracellular second messengers in a large variety of cells including plant, protozoan and mammalian.

Recent investigations have demonstrated the physiological and pathophysiological roles of CD38(NADase)/cADPR signaling in the regulation of many cell functions

including insulin secretion, egg fertilization and cell proliferation.

In addition, the importance of redox status as a regulator of ADPR cyclase was demonstrated in some tissues or cells; in fact, the production of cADPR and the subsequent calcium release are increased by intracellular oxidants. In pulmonary arteries, the cellular redox state has been indicated as a regulator of hypoxic pulmonary vasoconstriction, and recent studies conducted with O_2^- generation systems indicate that cADPR signaling is involved in the regulation of calcium mobilization in vascular smooth muscle during oxidative stress (Wilson et al. 2001; Zhang and Li 2006).

Furthermore, in hypoxia-induced pulmonary vasoconstriction, CD38/cADPR signaling contributes to airway hyperesponsiveness, a hallmark of asthma (Deshpande et al. 2005).

Another exciting contribution highlighting the versatility of NAD^+ is the discovery of SIRT enzymes, NAD-dependent histone deacetylases which modulate aging, senescence, apoptosis and energy metabolism in mammalian cells. The mechanisms that regulate intracellular modulation of SIRT enzymes are still unclear, but a novel role for CD38 as a major regulator of NAD^+ levels and SIRT activity has been reported (Aksoy et al. 2006b).

It is important to note that all the NAD^+ catabolizing enzymes known so far produce nicotinamide in addition to ADPR, cADPR, NaADPR, poly(ADP)ribose and deacetylated proteins/histones. The nicotinamide is an inhibitor of all the enzymes involved in these reactions. It is possible to hypothesize that changes in Nam concentration regulate the metabolic fluxes occurring in these NAD-mediated pathways, which are involved in the control of crucial cellular events.

At the start of this decade, Nam was adopted in therapeutic trials in order to prevent cancer recurrence and insulin-dependent diabetes (Gale 2003; Kaanders et al. 2002).

The biological relevance of this ubiquitous inhibitory effect, which acts on all the NAD^+ catabolizing enzymes, remains unclear; but it could be due to the vital role that NAD^+ homeostasis exerts in cellular life.

The comprehensive molecular characterization of NAD metabolic pathways over the past few years has further extended the understanding of the multiple roles of pyridine nucleotides in cellular function and the involvement in different pathways.

Understanding how CD38 catalytic properties and kinetic constants could be modulated in physiological and pathological conditions represents an important goal in order to define appropriate pharmacological therapy.

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