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Pyridine dinucleotide biosynthesis in archaebacteria: presence of NMN adenylyltransferase in *Sulfolobus solfataricus*

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Abstract The enzyme NMN adenylyltransferase, leading to NAD synthesis, has been observed for the first time in soluble extracts from the extreme acidothermophilic archaeon *Sulfolobus solfataricus*. Comparison of its molecular and kinetic properties with those of the enzyme isolated from prokaryotes and eukaryotes revealed significant differences, knowledge of which may contribute to the understanding of metabolic evolutionary mechanisms. The thermophilic enzyme shows a molecular mass of about 66,000 and an isoelectric point of 5.4. The K_m values for ATP, NMN and nicotinic acid mononucleotide are 0.08 μM, 1.4 μM and 17 μM, respectively. The enzyme shows a remarkable degree of thermophilicity, with an activation energy of 95 kJ/mol.

Key words: Archea; Thermophile; NAD; NMN adenylyltransferase

1. Introduction

Extensive studies on NAD metabolism have been conducted both in eukaryotic and prokaryotic systems (for a review cf. [1]). Most of the organisms that have been considered are capable of synthesizing NAD de novo, through different pathways leading to the formation of quinolinic acid as a common intermediate. In addition, several salvage pathways, known as the pyridine nucleotide cycles (PNC), have been described to allow recycling of NAD degradation products [2]. Both in the de novo and salvage pathways the final step consists in the transfer of the adenylyl moiety of ATP to the phosphoryl group of NMN or NaMN to form NAD or NaAD, respectively. On the other hand, a well known pathway for NAD catabolism is via mono- and poly-(ADP-ribosyl)ation reactions, the former occurring mainly in the cytoplasm and having signal transduction functions, the latter being involved in nuclear processes, like DNA repair, cell differentiation and gene expression [3]. Recently a wide group of NAD-catabolizing enzymes, namely NADases, that hydrolyze NAD to ADP-ribose and nicotinamide, has been identified in many organisms. The occurrence of a NADase able to convert NAD to cyclic-ADPR, which has been shown to be a potent Ca^{2+}-releasing factor, has been reported, suggesting a direct role of NAD catabolism in Ca^{2+} homeostasis [4,5]. Such a central role of NAD in cellular metabolism, besides its role as a coenzyme in cellular redox reactions, suggests that its biosynthetic pathway must be a highly regulated one. In this regard the enzyme NMN adenylyltransferase (EC 2.7.7.1), which catalyzes the last step of NAD biosynthesis, could play an important role in the regulation of intracellular NAD levels with respect to the cellular needs. This enzyme activity has been detected both in procaryotes and in eucaryotes; in our laboratory the enzyme has been purified to homogeneity and extensively characterized in its molecular and kinetic properties from yeast and human placenta [6,7].

Very little is known concerning the pyridine nucleotide metabolism in archaebacteria, the third oldest line of living organisms, endowed both with a mixture of eubacterial and eucaryotic features and unique archaebacterial properties. Recently the presence of an ADP-ribosylating activity leading to NAD degradation has been demonstrated in *Sulfolobus solfataricus*, a thermoacidophilic archaebacterium living in acidic solfatara fields [8]. This finding, pointing to the existence of a PNC in archaebacteria, prompted us to investigate on the presence of the enzyme NMN adenylyltransferase in this organism. The present report deals with data showing for the first time the presence of a NMN adenylyltransferase activity in *Sulfolobus solfataricus*, together with its major molecular and catalytic properties.

2. Materials and methods

Sulfolobus solfataricus, strain DSM 1617, grown on glucose and harvested during the stationary phase, was kindly provided by Prof. M. Rossi (Istituto di Biochimica delle Proteine ed Enzimologia, CNR, Napoli), as lyophilized cells.

2.1. NMN adenylyltransferase activity assay

The activity was routinely measured with a two-steps assay. In the first step the incubation mixture, containing 100 mM HEPES buffer, pH 7.4, 20 mM $MgCl_2$, 1 mM ATP and 1 mM NMN, was preheated at 70°C for 3 min before adding the appropriate amount of enzyme fraction to a final volume of 150 μl. After 10 min at 70°C the incubation mixture was brought to room temperature to stop the reaction and clarified by centrifugation. A 100-μl aliquot of the supernatant was used for the spectrophotometric quantitation of the NAD formed, by using ethanol and yeast alcohol dehydrogenase [9]. For kinetic analysis bovine serum albumin was included in the incubation mixture, at a final concentration of 0.1 mg/ml, to prevent dilution-inactivation of the enzyme. After 5 min incubation at 70°C the reaction was stopped with 0.4 M ice-cold $HClO_4$, centrifuged and the supernatant neutralized with 0.2 M K_2CO_3. The NAD or NaAD formed were quantitated by HPLC separation [10]. One enzyme Unit was defined as the amount of NMN adenylyltransferase catalyzing the formation of 1 μmol NAD in 1 min at 70°C. The thermal hydrolysis of the substrates and the product of the reaction was negligible at 70°C, as demonstrated by running appropriate blanks (either in the presence or in the absence of cell extracts).

