

Synechocystis sp. slr0787 protein is a novel bifunctional enzyme endowed with both nicotinamide mononucleotide adenylyltransferase and ‘Nudix’ hydrolase activities

Nadia Raffaelli^a, Teresa Lorenzi^a, Adolfo Amici^a, Monica Emanuelli^a, Silverio Ruggieri^b, Giulio Magni^{a,*}

^aIstituto di Biochimica, Facoltà di Medicina e Chirurgia, University of Ancona, via Ranieri, 60100 Ancona, Italy

^bDipartimento di Biotecnologie Agrarie ed Ambientali, University of Ancona, via Ranieri, 60100 Ancona, Italy

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Abstract *Synechocystis* sp. slr0787 open reading frame encodes a 339 residue polypeptide with a predicted molecular mass of 38.5 kDa. Its deduced amino acid sequence shows extensive homology with known separate sequences of proteins from the thermophilic archaeon *Methanococcus jannaschii*. The N-terminal domain is highly homologous to the archaeal NMN adenylyltransferase, which catalyzes NAD synthesis from NMN and ATP. The C-terminal domain shares homology with the archaeal ADP-ribose pyrophosphatase, a member of the ‘Nudix’ hydrolase family. The slr0787 gene has been cloned into a T7-based vector for expression in *Escherichia coli* cells. The recombinant protein has been purified to homogeneity and demonstrated to possess both NMN adenylyltransferase and ADP-ribose pyrophosphatase activities. Both activities have been characterized and compared to their archaeal counterparts.

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Key words: Adenylyltransferase; Nudix hydrolase; NAD biosynthesis; Cyanobacterium; Expression

1. Introduction

In the NAD biosynthetic pathway, the enzyme NMN adenylyltransferase catalyzes the reaction $\text{NMN} + \text{ATP} \rightleftharpoons \text{NAD} + \text{PPi}$. The archaeal NMN adenylyltransferase has recently been characterized and its gene has been identified and cloned from the thermophilic archaeon *Methanococcus jannaschii* [1]. To date, the archaeal gene is the only known gene for this enzyme. When the archaeal protein sequence was analyzed using the gapped BLAST service [2], the only significant similarity was found with the cyanobacterial *Synechocystis* sp. slr0787 orf-encoded hypothetical protein [3]. The protein is composed of 339 amino acid residues, and its N-terminal half was found to align with a high score to the entire *M. jannaschii* NMN adenylyltransferase sequence of 168 residues. On the other hand, the C-terminal half was highly similar to *M. jannaschii* ADP-ribose pyrophosphatase, recently identified by Sheikh et al. [4]. The highly conserved signature sequence $\text{GX}_5\text{EX}_7\text{RE(I/L/V)XEEEX}_2\text{(I/L/V)}$, which designates the ‘Nudix’ hydrolase family [5], is also present in the C-terminal domain of the slr0787 protein. ‘Nudix’ hydrolases are able to cleave substrates containing an X-linked nucleoside diphosphate (X = phosphate, sugar, nucleoside mono/diphosphate, etc.). Their metabolic role has been related to the control of the cellular concentration of toxic compounds

and physiological metabolites whose accumulation could be harmful for the cell [5]. In this paper we describe the cloning and expression of the slr0787 gene, and the purification and characterization of the slr0787 protein. It is demonstrated that this protein is a bifunctional enzyme, endowed with NMN adenylyltransferase and ‘Nudix’ hydrolase activities. The major catalytic properties of the two activities are presented.

2. Materials and methods

2.1. Materials

Cosmid clone cs1377, containing the slr0787 gene, was kindly provided by Dr. Kaneko, Kazusa DNA Research Institute (Japan). Primers were obtained from PRIMM (Milan, Italy). Restriction enzymes were from Promega Biotec (Madison, WI, USA), and T4 DNA ligase, PCR reagents, and enzymes were from Boehringer Mannheim GmbH (Mannheim, Germany). The basic molecular biology procedures for bacterial growth, plasmid preparation and transformation of competent cells described in [6] were followed. Ampicillin and nucleotides were purchased from Sigma. Bradford reagent for protein assay and molecular weight standards were from Bio-Rad.

