FEBS Letters 377 (1995) 530–534 FEBS 16437

# Identification and purification of a bovine liver mitochondrial $NAD^+$-glycohydrolase

Ju Zhang[a], Mathias Ziegler[b], Rainer Schneider[a,*], Helmut Klocker[c], Bernhard Auer[a], Manfred Schweiger[b]

[a]*Institute of Biochemistry, University of Innsbruck, Peter Mayr Straße 1a, A-6020 Innsbruck, Austria*
[b]*Institut für Biochemie, Freie Universität Berlin, Berlin, Germany*
[c]*Department of Urology, University of Innsbruck, Innsbruck, Austria*

Received 3 November 1995

**Abstract Nonenzymatic ADP-ribosylation of mitochondrial proteins is thought to play a role in the regulation of $Ca^{2+}$ efflux from mitochondria. It has been shown that intramitochondrial ADP-ribose is generated by a specific $NAD^+$glycohydrolase, which catalizes hydrolysis of $NAD^+$ to ADP-ribose and nicotinamide. We purified this enzyme from bovine liver mitochondrial membranes. The final preparation had a 1660-fold purified enzyme activity and contained a main protein band with an apparent molar mass of 32,000 in a SDS-polyacrylamide gel. The identity of this protein band with $NAD^+$-glycohydrolase was verified by renaturation of its enzymatic activity. Partial amino acid sequence information was obtained from two enzyme fragments after proteolytic cleavage of the protein band in the SDS-polyacrylamide gel. Searches in protein databases revealed that an arginine ADP-ribosyl hydrolase harbours two stretches of amino acids that are highly similar to the partial $NAD^+$-glycohydrolase sequences.**

*Key words:* $NAD^+$-glycohydrolase; ADPR-arginine hydrolase; Mitochondrial membrane; Purification

## 1. Introduction

The level of cytosolic free $Ca^{2+}$ is a key factor of cellular regulation [1]. Extracellular signals often activate membrane $Ca^{2+}$ channels through the synthesis of second messengers that trigger $Ca^{2+}$ release from internal stores like inositol 1,4,5-trisphosphate or the very recently discovered cyclic ADP-ribose (ADPR) [2,3]. However, the molecular mechanisms of $Ca^{2+}$ uptake and release of mitochondria are not well understood yet. Release of $Ca^{2+}$ from intact rat liver mitochondria can be induced by the oxidation of mitochondrial pyridine nucleotides by hydroperoxides [4]. It has been shown that an increased generation of endogenous hydroperoxides is associated with a high level of oxidized pyridine nucleotides [5]. However, the oxidation of pyridine nucleotides is not sufficient to induce the release of $Ca^{2+}$ from mitochondria. In addition, hydrolysis of $NAD^+$ to nicotinamide and ADP-ribose by $NAD^+$glycohydrolase is required [6]. It has been reported that ADPR generated by this enzyme might lead to ADP-ribosylation of acceptor proteins in the inner mitochondrial membrane in a nonenzymatic reaction [7]. Specific ADP-ribosylation of an ADPR acceptor protein may then trigger the release of $Ca^{2+}$ from mitochondria through regulation of a $Ca^{2+}$ channel. The respective $NAD^+$glycohydrolase was assigned to the inner mitochondrial membrane and a 60- to 70-fold enriched preparation from rat liver showed a main protein band with a molar mass of 62,000 [8]. The putative ADPR acceptor protein was proposed to be a protein of the inner mitochondrial membrane which has a molar mass of 30,000 [6]. A recent report suggested that the mitochondrial $NAD^+$-glycohydrolase is located in the outer mitochondrial membrane [9]. This localization is quite controversial as it would call into question the role of $NAD^+$-glycohydrolase in the hydrolysis of intramitochondrial pyridine nucleotides. In case of a localization in the outer membrane $NAD^+$ could only be hydrolysed after its release through an inner membrane pore. Formation of such pores has been observed upon oxidative stress [10]. However, their involvement in $NAD^+$ hydrolysis appears unlikely, because pore formation is not required for the hydroperoxide-induced $Ca^{2+}$ release from rat liver mitochondria [11]. Thus, the studies published so far which investigated the role of mitochondrial $NAD^+$-glycohydrolase in the $Ca^{2+}$ homeostasis of these organelles are not conclusive. This prompted us to investigate in detail the biochemical and molecular basis of this regulatory system. In this first report we present the purification of the $NAD^+$-glycohydrolase activity from bovine liver mitochondrial membrane and its unequivocal assignment to a protein with a molar mass of 32,000. Moreover, we obtained first molecular data of this enzyme, namely partial amino acid sequence information which might indicate a relationship of $NAD^+$-glycohydrolase to an arginine-ADPR hydrolase.

