Biochem. J. (1995) **310**, 395–400 (Printed in Great Britain)



# NMN adenylyltransferase from bull testis: purification and properties

Enrico BALDUCCI,* Giuseppe ORSOMANDO,* Valeria POLZONETTI,* Alberto VITA,* Monica EMANUELLI,† Nadia RAFFAELLI,† Silverio RUGGIERI,† Giulio MAGNI† and Paolo NATALINI*‡

*Dipartimento di Biologia M. C. A., Università di Camerino 62032 Camerino (MC), Italy and †Istituto di Biochimica, Facoltà di Medicina e Chirurgia, Università di Ancona, 60100 Ancona, Italy

The purification procedure of NMN adenylyltransferase from bull testis presented here consists of a heat step and an acidic precipitation followed by four chromatographic steps, including dye ligand, adsorption and hydrophobic chromatography. The final enzyme preparation subjected to non-denaturating and denaturating PAGE with silver nitrate staining exhibited a single band. At this step the enzyme appeared to be homogeneous. The $M_r$ value of the native enzyme calculated by gel filtration was about 133000. The protein appeared to possess a quaternary structure with four subunits of apparent $M_r$ 33000 without disulphide interchain bonds. Isoelectric experiments gave a pI of 6.2, and pH studies showed the possible presence of an acidic group in the active site having a p$K_a$ of 4.9. Analysis of the amino acid composition showed the presence of more acidic residues than basic ones, according to the pI value calculated by Mono P FPLC. The $E_a$ calculated by Arrhenius plot gave an apparent value of 55.7 kJ/mol. The $K_m$ values for NMN, ATP, $NAD^+$ and $PP_i$ were 0.11, 0.023, 0.37 and 0.16 mM respectively. The polyclonal antiserum produced against the NMN adenylyltransferase reacted with the purified enzyme at different dilutions and recognized the enzyme in the homogenate as well.

## INTRODUCTION

During the last decade attempts have been made to elucidate the biological role of $NAD^+$, in addition to its well-known one of respiratory coenzyme in oxidation–reduction systems. At the end of the fifties, Morton [1] discovered a relationship between intracellular $NAD^+$ levels and cell proliferation. Much more recently several authors have focused their interest on ADP-ribosylation reactions. Several enzymes that utilize $NAD^+$ as substrate split the molecule into ADP-ribose and nicotinamide, which is eventually recycled back to the dinucleotide, after deamidation to nicotinic acid, through specific reaction sequences described by Gholson [2] and known as the pyridine nucleotide cycle. Of the enzymes that utilize $NAD^+$, the best-characterized are poly(ADP-ribose) polymerase (ADPRP) [3], ADP-ribosyltransferases from bacterial toxins [4] and $NAD^+$ glycohydrolase [5]. Fairly recently another enzyme has been purified, ADP-ribosyl cyclase [6], which after hydrolysing $NAD^+$ to ADP-ribose and nicotinamide catalyses the cyclization of ADP-ribose. A strong interdependence between the cyclic ADP-ribose concentration and mobilization of $Ca^{2+}$ in cells has been demonstrated [7]. This observation raises the question of the role of a new intracellular metabolite for the cyclic ADP-ribose [8].

Quantitative measurements of $NAD^+$ levels in cell lines have suggested a correlation, which is not yet fully understood, between $NAD^+$ metabolism, cell division and differentiation. Increasing interest has been focused on the enzymes described above, characterizing them from different points of view, cloning their genes, identifying new acceptor proteins and trying to clarify how they are involved in triggering $NAD^+$ catabolism in physiological and non-physiological conditions. However, less interest has been focused on NMN adenylyltransferase (NMNAT; EC 2.7.7.1), an enzyme involved in $NAD^+$ metabolism. First partially purified from yeast by Kornberg [9], NMNAT reversibly catalyses the formation of $NAD^+$ with the simultaneous production of $PP_i$ from ATP and NMN.

Both ADPRP and NMNAT are localized within the nucleus, but whereas ADPRP is associated with the internucleosomal DNA linker [10], the nuclear localization of NMNAT is quite unclear, although it appears to be strongly associated with the nuclear matrix [11]. Therefore $NAD^+$ turnover is greatly suppressed in enucleated cells [12], the nuclear events being responsible for both breakdown and resynthesis of the coenzyme $NAD^+$. Since NMNAT represents a key route in $NAD^+$ synthesis, its important role as modulator of intracellular $NAD^+$ levels should be recognized.

