JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7718–7723 Vol. 179, No. 24
0021-9193/97/$04.00+0

Characterization of Nicotinamide Mononucleotide Adenylyltransferase from Thermophilic Archaea

NADIA RAFFAELLI,[1] FRANCESCA M. PISANI,[2] TERESA LORENZI,[1] MONICA EMANUELLI,[1] ADOLFO AMICI,[1] SILVERIO RUGGIERI,[3] AND GIULIO MAGNI[1]*

Istituto di Biochimica, Facoltà di Medicina e Chirurgia,[1] and Dipartimento di Biotecnologie Agrarie ed Ambientali, Facoltà di Agraria,[3] Università di Ancona, Ancona, and Istituto di Biochimica delle Proteine ed Enzimologia, Consiglio Nazionale delle Ricerche, Naples,[2] Italy

Received 23 June 1997/Accepted 14 October 1997

The enzyme nicotinamide mononucleotide (NMN) adenylyltransferase (EC 2.7.7.1) catalyzes the synthesis of NAD^+ and nicotinic acid adenine dinucleotide. It has been purified to homogeneity from cellular extracts of the thermophilic archaeon *Sulfolobus solfataricus*. Through a database search, a highly significant match was found between its N-terminal sequence and a hypothetical protein coded by the thermophilic archaeon *Methanococcus jannaschii* MJ0541 open reading frame (GenBank accession no. U67503). The MJ0541 gene was isolated, cloned into a T7-based vector, and expressed in *Escherichia coli* cells, yielding a high level of thermophilic NMN adenylyltransferase activity. The expressed protein was purified to homogeneity by a single-step chromatographic procedure. Both the subunit molecular mass and the N-terminal sequence of the pure recombinant protein were as expected from the deduced amino acid sequence of the MJ0541 open reading frame-encoded protein. Molecular and kinetic properties of the enzymes from both archaea are reported and compared with those already known for the mesophilic eukaryotic NMN adenylyltransferase.

NAD^+ synthesis can be accomplished either via de novo pathways or through preformed pyridine ring salvage routes (13). All such pathways converge to the reaction nicotinamide mononucleotide (NMN) (or nicotinic acid mononucleotide) + ATP ↔ NAD^+ (or nicotinic acid adenine dinucleotide) + PP_i, which is catalyzed by the enzyme NMN adenylyltransferase (EC 2.7.7.1). Interestingly, this is the only enzyme in the biosynthetic pathway to be located in the cell nucleus (15). Numerous examples of a fluctuation of NMN adenylyltransferase activity during the DNA synthesis phase of the cell cycle have been reported (14, 23, 32). More recently, it has been proposed that the nuclear localization of the enzyme could be related to the consistent demand for NAD^+ as a substrate for nuclear poly(ADP) ribosylation reactions (29, 34), thus suggesting a major role for the enzyme in cellular metabolism. In prokaryotes, the NMN adenylyltransferase gene, designated *nadD*, was mapped and demonstrated to be essential for viability (16). However it remains one of the few genes involved in the NAD^+ biosynthetic pathway not to have been cloned and sequenced from any organism. The enzyme has been identified and characterized in its catalytic properties from several prokaryotic sources (6, 10, 26). It has been purified to homogeneity and extensively characterized by us from yeast, bull testis, and human placenta (4, 12, 24), but its instability and relatively low concentration in cell extracts precluded obtaining enzyme preparations suitable for gene isolation. We have previously reported the presence of NMN adenylyltransferase in the thermophilic archaeon *Sulfolobus solfataricus* (28). Purification to homogeneity of the *S. solfataricus* enzyme and determination of its N-terminal sequence allowed us to recognize the MJ0541 open reading frame (ORF) from the *Methanococcus jannaschii* genome sequence as the NMN adenylyltransferase gene. This report describes the characterization of the enzyme from thermophilic archaea, including the first documentation of the identification, cloning, and expression of the NMN adenylyltransferase gene.

* Corresponding author. Mailing address: Istituto di Biochimica, Facoltà di Medicina e Chirurgia, Via Ranieri, 60131 Ancona, Italy. Phone: 39 71 2204678. Fax: 39 71 2802117. E-mail: magnig@anvax1.unian.it.