## 2. Materials and methods

### 2.1. Materials

DEAE-52 cellulose was purchased from Whatman, heparin Sepharose CL-6B from Pharmacia and reactive green-19 agarose from Sigma. Hydroxyapatite was synthesized according to the method of Bernardi [12]. Polyethylenimine cellulose CEL 300 PEI/$UV_{254}$ thin-layer chromatography plates (20 × 20 cm) were from Machery-Nagel. Triton X-100, Triton X-114 and bovine serum albumin (BSA) were obtained from Boehringer-Mannheim, 1,$N^6$-etheno-$NAD^+$ ($\varepsilon NAD^+$), $\beta NAD^+$, ADP-ribose, AMP, ADP and nicotinamide from Sigma, [adenine-2,8-$^3$H]$NAD^+$ (4.2 Ci/mmol) from New England Nuclear (NEN), and the protein determination Kit Micro BCA from Bio-Trade. All other chemicals were of analytical grade.

### 2.2. Protein determination

Protein concentrations were determined using the Micro BCA kit

*Corresponding author. Fax: (43) (512) 507-2894.

*Abbreviations:* ADPR, ADP-ribose; DEAE, diethylaminoethane; DTT, dithiothreitol; PAG, polyacrylamide gel, PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.


*SSDI* 0014-5793(95)01366-0

which is applicable to solutions containing Triton X-100 according to the suggestion of the supplier.

*2.3. Fluorimetric activity assay*

Hydrolysis of $\varepsilon NAD^+$ results in an about 10-fold increase of fluorescence [13,14]. This change of fluorescence was used to monitor NADase activity [15]. Enzyme preparations were incubated for 0.5 h at 37°C in a buffer containing a saturating concentration of $NAD^+$ (100 mM Tris-HCl pH 7.3, 0.2 mg/ml BSA, 0.1% Triton X-100, 1 mM $\beta NAD^+$ and 0.1 mM $\varepsilon NAD^+$) in a total volume of 50 μl. After the incubation 950 μl of $H_2O$ were added and the fluorescence was measured in a fluorescence spectral photometer (Perkin Elmer 650). The samples were excited with 300 nm light, emission was measured at a wavelength of 412 nm.

*2.4. Identification of $NAD^+$-glycohydrolase as an integral membrane protein*

The method reported by Findlay [16] was applied to test if $NAD^+$-glycohydrolase is an integral or a peripheral membrane protein. Triton X-114 was added to an enzyme solution to a final concentration of 2.5% in 10 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl at 0–4°C. Thereafter the mixture was warmed to 25–30°C and centrifuged to obtain a watery and a Triton X-114 phase. In this two phase system the enzyme activity was recovered from the Triton X-114 phase thus identifying it as an integral membrane protein.

*2.5. Purification of $NAD^+$glycohydrolase from bovine liver mitochondria*

*2.5.1. Preparation of mitochondrial membranes.* Mitochondria from fresh bovine liver were isolated according to the procedure described by Bustamante et al. [17]. Mitochondria from about 800 g bovine liver suspended in about 250 ml of 30 mM Tris-HCl buffer, pH 8.5, containing 1 mM DTT, 0.2 mM PMSF were extracted by passing them three times through a precooled French Pressure Cell Press at a pressure of 10,000 psi (680 atm). The extract was centrifuged at 215,000 × *g* for 40 min at 4°C, the supernatant was discarded and the pellet was resuspended in 250 ml of the same buffer with a motor-driven 60 ml glass homogenisator (Potter S, B. Braun) at 4°C. This centrifugation–resuspension step was repeated four times.