In addition to yeast, this enzyme has been studied in different cells and tissues such as *Escherichia coli* [13], rat liver [14,15], mouse liver [16], pig liver [17], ground squirrel retina [18], chicken erythrocytes [19], tumour cells [20] and human placenta [21]. Unfortunately, no studies on the homogeneous enzyme have been reported for a long time, mainly because of difficulties in the purification procedure. Our group was the first to purify NMNAT to homogeneity from yeast [22]. The present paper reports a purification procedure for NMNAT from bull testis and a kinetic characterization and description of the main molecular and immunological properties of the protein.

## EXPERIMENTAL

### Materials

All nucleotides and nucleosides were obtained from Sigma. Phenyl-Sepharose, TSK-phenyl-5PW, Superose 12 HR 10/30 and Mono P FPLC columns were from Pharmacia. Matrex gel Green A and PM30 membranes were from Amicon Corp., and hydroxyapatite and $M_r$ markers were from Bio-Rad. All other chemical reagents and materials were of the highest quality commercially available and were used without further purification.

### Enzyme purification

Testes were obtained from a bull immediately after its death and external membranes were removed as quickly as possible. The

Abbreviations used: NMNAT, NMN adenylyltransferase; ADPRP, poly (ADP-ribose) polymerase; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

‡ To whom correspondence should be addressed.

starting tissue, about 1600 g, was cut into small pieces and homogenized three times, 30 s each with 10 s intervals in 100 mM buffer A (potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EDTA and 1 mM PMSF) in a Waring Blender at high speed. The homogenate so obtained was kept for 10 min at 56 °C in a swinging bath. The temperature was then rapidly lowered to 4 °C and the suspension subsequently centrifuged at 13000 *g* for 20 min. To the supernatant, which was maintained at 4 °C, was added 1 M cold acetic acid dropwise to bring the pH to 5. After 20 min of stirring, the suspension was centrifuged at 25000 *g* for 20 min.

The pellet containing the enzyme activity was resuspended in a small volume of 100 mM buffer A containing 3 M KCl. It was then centrifuged at 25000 *g* for 20 min. This fraction, called pH 5, was loaded on to a phenyl-Sepharose column, previously equilibrated with 100 mM buffer A containing 3 M KCl, and washed with 100 mM buffer A containing 2 M KCl. The enzyme activity was eluted by a linear gradient, 2–0 M KCl in 100 mM buffer A. The active fractions were pooled and concentrated through an Amicon PM30 membrane.

Before the dye–ligand chromatography, the pool was diluted to decrease the ionic strength to a conductivity reading of about 13 mS/cm at 4 °C. The diluted phenyl-Sepharose pool fraction was applied to a Green A column, previously equilibrated with 100 mM buffer A. Washing was carried out using 100 mM as well as 10 mM buffer A until unbound proteins were removed from the column. Enzyme activity was eluted with a linear gradient of 0–1.2 M KCl in 10 mM buffer A. The pooled active fractions were directly loaded on to a hydroxyapatite column equilibrated with 5 mM buffer A. The column was washed with the 5 mM buffer A containing 2 M KCl, and then eluted with a linear gradient between 5 and 60 mM potassium phosphate buffer, pH 7.4, containing 2 M KCl. The active pool, concentrated through an Amicon PM30 membrane, was injected in 1 ml aliquots on to a TSK-phenyl-5PW FPLC column previously equilibrated with 50 mM sodium phosphate buffer, pH 6.8, containing 2 M NaCl. Elution was performed at room temperature with a discontinuous gradient 2–0 M NaCl in 50 mM sodium phosphate buffer, pH 6.8, at a flow rate of 1 ml/min.

The active fractions were analysed by SDS/PAGE, pooled, concentrated and stored at 4 °C. At this point the enzyme was considered to be homogeneous, and each analysis described below was carried out utilizing this final step.

## Assay of NMNAT activity

The standard assay was performed in a 1 ml 1 cm-pathlength quartz cuvette and was a continuous spectrophotometric coupled test directly developed from the Kornberg method [14]. In a final volume of 0.85 ml, the incubation mixture contained: 0.24 ml of 100 mM Hepes, pH 7.4, containing 40 mM $Mg^{2+}$, 0.1 ml of 12.5 mM ATP, 0.02 ml of 50 mM NMN, 0.05 ml of yeast alcohol dehydrogenase (156 units/ml), 0.39 ml of ethanol reagent composed of 0.1 M ethanol and 35 mM semicarbazide/HCl, pH 7.4, and the appropriate amount of NMNAT enzyme. The reaction, thermostatically controlled at 37 °C, was usually started by adding the NMN substrate. The increase in absorbance was recorded continuously at 340 nm in a Kontron model Uvikon spectrophotometer.