2.2. Cloning of the *Synechocystis* slr0787 gene

The synthetic oligonucleotides d(CGCGGGATCCATGCAAAC-TAAATATCAATAC) and d(CGGCTGCAGCTAAACTTTGCT-GACAAAATG) were used as primers in PCR in order to both amplify the slr0787 gene and incorporate a *Bam*HI site at the start of the gene, and a *Pst*I site at its end. PCR was performed using 0.1 µg of cosmid clone cs1377 as the template, with 10 pmol of each primer in a final volume of 50 µl. Each cycle was set for 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min elongation at 72°C, and 30 reaction cycles were carried out. The amplified gene was purified, digested with *Bam*HI and *Pst*I, and ligated into the *Bam*HI and *Pst*I restriction sites of plasmid pT7-7 to obtain the construct pT7-7-slr0787. The nucleotide sequence of the insert was confirmed by direct sequencing. The construct was used to transform *Escherichia coli* TOP10 for plasmid preparation and *E. coli* BL21 (DE3) for protein expression.

2.3. Purification of the slr0787 protein

2.3.1. Growth and expression. Single colonies of *E. coli* strain BL21 (DE3) containing pT7-7-slr0787 were inoculated into 1 l of Luria-Bertani medium containing 0.1 mg/ml ampicillin and grown at 37°C to saturation (OD_{600} 1.8). The culture was then induced with 1 mM IPTG and grown for an additional 5 h.

2.3.2. Crude extract. All steps were performed at 4°C. Induced cells were harvested by centrifugation at $5000 \times g$ for 10 min and resuspended in two volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM MgCl_2 , 1 mM DTT, 1 mM EDTA, 5% glycerol), containing 1 mM phenylmethylsulfonyl fluoride and 0.002 mg/ml leupeptin, anti-pain and chymostatin. The suspension was sonicated five times for 3 min, with 5 min intervals, and centrifuged at $15000 \times g$ for 30 min. The supernatant (11 ml) represented the crude extract.

2.3.3. Ammonium sulfate fractionation. Crude extract was brought to 30% saturation ammonium sulfate, allowed to equilibrate for 1 h, and centrifuged at $39000 \times g$ for 20 min. The supernatant was ad-

*Corresponding author. Fax: (39) (71) 2802117.
E-mail: magnig@popcsi.unian.it

justed to 48% saturation ammonium sulfate, and after 3 h it was centrifuged as above. The precipitate containing the slr0787 protein was dissolved in 10 ml buffer A.

2.3.4. Phenyl agarose. The active preparation from the previous step was brought to 1.7 M ammonium sulfate and applied to a 1.4 cm×8 cm phenyl agarose (Sigma) column, equilibrated with buffer A containing 1.7 M ammonium sulfate. After a wash with the same buffer, a linear 1.7–0 M ammonium sulfate in buffer A gradient (50 ml plus 50 ml) was applied. A flow rate of 1 ml/min was maintained.

2.3.5. Matrex gel green A. Active fractions from the phenyl agarose step were pooled (22 ml) and directly loaded onto a 1.4 cm×5 cm Matrex gel green A (Amicon Corp.) column, equilibrated with buffer A containing 0.5 M NaCl. After a wash with 40 ml of the same buffer, the slr0787 protein was eluted with buffer A, containing 3 M NaCl. A flow rate of 1 ml/min was maintained. Active fractions (30 ml) were pooled, concentrated to 10 ml on an Amicon ultrafiltration cell with a YM30 membrane and dialyzed against 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.3 M ammonium sulfate. The final preparation was stored at –20°C. No loss of activity was observed under these conditions for several months.