MATERIALS AND METHODS

Materials. Lyophilized cells of *S. solfataricus* MT-4 were kindly provided by A. Gambacorta (Istituto per la Chimica di Molecole di Interesse Biologico, CNR, Napoles, Italy). *M. jannaschii* genomic clone AMJAJ54, containing the MJ0541 gene, was purchased from the American Type Culture Collection (Rockville, Md.). Oligonucleotide primers were obtained from PRIMM (Milan, Italy). Restriction enzymes were obtained from Promega Biotec (Madison, Wis.), and T4 DNA ligase, PCR reagents, and enzymes were obtained from Boehringer Mannheim GmbH. The basic molecular biology procedures for bacterial growth, plasmid DNA purification, and preparation and transformation of competent cells described by Sambrook et al. (30) were followed.

Purification of *S. solfataricus* NMN adenylyltransferase. Unless otherwise stated, all steps were performed at room temperature. The following buffers were used: buffer A (10 mM Tris-HCl [pH 8.4], 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 2.5 mM $MgCl_2$, 10% glycerol), buffer B (50 mM Tris-HCl [pH 7.4], 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT), buffer C (potassium phosphate buffer [pH 6.8], 1 mM $MgCl_2$, 1 mM DTT), and buffer D {50 mM Tris-HCl [pH 7.4], 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 0.25 mM 3-[(3-cholamidopropyl) dimethylammonio] 2-hydroxy-1-propanesulfonate [CHAPSO]}.

(i) Crude extract preparation. *S. solfataricus* cells were grown on glucose as the carbon source and were collected at the stationary phase. In a typical preparation, 6 g of lyophilized cells (about 30 g in wet weight) was allowed to rehydrate in 120 ml of buffer A containing 1 M NaCl. After standing for 30 min at 4°C, 180 g of sea sand was added, and cells were broken in a refrigerated Brown homogenizer for 5 min. Sea sand was removed by filtration, and the clarified homogenate was centrifuged at 60,000 × *g* for 120 min at 4°C. The resulting supernatant represented the crude extract.

(ii) DEAE-Sepharose chromatography. The crude extract (110 ml) was dialyzed overnight against 10 liters of buffer A and applied to a DEAE-Sepharose Fast Flow (Pharmacia) column (5.0 by 20 cm) equilibrated with the same buffer. After being washed with 1 bed volume of buffer A, bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in 1,200 ml of buffer A at a flow rate of 10 ml/min.

(iii) Matrex Gel Red A chromatography. Active fractions from the DEAE-Sepharose were pooled (280 ml) and directly loaded onto a Matrex Gel Red A (Amicon Corp.) column (2.5 by 40 cm) previously equilibrated with buffer B. A flow rate of 4 ml/min was maintained. After a wash with buffer B plus 0.5 M NaCl at the same flow rate, the enzymatic activity was eluted with a linear gradient of NaCl from 0.5 to 3 M in 800 ml of buffer B at a flow rate of 2.5 ml/min.

(iv) Hydroxylapatite chromatography. The active pool from the previous step (350 ml) was concentrated to 30 ml on an Amicon ultrafiltration cell with a YM30 membrane and loaded at a flow rate of 1 ml/min onto a hydroxylapatite

TABLE 1. Purification of *S. solfataricus* NMN adenylyltransferase[a]

Fraction	Total protein (mg)	Total activity (U)	Sp. act. (U/mg)	% Yield	Purification factor
Crude extract	1,106	2.5	0.0022	100	
DEAE-Sepharose	390	2.7	0.007	108	3.2
Matrex Gel Red A	19.1	3.0	0.157	120	71.4
Hydroxylapatite	0.41	1.4	3.4	56	1,545
TSK Phenyl-5PW	0.082	0.72	8.8	29	4,000

[a] The purification procedure and the enzyme assay were performed as described in Materials and Methods.

(Bio-Rad) column (1.4 by 12 cm) equilibrated with 10 mM buffer C. After a wash with 60 mM buffer C, a linear 60 to 600 mM potassium phosphate gradient (65 ml plus 65 ml) was applied. The active pool (30 ml) was made 0.25 mM with CHAPSO and concentrated to 7 ml by ultrafiltration as previously described.