*2.5.2. Solubilization of mitochondrial membranes.* After the last centrifugation step the mitochondrial membranes were homogenized in solubilization buffer (30 mM Tris-HCl, pH 8.5, 1 mM DTT, 0.2 mM PMSF and 2% Triton X-100), with a motor-driven 60 ml glass potter at 4°C, the resulting solution was placed on ice for 1 h and then centrifuged at 215,000 × *g* for 50 min. The extract was collected and the solubilization procedure was repeated with the pellet four times. In each extraction cycle the volume of the extraction buffer was reduced. This repeated extraction yielded a total extract of 360 ml containing about 8 mg protein/ml.

*2.5.3. DEAE-52 cellulose chromatography I.* About 3 g of solubilized mitochondrial membrane protein was applied at a flow rate of 50 ml/h to a DEAE-52 column (20 × 5 cm) equilibrated in solubilization buffer. The $NAD^+$-glycohydrolase activity was found in the flow-through.

*2.5.4. Hydroxyapatite chromatography.* The buffer system of the pooled active fractions from the DEAE-52 cellulose column I was adjusted by adding 65 μl of 0.5 M sodium phosphate buffer, pH 6.2, and 11 μl of 1 M NaCl per ml of the protein solution. The adjusted solution was applied to a hydroxyapatite column (4 × 2.5 cm) equilibrated in 30 mM $Na_2HPO_4/NaH_2PO_4$ buffer pH 7.3, containing 10 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 2% Triton X-100. The column was run by gravital force. The $NAD^+$glycohydrolase activity was again in the flow-through of the column.

*2.5.5. Heparin Sepharose CL-6B chromatography.* Combined fractions of the hydroxyapatite column were dialysed for 5–6 h against 10 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.3, containing 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 2% Triton X-100 and applied to a heparin sepharose CL-6B column (1.5 × 1.4 cm) equilibrated in the same buffer at a flow rate of 36 ml/h. $NAD^+$-glycohydrolase activity was retained on the column. The column was washed with 10 column volumes of application buffer followed by a 0–70 mM NaCl salt gradient in application buffer containing 0.1% Triton X-100 which was applied over a period of 60 min at a flow rate of 18 ml/h. For elution a 70–300 mM NaCl gradient in the same buffer was applied over a period of 80 min at the same flow rate. The enzyme activity eluted at a salt concentration of 150 mM.

*2.5.6. Denaturation–renaturation step.* Active fractions from the heparin sepharose eluate were combined and dialysed against 10 mM Tris-HCl buffer, pH 8.5, containing 0.1% Triton X-100 and proteins were denatured by addition of SDS to a final concentration of 0.5%. Thereafter, the protein solution was adjusted to 6% Triton X-100 in order to allow renaturation of a portion of the proteins.

*2.5.7. DEAE-52 cellulose chromatography II.* The renatured protein solution was applied to a second DEAE-52 cellulose column (12.2 × 2.5 cm) equilibrated in 10 mM Tris-HCl buffer, pH 8.5, containing 0.1% Triton X-100. $NAD^+$-glycohydrolase was found in the void volume of the column.

*2.5.8. Reactive green-19 agarose chromatography.* Active fractions from DEAE-52 cellulose column II were combined and the buffer system adjusted to reactive green-19 agarose chromatography by addition of 64 μl of 0.5 M sodium phosphate buffer, pH 6.6, per ml of the protein solution. The resulting protein solution was applied to a small reactive green-19 agarose column (1 × 0.8 cm) equilibrated in 30 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.3, containing 2% Triton X-100. The column was washed first with 10 column volumes of application buffer, second with 5 volumes of 10 mM $Na_2HPO_4/NaH_2PO_4$ buffer, pH 7.3, containing 0.2% Triton X-100 and third with 10 column volumes of 0.1 M NaCl in the same buffer. $NAD^+$-glycohydrolase activity was eluted by raising the NaCl concentration to 0.6 M.