Alternatively, enzyme activity was measured by HPLC quantification of the reaction substrates and products. The assay conditions were as described above for the spectrophotometric assay without the ancillary system (ethanol reagent and yeast alcohol dehydrogenase). After incubation for 10 min at 37 °C, a 100 $\mu$l aliquot of the reaction mixture was withdrawn and added to 50 $\mu$l of ice-cold 1.2 M $HClO_4$ to stop the reaction. After 15 min on ice, the mixture was centrifuged for 1 min at 12000 *g*. A 130 $\mu$l aliquot of the $HClO_4$ supernatant was subsequently neutralized by addition of 35 $\mu$l of 0.79 M $K_2CO_3$, kept on ice for 15 min and centrifuged as described above. An aliquot of the neutralized $HClO_4$ supernatant was injected on to an HPLC system (Beckman System gold) utilizing an LC-18 reverse-phase column (250 mm × 4.6 mm internal diameter). The column was eluted with the following gradient, modified as described by Stocchi et al. [23]: 9 min at 100 % buffer 1, 6 min at 12.5 % buffer 2, 2.5 min at 45 % buffer 2, and 2 min at 100 % buffer 2; finally, after 5 min at 100 % buffer 2, the gradient was returned to 100 % buffer 1 for 5 min; buffer 1 is 0.1 M potassium phosphate buffer, pH 6, and buffer 2 is buffer 1 containing 20 % (v/v) methanol. The eluate absorbance was monitored at 254 nm with a model 166 variable-wavelength detector connected to integrating software. The amount of both the product formed and the residual substrates was determined from the peak areas of the HPLC-separated compounds, with reference to the appropriate standards. With this gradient all substrates, products and most of their derivatives and degradation products were separated and quantified.

Activity with $\beta$-NMNH as the substrate was determined by either a coupled spectrophotometric test developed by ourselves or HPLC. For the spectrophotometric test, the incubation mixture contained, in a final volume of 0.85 ml, 0.47 ml of 100 mM Hepes buffer, pH 7.4, containing 40 mM $MgCl_2$, 0.1 ml of 12.5 mM ATP, 0.05 ml of 12.5 mM $\beta$-NMNH, 0.1 ml of 23 mM pyruvate, 0.1 ml (0.5 unit) of lactate dehydrogenase and the appropriate amount of purified NMNAT. The reaction, which was started by adding lactate dehydrogenase, was followed at 37 °C by measuring the decrease in $A_{340}$ with time.

The incubation mixture for the HPLC assay contained, in a final volume of 0.4 ml, 0.220 ml of 100 mM Hepes buffer, pH 7.4, containing 40 mM $MgCl_2$, 0.050 ml of 12.5 mM ATP, 0.025 ml of 12.5 mM $\beta$-NMNH and the appropriate amount of NMNAT. After 10 min of incubation at 37 °C, the reaction was stopped by $HClO_4$ at different times and samples were processed as previously described. Specific modifications of the standard conditions are described in the appropriate Figure legends.

One enzyme unit is defined as the amount of enzyme that catalyses the synthesis of 1 $\mu$mol of $NAD^+$/min at 37 °C.

## PAGE

The homogeneity of the purified enzyme was determined by discontinuous slab gel electrophoresis conducted in 0.015 M sodium borate, pH 9. Approx. 0.06 unit was loaded and run at 2 mA per lane. After the run, the gel was stained with silver nitrate [24]. One lane was used for protein detection and the other was sliced into 2 mm sections to test for enzyme activity. Each slice was incubated overnight in 200 $\mu$l of 100 mM Hepes buffer, pH 7.4, containing 40 mM $MgCl_2$. The slices were then assayed for enzyme activity using the standard procedure described above.

Electrophoresis under denaturing conditions (SDS/PAGE) was performed to estimate subunit molecular mass. About 3 $\mu$g of the purified NMNAT was boiled at 100 °C in a denaturing mixture, applied to an 8 cm-long 12 % (w/v) polyacrylamide slab gel and run at 30 mA as described by Laemmli [25].