(v) TSK Phenyl-5PW chromatography. The active pool was applied in 1-ml aliquots, at a flow rate of 1 ml/min, to a fast protein liquid chromatography (FPLC) column of TSK gel Phenyl-5PW (Pharmacia) equilibrated with buffer D containing 2 M NaCl. The column was washed with 10 ml of the same buffer and eluted with a linear gradient of decreasing NaCl concentration (2 to 0 M) in buffer D. Active fractions were pooled and concentrated by ultrafiltration.

Cloning of the MJ0541 gene. The synthetic oligonucleotide primers 5′-CTA GAATTCGCTTGAGAGGGTTTATAATTGGT-3′ and 5′-CTAAAGCTTTTA TTTGTCTGTCTGAGCTAA-3′ were used in a PCR to amplify the MJ0541 ORF and to insert the *Eco*RI and *Hin*dIII restriction sites at its 5′ and 3′ ends, respectively. PCR was performed with 0.3 μg of genomic AMJAJ54 clone as the template, with 150 pmol of each primer in a final volume of 100 μl. Each cycle was set for 1 min of denaturation at 94°C, 1 min of annealing at 45°C, and 1 min of elongation at 72°C, and 30 reaction cycles were carried out in a DNA thermal cycler. The 527-bp product was purified from an agarose gel, digested with *Eco*RI and *Hin*dIII, and cloned into *Eco*RI-*Hin*dIII-digested pT7-7 plasmid vector (33) to obtain the construct pT7-7MJ0541. The nucleotide sequence of the insert was confirmed by direct sequencing. The construct was used to transform *Escherichia coli* TOP10 (Invitrogen) for plasmid preparation and *E. coli* BL21 (DE3) for protein expression.

Purification of the MJ0541 ORF-encoded protein. (i) Growth and expression. Single colonies of strain BL21 (DE3) harboring the pT7-7-MJ0541 plasmid were inoculated into 50 ml of Luria-Bertani medium (supplemented with ampicillin at 100 μg/ml). After overnight growth at 37°C, 0.5 ml of the saturated culture was inoculated into 1 liter of fresh medium containing ampicillin. When the cells had again reached the stationary phase (A_{600}, 1.8), they were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM [final concentration]) and grown for an additional 4 h.

(ii) Crude extract. All steps were carried out at 4°C. The induced cells were harvested by centrifugation at 10,000 × *g* for 10 min, washed once with 50 mM Tris (pH 7.5), and resuspended in 50 ml of 50 mM Tris (pH 7.5)–1 mM $MgCl_2$–0.5 M NaCl–1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by passage through a French pressure cell at about 100 mPa, and crude extract was clarified by centrifugation at 15,000 × *g* for 30 min.

(iii) Hydroxylapatite. After addition of NaCl to a final concentration of 1.5 M, the crude extract was loaded onto a column (2.5 by 31 cm) of hydroxylapatite (Bio-Rad) equilibrated with 10 mM buffer A (potassium phosphate buffer [pH 6.8], 1 mM $MgCl_2$). After being washed with 800 ml of 60 mM buffer A, a linear 60 to 600 mM buffer A gradient (1 liter plus 1 liter) was applied. Fractions containing the MJ0541 gene-encoded protein were combined, concentrated by ultrafiltration, dialyzed against 50 mM Tris (pH 7.5), and stored at −20°C.

NMN adenylyltransferase assay. The routine reaction mixture contained 100 mM HEPES (pH 7.4), 13 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM NMN, and the appropriate amount of the enzyme preparation in a final volume of 150 μl. The reaction was carried out for 10 min at 70°C, and the NAD^+ formed was determined either spectrophotometrically or with a high-pressure liquid chromatography-based assay, as previously described (28). The high-pressure liquid chromatography-based assay was modified with a 7.5-cm by 4.6-mm-inside-diameter Supelcosil LC-18-DB 3-μm-particle-size reversed-phase column. The elution conditions were 2.5 min at 100% buffer A (0.1 M potassium phosphate [pH 6.0]), 1 min at up to 100% buffer B (buffer A containing 20% methanol), and holding at 100% buffer B for 3 min. Finally, the gradient returned to 100% buffer A in 1 min. The column was flushed with buffer A for 2 min prior to the next run. The flow rate was 1 ml/min.