*2.6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed as described by Laemmli [18] in 15% gels or in 12.5–17.5% gradient gels as indicated.

*2.7. $NAD^+$-glycohydrolase renaturation in SDS-polyacrylamide gels*

SDS-PAGs were produced at least 1 day prior to use to allow complete decomposition of radicals. The runs were performed under standard conditions, but the gels were cooled during the run. After the run, the lanes were cut into 1 mm slices. Each slice was put into an 1.5 ml tube and homogenized with a plastic pestle in 60 μl of 0.25 M Tris-HCl buffer, pH 7.3, containing 6% Triton X-100. 10 μl of 500 μM $\varepsilon NAD^+$ solution and 5 μl of 10 mM $\beta NAD^+$ solution were added to each tube, and the samples were then incubated for 1 h at 37°C. Thereafter, 900 μl of $H_2O$ was added, polyacrylamide fragments were settled by centrif-

Table 1
Purification of $NAD^+$glycohydrolase from bovine liver mitochondrial membranes

| Purification steps | Amount of protein (mg) | Total enzyme activity (μmol × $min^{-1}$) | Specific enzyme activity (μmol × $min^{-1}$ × $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude mitochondria proteins | 11320 | 116.5 | 0.01 | 100 | 1 |
| Washed mitochondria membranes | 2900 | 116.5 | 0.04 | 100 | 4 |
| DEAE-52 cellulose I | | – | – | – | – |
| Hydroxylapatite | | – | – | – | – |
| Heparin sepharose CL-6B | 4.0 | 25.64 | 6.41 | 22 | 641 |
| DEAE-52 cellulose II | | – | – | – | – |
| Reactive green-19 agarose | 0.56 | 9.32 | 16.64 | 8 | 1664 |

The purification procedure was performed starting with 800 g of bovine liver. Protein concentrations were determined using the Micro BCA kit which is applicable to solutions containing Triton X-100. $NAD^+$-glycohydrolase activity was determined using the fluorimetric assay.

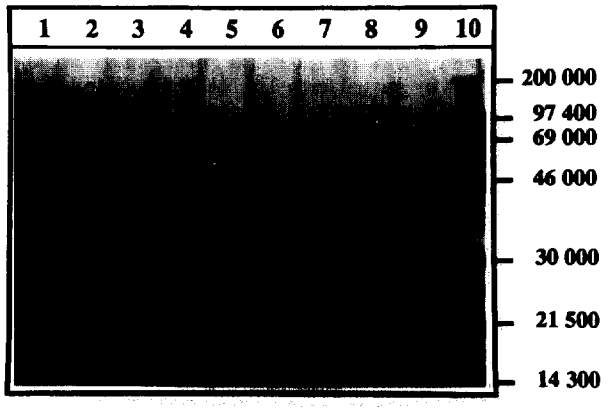


Fig. 1. SDS-PAG of Reactive green-19 agarose eluate fractions. Eluate fractions of the reactive green-19 column were separated in a 15% polyacrylamide gel and silver stained. Lanes 1–9 = eluate fractions, lane 10 = marker proteins. The enzyme activity of eluted fractions 1–9 determined with the fluorimetric assay were 0, 0, 0, 7.1, 12.4, 15.1, 15.3, 11.3 and 7.6 arbitrary units, respectively.

ugation and the fluorescence of the supernatants was measured. The apparent molecular masses of both the protein band and the enzymatic activity were identical in the SDS-PAGE.

## 3. Results

Purification of the $NAD^+$-glycohydrolase from mitochondrial membranes of bovine liver was routinely started with 32–40 g mitochondria prepared from about 800 g of bovine liver. This amount of mitochondria yielded usually about 11 g of mitochondrial protein after extraction with Triton X-100. As initial experiments using Triton X-114 phase partition indicated that the $NAD^+$-glycohydrolase behaves like an integral membrane protein, we first isolated mitochondrial membranes and further removed membrane associated proteins by a washing step. Solubilization of the remaining membrane fraction resulted in a clear, yellow solution which contained about 3 g of protein. This solution was subjected to 5 chromatographic purification steps.