2.4. Assays of enzymatic activities

An HPLC-based assay was used to measure the 'Nudix' hydrolase activity. The reaction mixture (150 µl) contained 50 mM HEPES, pH 8.2, either 5 mM MgCl₂ or MnCl₂, 0.2 mM ADP-ribose (or other substrate when indicated) and an appropriate amount of slr0787 enzyme. After 5 or 10 min incubation at 37°C, the reaction was stopped by adding 75 µl of cold 1.2 M HClO₄. After 10 min on ice the samples were centrifuged for 1 min at 12000×g; 150 µl of the supernatant was neutralized with 40 µl of cold 0.8 M K₂CO₃, kept on ice for 10 min and centrifuged as described above. The supernatants were used to measure the product formed by HPLC analysis. When reduced pyridine nucleotides were used as the substrates, reactions were stopped by boiling for 3 min and the samples were injected into the HPLC system after neutralization with 2.6 µl of 1.2 M HClO₄. For HPLC analysis, either a 7.5 cm or a 12.5 cm Supelcosil LC-18-DB, 3 µm particle size, reversed-phase column was used. Elution conditions were as reported in [7] and [1], with slight modifications. Controls without the enzyme were always processed in parallel in order to correct measurements; such corrections were crucial when Mn²⁺ was used as the divalent cation in the reaction mixture, due to a significant metal-ion catalyzed hydrolysis of several substrates [8].

For the determination of the NMN adenylyltransferase activity, the reaction mixture (150 µl) contained 50 mM HEPES, pH 8.2, 5 mM MgCl₂, 0.2 mM ATP, 0.2 mM NMN, and an appropriate amount of the slr0787 enzyme. After 5 or 10 min incubation at 37°C, NAD formed was measured either by the above HPLC-based assay, or spectrophotometrically [9].

In the assays for both enzymatic activities an appropriate amount of enzyme was used in order to provide a substrate consumption below 5% of the initial concentration after 10 min incubation. In addition, withdrawals from the assay mixtures at two different incubation times were always performed to ensure a linear time frame.

For kinetic analyses, the buffer used was 50 mM HEPES, pH 8.2. After 4 min preincubation at 37°C, reactions were initiated by the addition of pure slr0787 enzyme. The kinetic parameters for ADP-ribose, (2')phospho-ADP-ribose and IDP-ribose substrates were determined in the presence of 5 mM Mg²⁺ or 5 mM Mn²⁺ by analyzing reaction progress curves, obtained by measuring the product formed at different incubation times until total consumption of the substrate. *K_m* and *V_{max}* values were calculated by plotting data according to the integrated Michaelis-Menten equation [10]. Both the stability of the

enzyme and the lack of product inhibition during the reaction were ensured. Kinetic analysis of the 'Nudix' hydrolase activity in the presence of Ap₂A and ADP-mannose were performed by measuring the initial rates of AMP formation at substrate concentrations ranging from 10 to 200 µM, in the presence of 5 mM MnCl₂. For NMN adenylyltransferase kinetic measurements, the initial rates of NAD synthesis were determined as a function of the concentrations of ATP (20–200 µM) at various fixed concentrations of NMN (30–250 µM), in the presence of 5 mM MgCl₂. *V_{max}* and *K_m* values were obtained from the double reciprocal plots of substrate versus initial velocity.

One unit of enzyme activity represents the amount of enzyme catalyzing the formation of 1 µmol of product per min under the specified conditions.

2.5. Gel filtration, SDS-PAGE and N-terminal sequence analyses

Gel filtration of the pure slr0787 protein was carried out on a FPLC 1.77 cm×52 cm Sephacryl S-300 Superfine (Pharmacia) column, equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.5 M NaCl. Thyroglobulin (660 kDa), glutamic dehydrogenase (330 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66.2 kDa), dissolved in the equilibration buffer, were used as the standards. Protein samples were loaded and eluted at a flow rate of 0.4 ml/min, at 4°C.