Kinetic analyses were performed at 60°C; the reaction mixture (0.6 ml), which consisted of 100 mM HEPES (pH 7.4), 13 mM $MgCl_2$, 0.015 mg of bovine serum albumin per ml, and various amounts of NMN and ATP, was preheated at 60°C for 4 min. The reaction was initiated by the addition of 0.36 μg of *S. solfataricus* enzyme and 0.012 μg of *M. jannaschii* recombinant enzyme. Samples were withdrawn for NAD^+ measurement at 5-min intervals by removal into 0.6 M ice-cold perchloric acid. At any substrate concentration used, reactions were linear at least for up to 20 min. The activity values reported in Fig. 3 are the average of three independent measurements performed at 5-min intervals. One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min under the specified conditions.

Electrophoretic analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of either Laemmli (19) or Schagger and Von Jagow (31). In the latter case, a 10% total polyacrylamide concentration and a 3% concentration of cross-linker were used in the separation gel. To look for the presence of carbohydrate in the *S. solfataricus* protein, gels were subjected to periodate-Schiff staining (11) with ovalbumin and horseradish peroxidase as the standards.

Gel filtration. Gel filtration of the pure enzymes was carried out by FPLC with a Superose 12 HR 10/30 (Pharmacia) column equilibrated with 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1 mM $MgCl_2$, and 1 mM EDTA. For the *S. solfataricus* enzyme, chromatography was performed in both the presence and absence of 0.25 mM CHAPSO. Bovine serum albumin, ovalbumin, and carbonic anhydrase dissolved in the equilibration buffer were used as the standards. Proteins were loaded and eluted at a flow rate of 0.5 ml/min at room temperature.

Chromatofocusing. The isoelectric point of homogeneous *S. solfataricus* NMN adenylyltransferase was determined by FPLC chromatofocusing with a Mono P HR 5/5 (Pharmacia) column in the presence of 0.25 mM CHAPSO. The experimental conditions are described in reference 28.

Amino acid and sequence analyses. Three micrograms of pure *S. solfataricus* NMN adenylyltransferase was desalted by precipitation in 20% trichloroacetic acid. The pellet was washed once with 10% trichloroacetic acid and three times with ethanol-ether (1:1). Hydrolysis was carried out under a vacuum for 45 and 90 min at 155°C with 6 N hydrochloric acid containing 0.1% (wt/vol) thioglycolic acid. Amino acid analyses were then performed with a Chromakon (Kontron) instrument by an *o*-phthalaldehyde post column derivatization procedure. Cysteine was determined as cysteic acid after performic acid oxidation, as described by Moore (22).

N-terminal sequence analyses of the homogeneous proteins spotted on a polyvinylidene difluoride membrane were performed by automated Edman degradation with an automated protein sequencer (Applied Biosystems, Foster City, Calif.).

pH optimum. The activity of *S. solfataricus* NMN adenylyltransferase in the forward and reverse reaction was assayed at 70°C in the presence of buffers adjusted to the desired pH at 70°C. In order to check the buffering capacity at 70°C of the chosen salts, the pH values of the assay incubation mixtures were routinely measured. Control experiments were performed in order to evaluate the degradation of the substrates NMN, ATP, and NAD^+ incubated at 70°C at the different pH values. After 10 min of incubation at 70°C, at pH values ranging from 4.2 to 8.4, ATP remained completely stable, while NMN and NAD^+ were sensitive to hydrolysis at pH values above 7.8, being 50% degraded at pH 9.0.

Thermophilicity and thermostability. The temperature dependence of the enzyme activity of *S. solfataricus* and *M. jannaschii* was determined by assay of aliquots of the homogeneous enzymes in the standard mixture at temperatures ranging from 37 to 97°C.