Table 1 summarizes the purification procedure resulting in a 1660-fold increase of specific $NAD^+$-glycohydrolase activity with an overall yield of about 8%. $NAD^+$-glycohydrolase from bovine liver mitochondrial membrane is a highly hydrophobic, low abundance transmembrane protein. Its high tendency to associate with other very hydrophobic proteins severely hampered its purification beyond a certain stage. Probably, $NAD^+$-glycohydrolase became buried within hydrophobic protein aggregates and a conventional purification strategy was inapplicable. We therefore applied a 'negative purification' procedure including a DEAE-52 column and a hydroxyapatite column. DEAE-52 cellulose proved to be the best chromatography material for the first 'negative purification' step. The $NAD^+$-glycohydrolase passed through the column and the enzymatic activity was in the void volume. The majority of the other proteins, however, was efficiently retained on the column. The sample obtained from this and the subsequent flow through step on hydroxyapatite was purified and concentrated on Heparin sepharose CL-6B column. These three chromatographic steps resulted in a 640-fold enrichment of the enzymatic activity. The eluate apparently consisted of specific protein aggregates, because further purification steps with this sample, for example on Reactive green-19 agarose or on a 5′-AMP column did not change the pattern of protein bands as analysed by SDS-PAGE. Therefore, a denaturation–renaturation step was introduced in order to destroy the protein aggregates. By this procedure the $NAD^+$-glycohydrolase activity could be separated from other, possibly no longer correctly folded proteins. Thus, the eluate of the Heparin-sepharose was denatured by addition of SDS to a final concentration of 0.5%, which resulted in the disappearance of all $NAD^+$-glycohydrolase activity. Renaturation was achieved by the addition of Triton X-100 to a final concentration of 6%. After this procedure which recovered 80–100% of the enzyme activity two further chromatographic purification steps, namely a DEAE-52 cellulose and a Reactive green-19 agarose column were performed. Fig. 1 shows a silver stained SDS-PAG of the fractions eluted from the last column. Only one major protein band in the molar mass range above 20,000, namely a protein with a molar mass of about 32,000 was detected in the active fractions. This band correlated in staining intensity with the $NAD^+$-glycohydrolase activity of the fractions. In order to unequivocally assign the $NAD^+$-glycohydrolase activity to one of the protein bands in the final preparation we developed a renaturation procedure directly in the SDS-PAG which is based on the denaturation/renaturation step used in the purification. A gel lane was cut into 1 mm slices which were then homogenized under renaturation conditions and subsequently subjected to a fluorimetric $NAD^+$glycohydrolase assay. Fig. 2 demonstrates that in the final enzyme preparation only the protein band with a molar mass of 32,000 contained measurable $NAD^+$-glycohydrolase activity. The products of either a purified enzyme preparation