SDS-PAGE was carried out according to [11] or [12]. In the latter case, a 10% total polyacrylamide concentration and a 3% concentration of cross-linker were used in the separating gel. Proteins were stained with Coomassie brilliant blue R.

N-terminal sequence analysis of the homogeneous recombinant protein spotted on a polyvinylidene difluoride membrane was performed with an Applied Biosystem model 491 protein sequencer.

3. Results

3.1. Cloning, expression and purification of the slr0787 protein

Fig. 1 shows the alignment of the slr0787 sequence with both *M. jannaschii* NMN adenylyltransferase and ADP-ribose pyrophosphatase, as resulting from BLAST search. *M. jannaschii* NMN adenylyltransferase shows 25% identity with and 19% similarity to the first 183 residues of the cyanobacterial slr0787 protein; *M. jannaschii* ADP-ribose pyrophosphatase shares 32% identity and 22% similarity with the last 143 residues of the *Synechocystis* protein. To elucidate the catalytic activity of the slr0787 protein, its gene was isolated and cloned into a T7-based vector for expression. Cultures of BL21 (DE3) cells harboring pT7-7-slr0787 were grown, induced with IPTG and extracted as described in Section 2. The expression of the slr0787 protein was demonstrated by the appearance of a band of about 38.5 kDa (Fig. 2A, lane a), which was not detected in control extracts prepared from BL21 cells transformed with the non-recombinant plasmid (Fig. 2A, lane b). Cell extracts were assayed for the presence of ADP-ribose pyrophosphatase and NMN adenylyltransferase activities. High levels of both activities were found in extracts of BL21 cells transformed with pT7-7-slr0787. Such activities were not detectable in the controls. The recombinant protein was purified to homogeneity (Fig. 2B) and NMN

Table 1
Purification of the slr0787 bifunctional protein

Purification step	Total protein (mg)	NMN adenylyltransferase		ADP-ribose pyrophosphatase	
		specific activity (U/mg)	total activity (U)	specific activity (U/mg)	total activity (U)
Crude extract	77	0.34	26.2	0.07	5.4
Ammonium sulfate	28	0.67	18.8	0.16	4.5
Phenyl agarose	13	1.82	23.7	0.32	4.2
Green A	4.4	3.42	15.0	0.70	3.1

Both enzymatic activities were assayed as described in Section 2, in the presence of 5 mM Mg²⁺ ions.



Fig. 1. Comparison of the MJ0541 and MJ1149 amino acid sequences with the slr0787 protein. Comparison was performed through the gapped BLAST program [2]. Gaps are indicated by dashes. Identical and similar amino acids are indicated by vertical lines and colon, respectively. The conserved signature sequence of the 'Nudix' hydrolase family is highlighted in bold.

adenylyltransferase and ADP-ribose pyrophosphatase copurified throughout the overall purification procedure outlined in Table 1. The ratio of the two activities was significantly constant during the purification (Table 1), as expected for a bifunctional enzyme. The final preparation endowed with both activities was estimated to be more than 95% pure by SDS-PAGE (Fig. 2B, lane e). About 4.4 mg of pure protein was obtained from 1 l culture. The molecular mass of about 39.5 kDa resulting from SDS-PAGE was very close to the expected molecular mass calculated from the predicted amino acid sequence of the recombinant protein. Gel filtration experiments showed a native molecular mass of about 263 kDa, indicating that the native protein is an oligomer. Sequencing