The thermal stability was measured by incubation of enzyme solutions (0.073 mg/ml for the *S. solfataricus* enzyme and 0.006 mg/ml for the *M. jannaschii* protein) in 50 mM Tris-HCl (pH 7.4) at temperatures ranging from 70 to 95°C. At suitable times, aliquots of the incubated enzyme were withdrawn, cooled on ice, and assayed at 70°C. The pH dependence of the *S. solfataricus* enzyme thermal stability was studied in the pH range 5.0 to 8.5 with the following buffers: 100 mM sodium citrate (pH 5.0 to 6.7), 100 mM Tris-HCl (pH 6.1 to 7.5), and 100 mM sodium borate (pH 7.4 to 8.5) (pH values were measured at 85°C). Thermal stability at each pH was determined by incubation of the enzyme in the appropriate buffer for 60 min at 85°C, and enzyme activity was measured before and after heating to determine residual activity.

RESULTS AND DISCUSSION

Purification and molecular properties of *S. solfataricus* NMN adenylyltransferase. The purification procedure is outlined in Table 1. The crucial steps of the entire protocol are represented by the dye ligand chromatography on a Matrex Gel Red A column and the adsorption chromatography on a

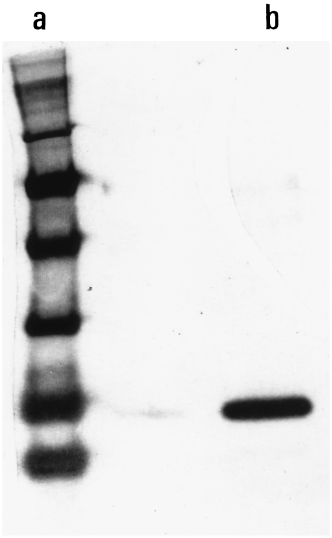

FIG. 1. Tricine-SDS-PAGE of homogeneous *S. solfataricus* NMN adenylyltransferase. Electrophoresis was performed as described in Materials and Methods. Proteins were stained with silver according to the method of Oakley et al. (25). Lane a, phosphorylase b (M_r, 97,400), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,400). Lane b, *S. solfataricus* NMN adenylyltransferase (1 μg).

hydroxylapatite column, each step resulting in about a 22-fold increase in specific activity. The enzyme was found to bind very strongly to Matrex Gel Red A; most of the contaminating proteins could be eluted by washing the column with 0.5 M NaCl, whereas NMN adenylyltransferase was eluted at 2.7 M NaCl. Such a high ionic strength did not prevent the enzyme from adsorbing to the subsequent hydroxylapatatite column, from which NMN adenylyltransferase could be eluted at 0.5 M phosphate. After the hydroxylapatite chromatography step, the enzyme preparation was rather unstable, and for further purification, it was beneficial to add 0.25 mM CHAPSO, which proved to be the best among several detergents tested (data not shown). Furthermore, the presence of CHAPSO in the equilibration and elution buffers of the TSK-Phenyl column was absolutely necessary in order to prevent an irreversible binding of the enzyme to the column matrix. The homogeneity of the final preparation was demonstrated by SDS-PAGE, as shown in Fig. 1. The purified enzyme migrated as a single band with a molecular mass of about 18,600 Da. The protein was devoid of any carbohydrate content, as revealed by the absence of periodate-Schiff staining of samples run in SDS-PAGE (see Materials and Methods). A native molecular mass of about 66,000 Da was estimated by gel filtration according to the method of Andrews (3). Data from SDS-PAGE and gel filtration chromatography might be consistent with both a trimer and a tetramer, but are not conclusive at present. Like NMN adenylyltransferases from yeast, bull testis, and human placenta (4, 12, 24), the thermophilic enzyme possesses a multimeric structure. However, the subunit molecular mass of the archaeal protein is considerable lower than that of yeast (24) and mammalian (4, 12) enzymes (50 and 33 kDa, respectively). Chromatofocusing experiments, performed as described in Materials and Methods, revealed a pI of 5.4.

The amino acid composition of the archaeal NMN adenylyltransferase is shown in Table 2. The data represent the nearest integer to the average of three analyses at 45- and 90-min hydrolyses. The (Asx plus Glx)/(Lys plus Arg) ratio was 1.9, in agreement with the acidic nature of the protein. The amino terminus was determined to be Met–Arg–Gly–Leu–Tyr-5–Pro–Gly–Arg–Phe–Gln-10–Pro–Phe–His–Leu–Gly-15–His–Leu–Asn–Val–Ile-20–Lys–Ile–Lys–Leu–Glu-25–Arg–Val–Asp–Asp–Pro-30–Ile–Ile.