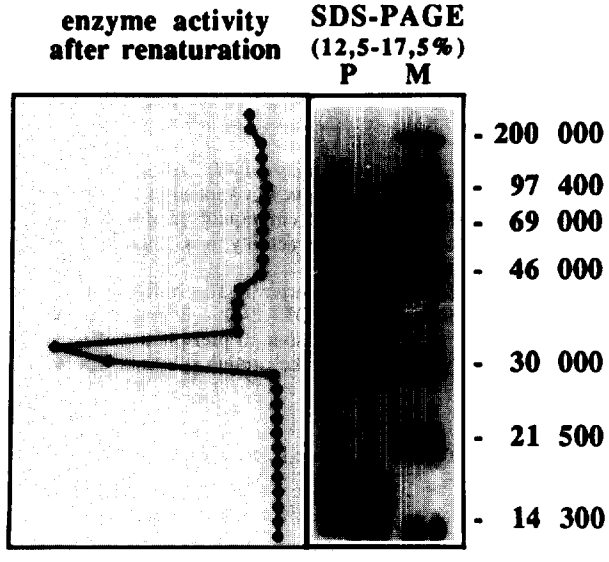


Fig. 2. Renaturation of the final enzyme preparation in a SDS-PAG. The final enzyme preparation was separated on a 12.5–17.5% PAG. After electrophoresis a lane containing the purified enzyme was cut into 1 mm slices, while the neighbouring reference lane containing an identical sample as well as molecular weight standards were stained with Coomassie blue. Lane P = final enzyme preparation; lane M = marker proteins. The enzyme activity after renaturation was determined with the fluorimetric assay. The 32,000 protein band in the SDS-PAG correlated exactly with the peak of enzyme activity after renaturation.

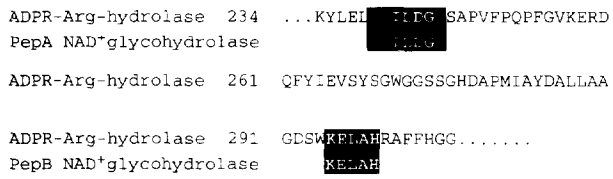


Fig. 3. Similarity between partial amino acid sequences of the $NAD^+$glycohydrolase and ADP-ribosyl arginine hydrolase from rat brain [21]. Numbers indicate the amino acid position in the ADP-ribosyl arginine hydrolase. Boxes highlight stretches of similar or identical amino acids. Amino acid similarity groups are as follows: HKR, EDQN, WFY, STGPA, C, ILVM. Inverse letters indicate similarity and inverse bold letters identity.

or of enzyme renatured from gel slices were analysed by thin-layer chromatography allowing the separation of $NAD^+$, ADPR, nicotinamide, ADP and AMP. In both cases the product of the enzymatic reaction was clearly identified as ADPR. The purified enzyme did not contain any contaminating activities of other $NAD^+$ metabolizing enzymes. Thus, the results of this product analysis demonstrate that the enzymatic activity residing in the purified protein band with a molar mass of 32 000 is an ADPR generating $NAD^+$-glycohydrolase.

Partial amino acid sequences of the protein band with the molar mass of 32,000 were determined after proteolytic digestion of the protein with LysC directly within the gel [19,20]. Sequencing of the generated fragments identified 2 partial protein sequences, namely (K)SILDGR and (K)ELAH. Searches in current databases for proteins containing such sequences revealed that the former sequence is not present in any of the known protein sequences, whereas the latter sequence is present in several other proteins, presumably because the probability of finding such a short sequence in the many known protein sequences is relatively high. For a more detailed database search we used the program FINDPATTERNS of the GCG program package (Genetics Computer Group, 575 Science Drive, Madison, WI 53711, USA) to screen for proteins containing sequences similar to both peptides with the restriction that such a double hit in one protein should occur within 300 amino acids, a length which corresponds to the putative length of the amino acid sequence of the purified protein with a molar mass of 32,000. Under these conditions the top scoring protein in all databases was a murine arginine ADPR hydrolase [21]. This protein has a molar mass of 39,000 and harbours 2 sequences which are highly similar to the sequences determined for the mitochondrial $NAD^+$-glycohydrolase and occur within a distance of 55 amino acids (Fig. 3). In this arginine ADPR hydrolase a sequence of RGILDGK corresponds to the sequenced peptide KSILDGR, namely 4 consecutive identical amino acids (ILDG) and 3 highly similar ones (two exchanges between R and K, both of which are highly basic amino acids; as well as one exchange of S-to-G, both of which are small and polar amino acids) are present. Furthermore, this enzyme harbours a sequence identical to the sequenced pentapeptide KELAH.