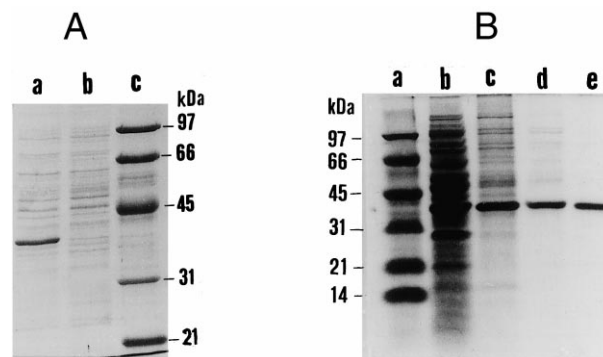


Fig. 2. Expression (A) and purification (B) of the slr0787 protein. A: SDS-PAGE (15% polyacrylamide gel) of BL21(DE3) cell extracts obtained from cells transformed with pT7-7-slr0787 and collected 5 h after IPTG induction (lane a), and with the plasmid without the insert (lane b). Cells were grown as described in Section 2. Extracts were prepared by suspending cells collected from 1 ml of the cultures in 100 µl buffer A (see Section 2), and disrupting them by vortexing with glass beads three times for 1 min, with 1 min intervals. About 10 µg of total proteins were applied to each lane. Marker proteins are shown in lane c. B: Tricine SDS-PAGE of fractions throughout the purification procedure. The lanes were loaded as follows: 2 µg of molecular mass markers (lane a), 15 µg of crude extract (lane b), 7.5 µg of the ammonium sulfate fraction (lane c), 3 µg of the phenyl agarose fraction (lane d), and 1.5 µg of the green A fraction (lane e).

of the first 20 residues of the pure recombinant protein yielded an exact match with the predicted sequence, confirming that the 39.5 kDa protein is that encoded by the cloned slr0787 gene.

3.2. Metal ion requirements

Both enzymatic activities of the bifunctional protein show metal ion requirement. Several ion species were tested for their ability to support the two activities, including Mn^{2+} , Mg^{2+} , Ca^{2+} , Co^{2+} , Ni^{2+} , Fe^{3+} , Zn^{2+} , Pb^{2+} , Cu^{2+} , Cd^{2+} and Hg^{2+} . Among them, only Mn^{2+} , Mg^{2+} , and Zn^{2+} were effective on the ADP-ribose pyrophosphatase activity. Mn^{2+} was the most effective, giving maximal activation at 5 mM. At 0.25 mM, Zn^{2+} and Mg^{2+} gave only 31% and 4.5% of the activity observed in the presence of Mn^{2+} , respectively. All the ion species tested, with the exception of Hg^{2+} and Cu^{2+} , were able to support the NMN adenylyltransferase activity: at 0.5 mM, Mn^{2+} , Mg^{2+} and Co^{2+} gave similar maximal activity, while the other ions were five-fold less effective.

Table 2
Effect of Mg^{2+} and Mn^{2+} ions on the substrate specificity of the slr0787 'Nudix' hydrolase activity

Substrate	Mg^{2+}		Mn^{2+}	
	specific activity (U/mg)	relative activity (%)	specific activity (U/mg)	relative activity (%)
(2')Phospho-ADP-ribose	1.65	100	2.75	100
ADP-ribose	0.70	42	2.20	80
IDP-ribose	0.80	48	1.70	62
NADPH	0.30	18	n.e. ^a	n.e.
ADP-mannose	0.01	< 1	0.60	22
ADP-glucose	0.02	< 1	0.80	29
Ap ₂ A	0		0.50	18
Ap ₃ A	0		0.02	< 1
NADH	0		n.e.	n.e.

All substrates were present at 2 mM concentration and were assayed as described in Section 2.

^aNADPH and NADH hydrolysis in the presence of Mn^{2+} could not be evaluated because of the highly interfering metal-catalyzed hydrolysis.

Table 3
Kinetic parameters of the slr0787 ‘Nudix’ hydrolase activity

Substrate/ion	k_{cat} (s^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)
ADP-ribose/ Mg^{2+}	0.4	0.27	1.7
(2')Phospho-ADP-ribose/ Mg^{2+}	1.1	0.20	5.4
IDP-ribose/ Mg^{2+}	0.6	1.0	0.6
ADP-ribose/ Mn^{2+}	1.5	0.26	5.9
(2')phospho-ADP-ribose/ Mn^{2+}	1.7	0.33	5.2
IDP-ribose/ Mn^{2+}	1.2	0.91	1.3
ADP-mannose/ Mn^{2+}	0.6	107	0.005
$\text{Ap}_2\text{A}/\text{Mn}^{2+}$	1.1	439	0.002

Kinetic analyses were performed as described in Section 2.