TABLE 2. Amino acid composition of *S. solfataricus* NMN adenylyltransferase

Amino acid	No. of residues/subunit
Asx	13
Thr	7[a]
Ser	13[a]
Glx	17
Gly	22
Ala	8
Val	8
Met	3
Ile	8
Leu	12
Cys[b]	2
Tyr	5
Phe	6
Lys	7
His	2
Arg	8
Trp	ND[c]
Pro	ND

[a] Value extrapolated to zero hydrolysis time.
[b] Determined as cysteic acid.
[c] ND, not determined.

Catalytic properties of *S. solfataricus* NMN adenylyltransferase. The effect of pH on the enzyme activity on both reaction directions (forward and reverse) was studied at 70°C in the pH range 4.0 to 9.0. As depicted in Fig. 2, the enzyme activity remained almost constant from pH 5.8 to 7.8. As for other thermophilic enzymes (20, 21), several ionic species were found to differently affect NMN adenylyltransferase activity. Among them, sulfate ions exhibited a powerful stimulatory effect; at 0.1 M $Na_2(SO_4)$, the enzyme activity was stimulated

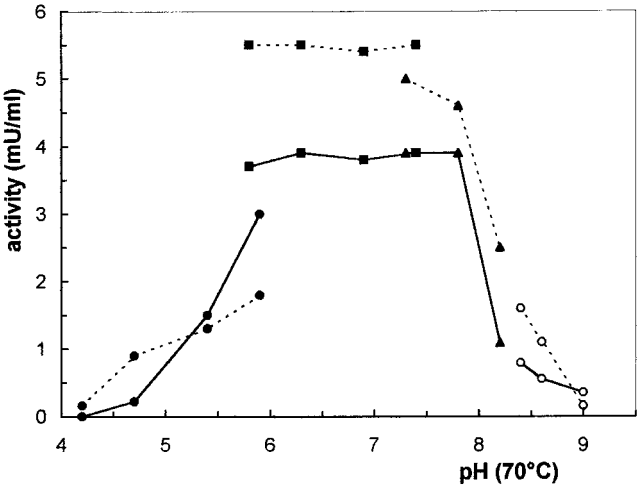

FIG. 2. Effect of pH on NMN adenylyltransferase activity. The activity was assayed in the forward (solid lines) and reverse (dashed lines) reactions in the presence of the following buffers, which were adjusted to the desired pH at 70°C: 50 mM sodium citrate (●), 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (■), 50 mM HEPES (▲), and 50 mM sodium borate (○). Activity was measured in milliunits per ml of enzyme solution.

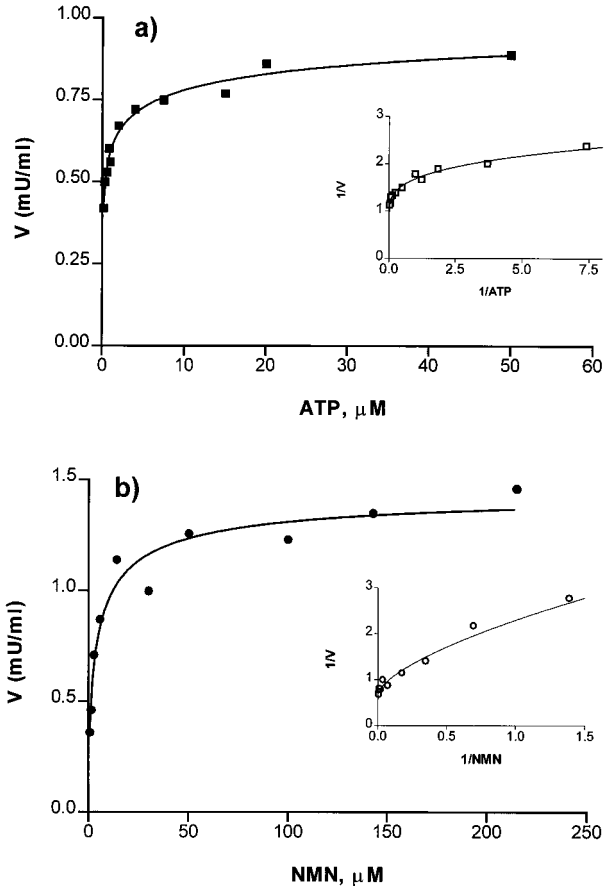

FIG. 3. NMN adenylyltransferase initial velocity (V) versus substrate concentration. (a) ATP as the variable substrate at 40 μM NMN. (b) NMN as the variable substrate at 50 μM ATP. The enzyme activity was determined as reported in Materials and Methods. Double-reciprocal plots are shown in the insets. Activity was measured in milliunits per ml of enzyme solution.