## FPLC

The FPLC system (LKB) consisted of a single model 2150 pump, a controller model 2152, a detector Uvicord SD model 2158 with a 279 nm filter, a two-channel recorder model 2210 and an

automatic fraction collector (Helirac model 2212). The samples were applied through an injection valve equipped with an external loop. All the samples were centrifuged or filtered on 0.45 $\mu$m nylon filter units (LIDA) before column injection.

### Chromatofocusing

Chromatofocusing experiments were carried out to determine the pI value. A Mono P HR 5/5 column (Pharmacia) connected to an FPLC system was used. The buffer system was 0.025 M methylpiperazine/HCl, pH 5.5, for the equilibration and Poly buffer 74 (Pharmacia), pH 4, as eluent (30 ml diluted 1:10 with distilled water). Fractions of volume 0.5 ml were collected at a flow rate of 0.5 ml/min and immediately tested for pH and enzyme activity.

### Determination of $M_r$

Using gel-filtration chromatography on an FPLC system with a Superose 12 HR 10/30 column (Pharmacia), the $M_r$ of the native purified enzyme was determined. After equilibration with 50 mM sodium phosphate buffer, pH 6.8, containing 0.5 M NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA and 1 mM dithiothreitol, 200 $\mu$l samples containing either purified enzyme or $M_r$ markers were applied. The elution was performed at a flow rate of 0.5 ml/min and fractions of volume 0.5 ml were collected.

### Amino acid analysis

After extensive dialysis against water to remove the buffer, duplicate samples of protein (2 $\mu$g) were hydrolysed for 30 and 45 min at 155 °C in 6 M HCl in sealed evacuated tubes as described by Hare [26]. The analysis was performed on a ChromaKon 500 (Kontron instruments) amino acid analyser and the amino acids were detected fluorimetrically after derivatization with *o*-phthaldehyde. Cysteine plus cystine was determined separately as cysteic acid after performic acid oxidation.

### Rabbit immunization

One New Zealand White rabbit received about 200 $\mu$g of purified bull testis NMNAT in 600 $\mu$l of 100 mM potassium phosphate buffer, pH 7.4, containing 1 % (w/v) SDS vigorously emulsified with the same volume of Freund's complete adjuvant. The total dose was given in six subdermal injections on sites on both sides of the abdomen in small volumes. After 4 weeks the animal was boosted with a similar dose of antigen again given as an emulsion. A third boost with 50 $\mu$g of protein was injected intramuscularly a week after the second one. Serum samples were collected at various times after immunization, and antibody presence was determined using Western blots.

### Purification of antibody

Antibodies were purified on a Protein A–Sepharose CL-4B column equilibrated in 100 mM $Na_2CO_3$ buffer, pH 8.3, containing 0.5 M NaCl. Before being loaded on to the column, the sera were extensively dialysed against the same buffer. The sample was eluted in isocratic conditions with 100 mM glycine/HCl buffer, pH 2.5. Each sample after elution was buffered as early as possible with the $Na_2CO_3$ buffer described above in a 1:1 (v/v) ratio with the sample.

### Immunoblotting assay

The proteins resolved by electrophoresis were then electrophoretically transferred to nitrocellulose using a Bio-Rad transfer blott apparatus in 0.025 M Tris/glycine buffer, pH 9, with 20 % (v/v) methanol and 1 % (w/v) SDS at 25 mA overnight. The immunoreactive protein was detected using the standard procedure described by Towbin et al. [27]. Briefly, after being blocked in 0.1 % (w/v) BSA dissolved in 10 mM Tris/HCl, pH 8, containing 0.15 M NaCl and 0.05 % (v/v) Tween 20 (TBST), the nitrocellulose sheets were sequentially incubated with anti-NMNAT IgG at different dilutions in TBST and with IgG anti-rabbit alkaline phosphatase conjugate diluted 1:6000 in TBST. Development was performed with 66 $\mu$l of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 70 % dimethylformamide) and 33 $\mu$l of Nitro Blue Tetrazolium (50 mg/ml in 70 % dimethylformamide), both dissolved in 0.1 M Tris/HCl, pH 9.5, containing 0.1 M NaCl and 5 mM of $MgCl_2$ with several washes in TBST between each incubation.

### Protein determination

Quantitative determinations were carried out by the method of Bradford [28] using the Bio-Rad reagent and BSA as standard. Buffers that were identical with those containing the protein samples were used as blanks.

## RESULTS

### Stability

After 3 months at −20 °C in the presence of 0.5 % (v/v) Triton X-100 in 50 mM sodium phosphate buffer, pH 6.8, containing 0.5 M NaCl, the pure enzyme retained 100 % activity. This preservation of activity is strictly dependent on the presence of high salt concentrations which occur under any storage conditions. In contrast, 80 % of the original activity was present after 3 months under the same conditions at 4 °C.