3.3. pH optimum

Like the ‘Nudix’ hydrolases so far characterized [5], the ADP-ribose pyrophosphatase activity of slr0787 protein had an alkaline pH optimum. In HEPES buffer, the enzyme was maximally active at pH 8.2, the activity at pH 7.0 being only about 33% of that measured at pH 8.2. In borate buffer, the pH optimum was 10, with a 25% decrease of activity at pH 9.0. The pH optimum of the NMN adenylyltransferase activity was broad, ranging from pH 6.0 to 8.6.

3.4. Substrate specificity

An extensive study on the ‘Nudix’ hydrolase specificity was performed with the homogeneous enzyme in the presence of either Mg^{2+} or Mn^{2+} . Among the biochemicals tested as potential substrates, including purine and pyrimidine ribo- and deoxyribonucleoside diphosphates and triphosphates, ADP-ribose, cyclic ADP-ribose, IDP-ribose, ADP-glucose, GDP-glucose, UDP-glucose, ADP-mannose, GDP-mannose, UDP-acetylglucosamine, pyridine nucleotides, and diadenosine polyphosphates (Ap_nA , $n=2-5$), only the compounds listed in Table 2 were substrates of the ‘Nudix’ hydrolase activity. In the presence of Mg^{2+} , (2')phospho-ADP-ribose, ADP-ribose, IDP-ribose, NADPH, and to a markedly lower extent, ADP-mannose and ADP-glucose were hydrolyzed. In particular (2')phospho-ADP-ribose was hydrolyzed about two times faster than ADP-ribose and IDP-ribose. When Mg^{2+} was replaced by Mn^{2+} , the enzyme, in addition to the above nucleotides, catalyzed the hydrolysis of Ap_2A and Ap_3A . It is worth noting that NAD was not a substrate of the ‘Nudix’ hydrolase activity. As checked by HPLC, the hydrolysis of all the substrates tested yielded the corresponding 5'-mononucleotide as the product, i.e. in all cases the pyrophosphoric linkage was hydrolyzed. Table 3 compares the kinetic constants for the substrates tested. The kind of activating metal ion did not affect the K_{m} values for (2')phospho-ADP-ribose, ADP-ribose and IDP-ribose. However, V_{max} values were higher in the presence of Mn^{2+} . Data from Table 3 confirm that (2')phospho-ADP-ribose and ADP-ribose are the preferred substrates. Replacement of the adenine moiety with hypoxanthine slightly increases the K_{m} value, resulting in a catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) about four times lower. The substitution of ribose with mannose has a much larger effect on the catalytic efficiency, which is reduced by about 1000-fold, due to a significant increase of the K_{m} value. The $k_{\text{cat}}/K_{\text{m}}$ for Ap_2A is also very low.

The substrate specificity of slr0787 NMN adenylyltransferase activity was not affected by Mg^{2+} and Mn^{2+} ions. The enzyme exhibited a strict preference for the amidated form of NMN, NAD synthesis occurring at a rate 20 times faster than nicotinic acid adenine dinucleotide synthesis. Furthermore,

unlike NMN adenylyltransferases from other sources [13,14], the slr0787 enzyme did not catalyze the conversion of the reduced form of NMN to NADH. K_{m} values for NMN and ATP were 80 μM and 30 μM , respectively.

In order to investigate a possible interaction between the two catalytic sites, including channelling effects or any cross-action, both the substrates and the products of one enzyme activity were tested as possible effectors on the other and vice versa. The lack of any effect, together with the observation that the same kinetic parameter values were obtained for the NMN adenylyltransferase both in the presence and in the absence of 1 mM NaF, which fully inhibited the ‘Nudix’ hydrolase activity, suggests that the two catalytic domains work independently of each other.