## 4. Discussion

Even after 1660-fold enrichment of the enzymatic activity, a few protein bands in the molar mass range below 20,000 as well as some faint additional bands were present in the final preparation besides the prominent protein band at a molar mass of 32,000. This prevented a correlation of the enzymatic activity with a specific protein band. Fortunately, the successful application of the denaturation/renaturation procedure during purification indicated a potential use of a renaturation protocol for the efficient detection of the $NAD^+$-glycohydrolase activity after separation of the proteins by SDS-PAGE. Thus, a procedure was designed which led to the unequivocal assignment of the enriched activity to the molar mass range of about 30,000 to 35,000. This was an unexpected result since it was published that the $NAD^+$-glycohydrolase from rat liver mitochondria has a molecular weight of 62,000 [8]. In the molecular weight range around 62,000 we detected no enzymatic activity suggesting either quite different properties for the rat and the bovine mitochondrial $NAD^+$-glycohydrolases or an inadequate former assignment of the enzymatic activity.

The two partial amino acid sequences of $NAD^+$-glycohydrolase which were obtained after proteolytic digestion of the protein band and subsequent gas phase sequencing showed high similarities to two sequence stretches of another mammalian ADPR metabolizing enzyme, namely an arginine ADPR hydrolase from rat brain [21]. In our view, this finding corroborates our assignment of the enzyme to the protein with a molar mass of 32,000. Moreover, the occurrence of this protein band clearly correlated with the enzymatic activity in the fractions eluted from the Reactive green-19 column. Taken together the results demonstrate that beef liver mitochondria possess an $NAD^+$-glycohydrolase with a molar mass of approximately 32,000.

*Acknowledgements:* We are obliged to Dr. Friedrich Lottspeich for performing protein gas phase sequencing of the purified protein and thank Dr. Jörg Windisch for carefully reading the manuscript. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (SCHW532/2-1).

## References

[1] Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
[2] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–205.
[3] Kim, H., Jacobson, E.L. and Jacobson, M.K. (1993) Science 261, 1330–1333.
[4] Lehninger, A.L., Vercesi, A. and Bababunmi, E.A. (1978) Proc. Natl. Acad. Sci. USA 75, 1690–1694.
[5] Lötscher, H.-R., Winterhalter, K.H., Carafoli, E., and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330.
[6] Richter, C., Winterhalter, K.H., Baumhuter, S., Lötscher H-R. and Moser, B. (1983) Proc. Natl. Acad. Sci. USA 80, 3188–3192.
[7] Hilz, H., Koch, R., Fanick, W., Klapproth, K. and Adamietz, P. (1984) Proc. Natl. Acad. Sci. USA 81, 3929–3933.
[8] Moser, B., Winterhalter, K.H. and Richter, C. (1983) Arch. Biochem. Biophys. 224, 358–364.
[9] Boyer, C.S., Moore, G.A. and Moldéus, P. (1993) J. Biol. Chem. 268, 4016–4020.
[10] Crompton, M., Ellinger, H. and Costi, A. (1980) Biochem. J. 255, 357–360.
[11] Schlegl, J., Schweizer, M. and Richter, C. (1992) Biochem. J. 285, 65–69.
[12] Bernardi, G. (1971) in: Methods in Enzymology (Jacobi, W.B. ed.) vol. 22, pp. 325–339, Academic Press, New York.
[13] Barrio, J.R., Secrist III, J.A. and Leonard, N.J. (1972) Proc. Natl. Acad. Sci. USA 69, 2039–2042.
[14] Secrist III, J.A., Barrio, J.R., Leonard, N.J. and Weber, G. (1972) Biochemistry 11, 3499–3506.

[15] Yost, D.A. and Anderson, B.M. (1981) Anal. Biochem. 116, 374–378.
[16] Findlay, J.B.C. (1990) in: Protein Purification Applications (Harris, E.L.V. and Angal, S. eds.) pp. 59–82, IRL Press, Oxford.
[17] Bustamante, E., Soper, J.W. and Pedersen, P.L. (1977) Anal. Biochem. 80, 401–408.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Eckerskorn, C. and Lottspeich, F. (1989) Chromatographia 28, 92–94.
[20] Lottspeich, F. (1985) Trends Anal. Chem. 4, 244–246.
[21] Moss, J., Stanley, S.J., Nightingale, M.S., Murtagh Jr., J.J., Monaco, L., Mishima, K., Chen, H.C., Williamson, K.C. and Tsai, S.C. (1992) J. Biol. Chem. 267, 10481–10488.