### Absorption spectrum

The absorption spectrum of the pure enzyme showed a maximum at 276 nm and a minimum at 254 nm. The $A_{280}/A_{260}$ ratio was 1.3 and the absorbance of a 1 % solution at 280 nm was 7.85.

### Enzyme purification

For a detailed description of the procedure, see the Experimental section. NMNAT was extremely labile during the initial purification steps. Therefore, after the homogenization, the activity was checked in the presence of several proteinase inhibitors using

**Table 1 Purification of NMNAT from bull testis**

Activity in the homogenate was measured by HPLC (see the Experimental section) to overcome interference in the spectrophotometric assay because of the high turbidity of the sample. In subsequent steps a spectrophotometric coupled assay was used.

| Step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Enrichment (n-fold) | Yield (%) |
|---|---|---|---|---|---|
| Homogenate | 54107 | 30.3 | 0.00056 | – | 100 |
| Heat | 13636 | 30.0 | 0.0022 | 4 | 99 |
| pH 5.0 | 3939 | 26.0 | 0.0066 | 12 | 86 |
| Phenyl-Sepharose | 169 | 14.0 | 0.083 | 148 | 46 |
| Green A | 7 | 12.3 | 1.59 | 2839 | 41 |
| Hydroxyapatite | 1.5 | 8.9 | 6 | 10714 | 29 |
| TSK Phenyl-5PW | 0.222 | 3.7 | 16.7 | 29821 | 12 |

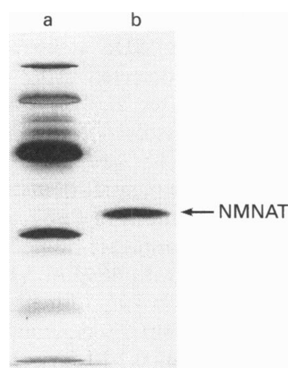


**Figure 1 SDS/PAGE of purified NMNAT**

The purified enzyme was subjected to SDS/PAGE (12% gels) in the presence of 2-mercaptoethanol as described in the Experimental section. Lane a contains the protein molecular-mass standards (given as $10^{-3} \times M_r$), which from top to bottom are phosphorylase *b* (97.4), BSA (66.2), ovalbumin (45), carbonic anhydrase (31), soya-bean trypsin inhibitor (21.5) and lysozyme (14.3). Lane b contains 3 μg of the purified NMNAT. After electrophoresis, the proteins were stained with silver nitrate [24]. The position of NMNAT is indicated.

**Table 2 Amino acid composition of NMNAT from bull testis**

The amino acid composition is given as mol/100 mol. Values are means of four determinations. Tryptophan and proline were not determined.

| Amino acid | Composition | Amino acid | Composition |
|---|---|---|---|
| Glx | 14.8 | Met | 1.5 |
| Asx | 9.4 | Ile | 5.9 |
| Cys | 1.3 | Leu | 8.9 |
| Thr | 4.4 | Tyr | 2.6 |
| Ser | 6.5 | Phe | 2.6 |
| Gly | 12.4 | Lys | 7.6 |
| Ala | 7.4 | His | 3.3 |
| Val | 5.2 | Arg | 6.1 |

the Boehringer-Mannheim proteinase inhibitor set. Of these, PMSF and E64 effectively preserved NMNAT activity. The whole procedure was therefore conducted in the presence of these serine proteinase inhibitors. The parameters of a typical enzyme purification starting from 1600 g of tissue are summarized in Table 1.

## $M_r$ and subunit structure

The native enzyme had an $M_r$ of $133000 \pm 10000$. This value, calculated by gel filtration as described in the Experimental section, was significantly similar to that for the enzyme from human placenta [21] but substantially different from those for enzymes obtained from other sources [17,19,29,30]. A single sharp band of $M_r$ $33000 \pm 1000$ was observed on SDS/PAGE in either the presence (Figure 1) or absence of 2-mercaptoethanol. Therefore the enzyme appears to be composed of four electrophoretically identical subunits not joined by interchain disulphide bridges. A single band was obtained after electrophoresis under non-denaturating conditions. The activity of NMNAT assayed in a parallel lane corresponded perfectly to the silver-stained band.