about fivefold. Experiments are in progress in order to search for a possible correlation of this finding with the ability of *Sulfolobus* spp. to gain energy through sulfur utilization (7). Unlike the mesophilic enzyme from *E. coli* (6), yeast (24), and mammalian sources (4, 12), homogeneous *S. solfataricus* NMN adenylyltransferase exhibited an apparent nonlinear kinetic behavior. Figure 3 shows the plots of the velocity of the enzyme reaction versus ATP and NMN substrate concentrations. The double-reciprocal plots shown in the insets are not linear. The downward curves might be consistent with a negative cooperativity, which was observed with respect to both substrates. Experimental conditions were chosen in order to keep the substrate consumption below 5% of the initial concentrations, thus ensuring the initial-rate kinetic conditions (1). Furthermore no product inhibition was detectable at the substrate concentrations used in the experiment. However, further kinetic analysis should be performed in order to calculate the specific kinetic parameters and to draw final conclusions about the significance of such an apparent nonlinear behavior.

Thermophilicity and thermostability of *S. solfataricus* NMN adenylyltransferase. The enzyme showed a continuous increase in activity at temperatures ranging from 37 to 97°C, and from the resulting Arrhenius plot, an activation energy of 98 kJ/mol was calculated, which well agrees with the value obtained for the partially purified enzyme (28).

FIG. 4. Comparison of the N terminus of *S. solfataricus* NMN adenylyltransferase (NMNAT) and the hypothetical protein from *M. jannaschii* coded by the MJ0541 ORF (MJ0541*orf*). Both identical and similar amino acids are shaded. Amino acids with similar properties are grouped according to the Dayhoff classification (9).

Catalytic activity was fully retained for at least 2 h at 80°C; the half-lives at 85 and 90°C were 75 and 30 min, respectively. Thermal stability was not pH dependent in a broad pH range extending from 6.2 to 7.5. A progressive increase of the enzyme activity half-life was observed in the presence of increasing concentrations of both NaCl and KCl, with the half-life at 85°C doubled at a 1 M salt concentration. On the contrary, in the presence of a 0.2 M concentration of salts such as KSCN, $NaNO_3$, and $NaClO_4$, which are indicated as chaotropic salts (5), 50% of the enzyme initial activity was lost after just 5 min at 85°C. This finding might indicate that hydrophobic interactions could play an important role in the enzyme thermal stability, as reported for other thermophilic proteins (18, 35).

Identification, cloning, and expression of *M. jannaschii* NMN adenylyltransferase gene. The N-terminal sequence of the *S. solfataricus* protein, the first sequence ever determined for the enzyme NMN adenylyltransferase, was instrumental in the identification of the archaeal enzyme gene. Similarity searches with the BLAST (2) network service at the National Center for Biotechnology Information showed that the N-terminal sequence of the *S. solfataricus* enzyme shares 65% identity with the N-terminal sequence of an *M. jannaschii* hypothetical protein encoded by the MJ0541 gene (Fig. 4). In addition, the molecular mass of 19.6 kDa predicted for the polypeptide encoded by the MJ0541 ORF closely corresponds to the subunit molecular mass of about 18.6 kDa obtained for the *S. solfataricus* enzyme.