2.2. Purification of NMN adenylyltransferase

All steps were carried out at 4°C. The buffers used during the purification procedure were: buffer (A) potassium phosphate buffer,

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Abbreviations: NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; DTT, dithiothreitol.

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pH 8.0, 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT; buffer (B) 50 mM Tris-HCl buffer, pH 7.4, 5 mM 2-mercaptoethanol.

Step I: Crude extract. Lyophilized *Sulfolobus solfataricus* cells (1 g) were resuspended in 20 ml of 100 mM buffer A. After standing for 30 min on ice with occasional shaking, the suspension was sonicated 3 times for 1 min using the maximum power setting of a Sonifier Cell Disruptor Model 185, then centrifuged at 15,000 × *g* for 20 min. The supernatant (19 ml) represented the crude extract.

Step II: DEAE-cellulose chromatography. The crude extract was dialyzed overnight against 3 l of 5 mM buffer A, loaded onto a column (2.5 × 7 cm) of DEAE-cellulose (DE-52), previously equilibrated with the same buffer and eluted at a flow rate of 4 ml/min. The flow-through containing the enzyme activity was collected and saved for the next step.

Step III: Red A chromatography. The DEAE-cellulose fraction (55 ml) was dialyzed overnight against 5 l of buffer B and then applied to a Matrex Gel Red A (Amicon) column (1.5 × 6 cm) equilibrated with the same buffer, at a flow rate of 1.5 ml/min. After washing with 1 M NaCl in buffer B at the same flow rate, the elution was performed with 100 ml of a linear gradient from 1 to 3 M NaCl in buffer B, at a flow rate of 1 ml/min. 1.5-ml fractions were collected and assayed for the enzyme activity. Active fractions were pooled (24 ml) and concentrated to 2.5 ml by ultrafiltration using an Amicon YM30 membrane. The concentrated final enzyme preparation could be stored at −20°C for several weeks without substantial loss of activity. This preparation was used for the molecular and kinetic characterization of the enzyme.

2.3. Polyacrylamide gel electrophoresis

The purity of the final enzyme preparation was judged by SDS-PAGE performed according to Schagger and von Jagow [11]. In the separating gel, a 10% total gel concentration and a 3% concentration of cross-linker were used. After electrophoresis, gels were silver stained according to Oakley et al. [12]. Gel densitometric analysis was performed at 560 nm with a DU40 spectrophotometer (Beckmann), equipped with a gel scanner.

2.4. Protein assay

Protein content was determined by the method of Bradford [13], using bovine serum albumin as the standard.

2.5. Molecular weight determination

The molecular weight of NMN adenylyltransferase was determined by gel filtration in FPLC on Superose 12 HR 10/30, equilibrated with 50 mM potassium phosphate buffer, pH 6.8, containing 0.5 M KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.5 mM EDTA. Carbonic anhydrase, bovine serum albumin and ovalbumin were used as standard proteins. Equilibration, loading and elution of the column were performed at a flow rate of 0.5 ml/min at room temperature.

2.6. Isoelectric point determination

Isoelectric point of NMN adenylyltransferase was determined by FPLC chromatofocusing on a Mono P HR 5/5 column equilibrated with 25 mM imidazole-HCl, pH 7.4. Elution was performed at room temperature, with 10-fold diluted Polybuffer 74, pH 4.0, at a flow rate of 1 ml/min. For the determination of the basic pI, the column was equilibrated with 75 mM Tris-acetic acid, pH 9.3 and the elution was performed with 45-fold diluted ampholyne in the pH range 7–9.5, adjusted to pH 7.0 with 1 N acetic acid.

2.7. Temperature optimum

Enzymatic activity was assayed at different temperatures, from 37°C to 90°C. Incubation mixtures were preheated at the assay temperatures before adding the enzyme and the temperatures kept constant with a thermostatic block. NMN adenylyltransferase activity was determined by HPLC. Non enzymatic hydrolysis of substrates and product was measured by performing control reactions in the absence of the enzyme.