**Table 3 Inhibition of bull testis NMNAT activity by metal ions**

NMNAT activity was assayed by HPLC as described in the Experimental section in the presence of various heavy metal ions.

| Metal ion | $IC_{50}$(mM) |
|---|---|
| $Hg^{2+}$ | 0.017 |
| $Cu^{2+}$ | 0.025 |
| $Ag^{+}$ | 0.037 |
| $Cr^{2+}$ | 0.1 |
| $Cd^{2+}$ | 0.2 |

## Isoelectric point

Chromatofocusing experiments revealed an isoelectric point of 6.2. This value was similar to those calculated for the enzymes from chicken [19] and human placenta [21].

## Amino acid composition

The amino acid analysis of the native enzyme presented in Table 2 showed an excess of glutamic acid and aspartic acid over other amino acid residues. A comparison of the amino acid composition of NMNAT from bull testis with that obtained for yeast and human placental enzymes revealed a strong correlation with regard to a relative excess of acidic amino acid residues.

## Effect of metal ions on NMNAT activity

Several heavy metal ions were tested for their ability to affect NMNAT activity. Only $Hg^{2+}$, $Cu^{2+}$, $Ag^{+}$, $Cr^{2+}$ and $Cd^{2+}$ inhibited enzyme activity (Table 3).

## Kinetic analysis

NMNAT activity exhibits linear behaviour with respect to different substrates in both the forward and backward direction of the reaction. The kinetic parameters calculated from the intercept replots are shown in Table 4. Inhibition studies were

**Table 4 Kinetic parameters of NMNAT from bull testis**

The data were obtained from the intercept replots using fixed substrate at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mM and variable substrate at concentrations of 0.05, 0.2, 0.5, 1 and 2 mM. Enzyme assay was performed by spectrophotometry for the forward reaction and by HPLC for the backward reaction and with deamidated substrates. The assay mixture contained a fixed amount (1 mM) of free $Mg^{2+}$. NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid–adenine dinucleotide.

| Reaction | Reactant | $K_m$ (mM) |
|---|---|---|
| Dinucleotide synthesis | | |
| | ATP | 23 |
| | NMN | 110 |
| | NaMN | 150 |
| | β-NMNH | 625 |
| Dinucleotide pyrophosphorolysis | | |
| | NAD | 370 |
| | NaAD | 910 |
| | $PP_i$ | 167 |

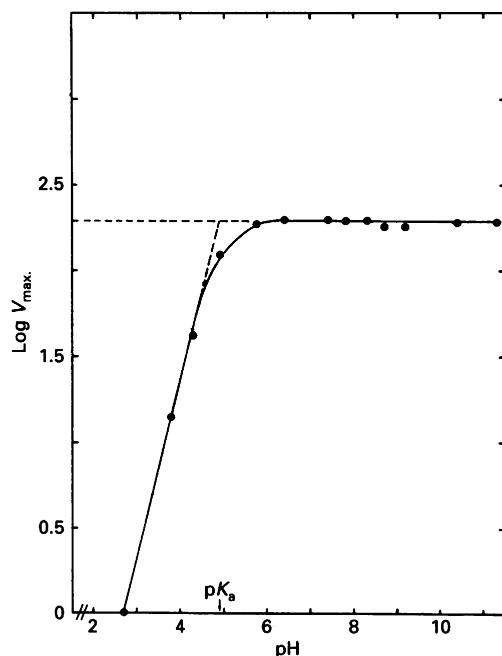


**Figure 2 pH-dependence of NMNAT activity**

Enzyme assay was carried out by HPLC in 0.028 M universal buffer, pH 2.7–11.3 (citric acid, $KHPO_4$, barbital and boric acid adjusted to the appropriate pH with 0.2 M NaOH).

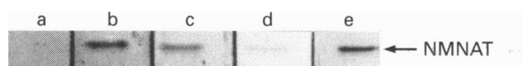


**Figure 3 Immunoblot analysis of NMNAT**

Samples (3 μg) of NMNAT were subjected to immunoblot analysis using IgG anti-NMNAT antibodies. Lane a is an immunoblot of purified NMNAT with preimmune IgG (94 μg); lanes b, c and d represent the immunoblot of purified NMNAT incubated with different dilutions of antibodies, respectively 1:50 (94 μg), 1:100 (47 μg) and 1:300 (15.6 μg); lane e is the immunoblot of the homogenate at a dilution of 1:50 (94 μg). A portion of the immunoblot is shown and the position of NMNAT is indicated.

performed with $PP_i$ at the following concentrations: 0.05, 0.1, 0.3, 0.6 and 1 mM. Double-reciprocal plots showed linear non-competitive inhibition by this product, and intercept replots with respect to both ATP and NMN gave the same $K_i$ value of 0.15 mM.