3.5. Thermophilicity and thermal stability

The study of the temperature dependence of both enzyme activities showed an optimum temperature for the NMN adenylyltransferase at 37°C; for ADP-ribose pyrophosphatase optimal temperature ranged from 50°C to 70°C. Thermal stability experiments performed by incubating the bifunctional protein at 0.42 mg/ml in 50 mM Tris-HCl, pH 8.0, 1 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.3 M ammonium sulfate, resulted in the complete loss of both activities after 30 min at 40°C.

4. Discussion

Overexpression of the *Synechocystis* sp. slr0787 gene in *E. coli* and characterization of the pure recombinant protein allowed us to demonstrate that the cyanobacterial gene encodes a novel bifunctional enzyme endowed with both NMN adenylyltransferase and ADP-ribose pyrophosphatase activities. The significant homology of the N-terminal and C-terminal domains of the cyanobacterial protein with the *M. jannaschii* NMN adenylyltransferase and ADP-ribose pyrophosphatase, respectively, suggests that the bifunctional enzyme derives from the fusion of the two archaeal genes. Therefore it is not surprising that the protein retains, although only partially, thermophilic properties, as observed in the present report for the ADP-ribose pyrophosphatase activity. Recent results obtained by analyzing the sequence of several genomes from different species revealed unexpected connections among microorganisms belonging to the three known kingdoms [15]. The results of our biochemical studies on this novel cyanobacterial bifunctional enzyme are in line with the hypothesis of a lateral gene transfer which took place from archaea to eubacteria [16].

A remarkable specificity of the slr0787 NMN adenylyltrans-

ferase towards the substrate NMN has been evidenced. In contrast to the archaeal NMN adenylyltransferases from *M. jannaschii* and *Sulfolobus solfataricus* [1], the cyanobacterial enzyme exhibits a linear kinetic behavior. The K_m values obtained for NMN and ATP are very similar to those observed for the human enzyme [17]. The slr0787 ADP-ribose pyrophosphatase is highly specific for ADP-ribose and (2')phospho-ADP-ribose, like the *M. jannaschii* enzyme [4]. However, the cyanobacterial enzyme shows a markedly higher affinity for these substrates. Furthermore, while the archaeal ADP-ribose pyrophosphatase is strictly Mg^{2+} -dependent, the bifunctional enzyme can replace Mg^{2+} with Mn^{2+} or Zn^{2+} . These properties make the slr0787 ADP-ribose pyrophosphatase more similar to rat liver ADP-ribose pyrophosphatase (ADP-ribose I) [18] than to the archaeal enzyme.

The bifunctional enzyme might represent a sophisticated biochemical tool for the regulation of the cellular NAD turnover. Indeed, the best substrates for the pyrophosphatase activity (namely ADP-ribose and (2')phospho-ADP-ribose) are also the main catabolites deriving from NAD(P) consumption. In prokaryotes, free ADP-ribose may be formed by the action of both NAD glycohydrolases and ADP-ribosyl glycohydrolases. Interestingly, endogenous ADP-ribosylation of eubacterial proteins has been observed only in photosynthetic bacteria: in *Rhodospirillum rubrum* the nitrogenase, a key enzyme in nitrogen fixation, is regulated by reversible ADP-ribosylation [19], and in *Synechocystis* sp. glutamine synthetase is subject to endogenous ADP-ribosylation [20]. Harmful non-enzymatic protein glycation by free ADP-ribose can easily occur even at micromolar concentrations of the nucleotide [21]; in addition, (2')phospho-ADP-ribose has been described to covalently modify proteins through both enzymatic and non-enzymatic reactions [22,23]. Therefore the intracellular level of these nucleotides must be under strict metabolic control. The bifunctional enzyme may act in the concerted modulation of the synthesis of NAD and the scavenging of its catabolic products.

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