3. Results and discussion

A 964-fold enriched NMN adenylyltransferase preparation was obtained from extracts of *Sulfolobus solfataricus* cells. Table 1 shows the enzyme purification procedure yielding a final preparation with a specific activity of 1.35 units/ml and an overall recovery of enzymatic activity greater than 100%. Such an increase of the total enzyme units throughout the purification process could arise from the removal of either an inhibitory factor or interfering activities present in the crude extract. In fact, measurement of the enzyme activity by the HPLC assay revealed that the very low value of specific activity in the crude extract was due to the action of endogeneous interfering activities, particularly phosphatases and hydrolases, consuming most of ATP and NMN substrates in the incubation mixtures. During the anion-exchange chromatography step most of the phosphatases were removed, whereas NMN was still degraded and converted to nicotinic acid. After the Red A chromatography step no endogeneous degradation of NMN and ATP could be observed, rendering the enzyme preparation suitable for further characterization. Protein silver staining, after SDS-PAGE, of the fractions corresponding to each purification step, showed a significant enrichment throughout the purification procedure of a band migrating in the 20,000 Da region. Densitometric analysis of the electrophoretic pattern of the final enzyme preparation indicated that such 'major' band represented 55% of the total protein stain.

The molecular weight of the native enzyme, estimated by gel filtration as described in section 2, was 66,000 ± 5,000. This value is noticeably lower than those found for the homogeneous enzymes from yeast and human placenta [6,7]. Chromatofocusing experiments revealed a single pI at pH 5.4, while a negligible fraction of the enzyme activity was eluted at pH 7.8. This finding is in contrast with the multiple pI values in the acidic range for both yeast and human enzymes; a pI of 5.5 has been reported for the crude enzyme from chicken erythrocytes, while multiple pI values were found for the partially purified enzyme from the same source [14]. The appearance of additional peaks in purified preparations has been ascribed to unspecific aggregations typical of nuclear non-histone proteins [15].

The determination of the K_m values for the substrates was carried out by measuring the initial rates of NAD synthesis as a function of the concentrations of both NMN and ATP. From the reciprocal plots, depicted in Fig. 1, the K_m values for NMN and ATP were calculated to be 1.4 μM and 0.08 μM, respectively. The V_{max} was 0.07 μmol NAD/minute/mg protein. Due to the high affinities of the thermophilic enzyme for its substrates, the K_m determination resulted particularly cumbersome. In fact, very low concentrations of substrate(s) – in the region of estimated K_m – had to be used and consequently, in order to maintain the fraction of consumed substrate within the limits of initial velocity conditions, only tiny amounts of NAD could be allowed to form and then had to be quantitated [16]. The specificity for the amide portion of the mononucleotide has been tested by comparing the utilization of nicotinate mono-

Table 1
Purification of NMN adenylyltransferase from *Sulfolobus solfataricus*

Step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification (fold)
Crude extract	342	0.48	0.0014	100	–
DE-52	30	1.34	0.045	279	32
Red A	1.2	1.62	1.35	337	964