### pH and temperature studies

As shown in Figure 2, NMNAT remained active until pH 3, inactivation below this extreme point becoming irreversible. After preincubation of the enzyme at the indicated pH values, activity was assayed within the pH range 3–11.3. The plot of log $V_{max.}$ versus pH indicates a decrease in velocity in the acidic region below pH 6, and from the intercepts of the plot the p$K_a$ value appears to be 4.9. The $E_a$ value determined from the Arrhenius plot is 54.7 kJ/mol.

### Antibody specificity

After purification, rabbit antibody raised against bull testis NMNAT was characterized by immunoblotting. The specificity of the anti-NMNAT is readily apparent (Figure 3). Only the NMNAT was stained on the immunoblot containing either the purified enzyme or the same amount of the homogenate. Different dilutions of IgG gave the same result.

## DISCUSSION

We undertook the purification of NMNAT to facilitate studies on the regulation of its catalytic activity and molecular properties. The seven-step purification scheme reported here allows us to obtain a highly purified and stable enzyme; purification was about 30000-fold relative to the specific activity in the homogenate, with a final specific activity of 16.7 units/mg. This is the most extensively purified preparation of NMNAT from any source.

The temperature studies gave an optimal temperature of about 52 °C with a plateau up to 56 °C, a value in agreement with that found by others for the enzyme from chicken [19]. The complete absence of inactivation of NMNAT after 10 min at 56 °C prompted us to introduce this step into the purification procedure. After the incubation at 56 °C, we noticed a vast improvement in the yield of catalytic protein after the last purification step and greater stability of enzyme activity. In addition, low-$M_r$ bands were demonstrated on SDS/PAGE of NMNAT purified in the absence of the heat step after 3 months of storage at 4 °C.

The purified human placental [21] and bull testis enzymes have similar subunit molecular masses which differ from that found for the yeast enzyme [22], but in every case the enzyme seems to possess a quaternary structure composed of four apparently identical subunits without interchain disulphide bridges. These similarities do not extend to the pI value. Yeast [22], chicken [17] and human NMNAT [21] exhibit multiple pIs. This behaviour could be the result of aggregation of the enzyme molecule. In contrast, the mammalian enzyme exhibits only one pI of about 6.2.

A p$K_a$ value of 5.76 was previously reported for pig liver NMNAT [31]; this result differs considerably from our findings. The value of 4.9 (Figure 2) calculated for the bull testis NMNAT suggests the presence of an acidic group in the catalytic site of the enzyme. The kinetic properties of NMNAT are consistent with its physiological role in $NAD^+$ metabolism. NMNAT can use either NMN or nicotinic acid mononucleotide, but a substantially lower $K_m$ for NMN indicates that the amidate pathway is predominant in bull testis. The values extrapolated from the double-reciprocal plots are consistent with a sequential mechanism.

To investigate the NMNAT mechanism further, a number of substrate analogues were examined for their ability to affect enzyme activity at various concentrations (0.1–1.0 mM). These analogues included pyrimidine and purine bases, oxy- and deoxy-nucleosides, oxy- and deoxy-nucleotides, cyclic AMP, several halogenated and methylated nucleobases, oxy- and deoxy-ribose, oxy- and deoxy-ribose 5-phosphate, fructose and fructose-1,6-bisphosphate. Only β-NMNH effectively depressed enzyme activity with a $K_i$ of 0.28 mM; α-NMN was completely ineffective, showing that nucleotide transfer is strongly stereospecific [32].

As for many anti-enzyme systems and for hen NMNAT [33], the polyclonal antibodies raised towards bull testis NMNAT were not directed against the active site, as the enzyme is fully active after immunoprecipitation (E. Balducci, G. Orsomando, V. Polzonetti, A. Vita, M. Emanuelli, N. Raffaelli, S. Ruggieri, G. Magni and P. Natalini, unpublished work). Moreover the antibody produced clearly recognizes the purified enzyme and NMNAT from the homogenate at different dilutions (Figure 3). The cross-reaction, which is also evident with commercial hog liver NMNAT, is completely lost when NMNAT purified from

yeast or human placenta is used (results not shown). These findings suggest that the enzyme has evolved with species variation in parallel with other immunologically similar proteins in tissues of one species.

In summary, this report is a detailed study of the enzymological properties of NMNAT. Clearly, more studies are required to understand fully the mechanism and regulation of NMNAT in bull testis, but the availability of a highly purified enzyme and its antibodies will enable the acquisition of a greater knowledge of the regulation of this important enzyme.

We thank Mrs. Giovanna Cimini for excellent technical assistance, Dr. Adolfo Amici for the amino acid analysis and CIGA (Centro Interdipartimentale Grandi Apparecchiature) of the University of Camerino. This work was supported by a grant from MURST (40%).

## REFERENCES

1 Morton, R. N. (1958) Nature (London) **181**, 540–543
2 Gholson, R. K. (1966) Nature (London) **212**, 933–935
3 Ushiro, H., Yokoyama, Y. and Shizuta, Y. (1987) J. Biol. Chem. **262**, 2352–2357
4 Price, S. R., Barber, A. and Moss, J. (1990) in ADP-Ribosylating Toxins and G Proteins: Insights into Signal Transduction (Moss, J. and Vaughan, M., eds.), pp. 397–424, American Society of Microbiology, Washington D.C.
5 Muller-Steffner, H., Schenherr-Gusse, I., Tarnus, C. and Schuber, F. (1993) Arch. Biochem. Biophys. **304**, 154–162
6 Lee, H. C. and Aarhus, R. (1991) Cell. Regul. **2**, 203–209
7 Clapper, D. L., Walseth, T. F., Dargie, P. J. and Lee, H. C. (1987) J. Biol. Chem. **262**, 9561–9568
8 Galione, A. (1993) Science **259**, 325–326
9 Kornberg, A. (1948) J. Biol. Chem. **176**, 1475–1476
10 Uhr, M. L. and Smulson, M. (1982) Eur. J. Biochem. **128**, 435–443
11 Balducci, E., Emanuelli, M., Magni, G. et al. (1992) Biochem. Biophys. Res. Commun. **189**, 1275–1279
12 Rechsteiner, M. and Catanzaritte, V. (1974) J. Cell. Physiol. **84**, 409–422
13 Dahmen, W., Webb, B. and Preiss, J. (1967) Arch. Biochem. Biophys. **120**, 440–450
14 Kornberg, A. (1950) J. Biol. Chem. **182**, 779–793
15 Grunicke, H., Keller, H. J., Liersh, M. and Benaguid, A. (1973) Adv. Enzyme Regul. **12**, 397–418
16 Hogeboom, G. and Schneider, W. (1952) J. Biol. Chem. **197**, 611–620
17 Ferro, A. M. and Kuehl L. (1975) Biochim. Biophys. Acta **410**, 285–298
18 Berger, S. J. and De Vries, G. W. (1982) J. Neurochem. **38**, 821–826
19 Cantarow, W. and Stollar, B. D. (1977) Arch. Biochem. Biophys. **180**, 26–33
20 Hudack, E. D., Eros, D. E. and Brummond, D. O. (1963) Exp. Cell. Res. **29**, 343–348
21 Emanuelli, M., Natalini, P., Raffaelli, N., Ruggieri, S., Vita, A. and Magni, G. (1992) Arch. Biochem. Biophys. **298**, 29–34
22 Natalini, P., Ruggieri, S., Raffaelli, N. and Magni, G. (1986) Biochemistry **25**, 3725–3729
23 Stocchi, V., Cucchiarini, L., Magnani, M., Chiarantini, L., Palma, P. and Crescentini G. (1985) Anal. Biochem. **146**, 118–124
24 Oakley, B. R., Kirsch, D. R. and Morris, N. R. (1980) Anal. Biochem. **105**, 361–363
25 Laemmli, U. K. (1970) Nature (London) **277**, 680–685
26 Hare, P. E. (1977) Methods Enzymol. **47**, 3–18
27 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
28 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
29 Adamietz, P., Klapproth, K. and Hilz, H. (1979) Biochem. Biophys. Res. Commun. **91**, 1232–1238
30 Kono, M., Shimizu, C., Matsui, T. and Matsuura, F. (1978) Nippon Suisan Gakkaishi **44**, 379–384
31 Atkinson, M. R., Jackson, J. F. and Morton, R. K. (1961) Nature (London) **192**, 946–948
32 Lowe, G. and Tansley, G. (1983) Eur. J. Biochem. **132**, 117–120
33 Cantarow, W. and Stollar, B. D.(1977) Arch. Biochem. Biophys. **180**, 34–40

Received 13 January 1995/12 April 1995; accepted 3 May 1995