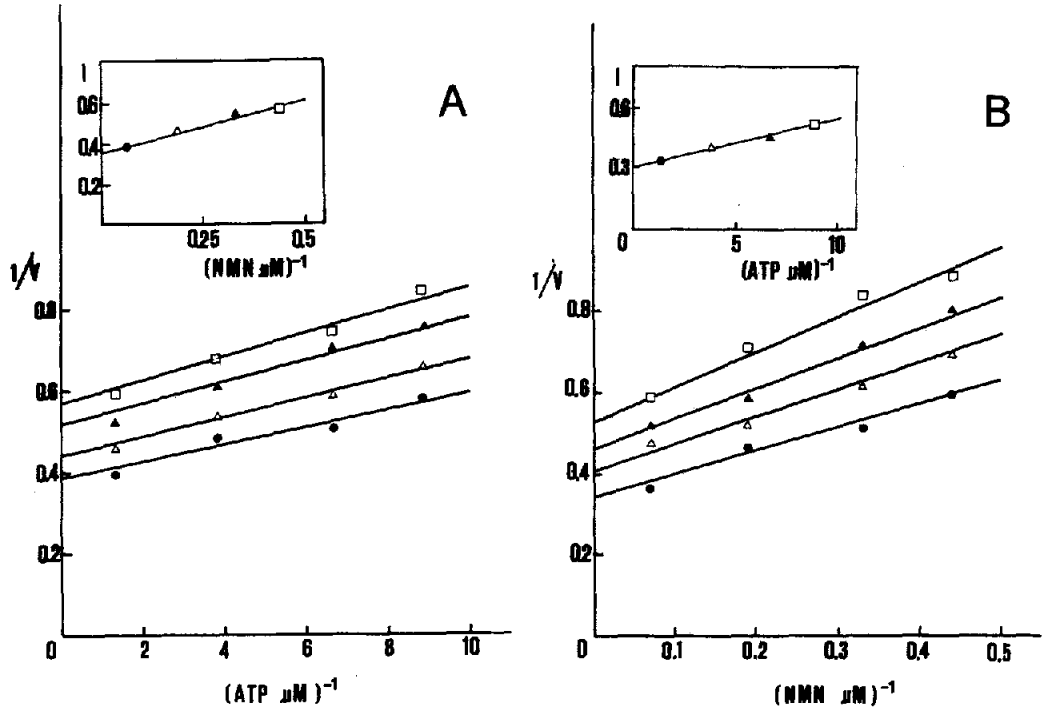

Fig. 1. Effect of NMN and ATP concentrations on NMN adenylyltransferase initial velocities. Assay mixtures contained: (A) concentrations of ATP varying from 0.11 μM to 0.75 μM at NMN concentrations of (●) 5.25, (△) 3.0, (▲) 2.25 and (□) 1.5 μM. (B) concentrations of NMN varying from 1.5 μM and 5.25 μM at ATP concentrations of (●) 0.75, (△) 0.26, (▲) 0.15 and (□) 0.11 μM. Insets are replots of intercepts.

nucleotide (NaMN) and NMN, as the substrates. The purified enzyme preparation could indeed catalyze NaAD synthesis from NaMN with a V_{max} of 0.39 μmol NaAD/min/mg protein and a K_m value for NaMN of 17 μM. Therefore the K_m for NaMN was higher than that for NMN, indicating a higher affinity of the enzyme for the amidated substrate, while the V_{max} was higher with NaMN. On the other hand the resulting V_{max}/K_m ratio for NMN is twice that for NaMN, indicating a relatively higher specificity of the archaeon enzyme for the amidated substrate. This finding could suggest that NAD biosynthesis in archaebacteria preferentially goes through the amidated pathway, while in procaryotes it seems to involve the deamidated form of NMN [1,17]. Comparison of kinetic parameters of NMN adenylyltransferase from *Sulfolobus solfataricus* with those of the enzyme from eucaryotic sources, shows that the thermophilic NMN adenylyltransferase exhibits higher affinities for the substrates NMN, ATP and NaMN, displaying K_m values of about one order of magnitude lower than those reported for the mesophilic enzymes. These values are considerably low even when compared with kinetic parameters found for other thermophilic enzymes [18,19], suggesting interesting kinetic properties of NMN adenylyltransferase from *Sulfolobus solfataricus*.

Fig. 2 shows the influence of the temperature on the NMN adenylyltransferase activity. Within the range 37°C–90°C the enzyme exhibits a continuous increase of its activity, displaying at 50°C only about 3% of the activity measured at 90°C. The Arrhenius activation energy is 95 kJ/mol, indicating a remarkable thermophilicity even when compared with that of other enzymes from thermophilic sources [20,21].

The thermophilicity and the thermostability features of the enzymatic protein appear to deserve further evaluation with regard to the possible biotechnological exploitation of the NMNAT enzyme, involved in the synthesis of a compound of such a widespread importance as the NAD coenzyme. On the other hand the study of the individual enzymes, involved in the intracellular degradation and resynthesis of NAD in extremophilic archaebacteria, may shed light on the significance of the role of ADP-ribosylation and of NAD homeostatic pathways both in the cellular response to extreme environmental conditions and in the evolutional process.

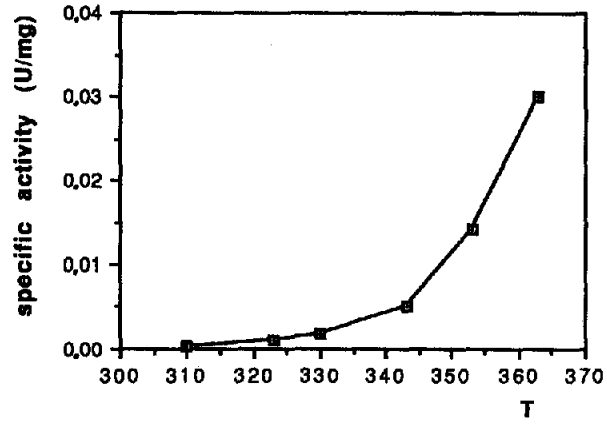

Fig. 2. Dependence of NMN adenylyltransferase activity on temperature. The enzyme was assayed under standard conditions at the indicated temperatures.

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