To confirm that the MJ0541 ORF indeed codes for NMN adenylyltransferase, the gene was isolated and cloned into a T7-based vector. *E. coli* BL21(DE3) cells harboring the recombinant plasmid were grown, induced with IPTG, and extracted as described under Materials and Methods. Cell extracts were assayed for NMN adenylyltransferase activity at 70°C, ensuring that *E. coli* NMN adenylyltransferase was inactive. Even in the absence of added IPTG, a high level of thermophilic NMN adenylyltransferase activity could be detected in BL21(DE3) cells transformed with the recombinant plasmid. At 4 h after IPTG induction, only a 20% increase in the total activity was observed, which then declined almost to the original levels. A major band with a molecular mass of about 21.5 kDa was detected in the extract obtained from cells collected 4 h after induction (Fig. 5, lane c). Such a protein band was absent from extracts of *E. coli* BL21(DE3) cells transformed with the not recombinant vector plasmid (Fig. 5, lane b).

Purification and properties of recombinant *M. jannaschii* NMN adenylyltransferase. The high levels of enzyme expression afforded a straightforward purification. NMN adenylyltransferase was purified to homogeneity with good yield (44%) by single-step adsorption chromatography on a hydroxylapatite column, as described under Materials and Methods. As for the *S. solfataricus* enzyme, the recombinant protein was eluted from the column at a phosphate concentration of 0.5 M. By achieving a 15-fold purification, about 3 mg of pure enzyme was obtained from 1 liter of culture, with a specific activity of 187 U/mg. The final preparation was estimated to be at least

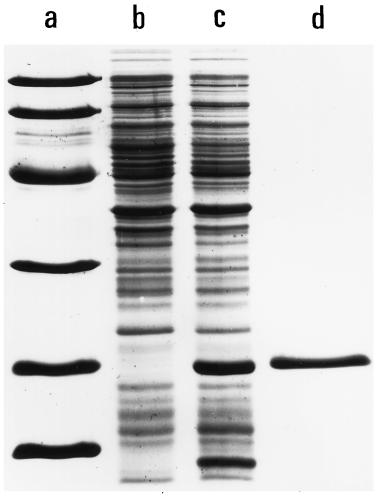

FIG. 5. Expression and purification of the MJ0541 ORF-encoded protein. SDS-PAGE (15% polyacrylamide gel) of BL21(DE3) cell extracts obtained by directly boiling the cells collected at 4 h after IPTG induction in SDS-PAGE loading buffer. Lane a contains 1 μg each of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lanes b and c contain the extracts of cells harboring pT7-7 and pT7-7MJ0541, respectively; lane d contains 2 μg of the purified MJ0541 ORF-encoded protein. Proteins were stained with Coomassie blue.

95% pure by SDS-PAGE (Fig. 5, lane d). The subunit molecular mass value of about 21.5 kDa resulting from SDS-PAGE agrees well with the expected molecular mass calculated from the predicted amino acid sequence of the recombinant protein. Gel filtration experiments showed a native molecular weight of about 72,000. To confirm the identity of *M. jannaschii* recombinant NMN adenylyltransferase, the pure protein was submitted to direct N-terminal sequencing. The sequence of the first 10 residues at the N terminus was Ala-Arg-Ile-Arg-Leu-Arg-Gly-Phe-Ile-Ile. While the first four residues were derived from the plasmid DNA translation (the N-terminal methionine was probably removed from the mature protein), the rest of the sequence was as predicted from the MJ0541 ORF sequence.

As in the case of the *S. solfataricus* enzyme, an apparent negative cooperativity with respect to NMN and ATP was also observed for the recombinant enzyme. Unlike the *S. solfataricus* NMN adenylyltransferase, the *M. jannaschii* enzyme displayed a higher specific activity and was not activated by sulfate ions. The temperature dependence of the recombinant enzyme activity showed an increase up to 90°C. The Arrhenius plot was biphasic, with a break point at about 67°C, suggesting a possible conformational change of the protein as reported for other thermophilic enzymes (8, 27). The activation energy levels calculated were 53 kJ/mol below 67°C and 3.9 kJ/mol above 67°C. Thermal stability studies revealed that the activity was fully retained for at least 6 h at 70°C, while at 80 and 90°C, the half-lives were 3 and 1 h, respectively.

To our knowledge, the *M. jannaschii* NMN adenylyltransferase gene is the first gene of this enzyme sequenced from any source. Computer-assisted similarity searches with the BLAST program (2) allowed us to identify the *Synechocystis* hypothetical protein srl0787 (17) as having the only sequence significantly similar to *M. jannaschii* NMN adenylyltransferase. Experiments are in progress in order to elucidate the significance of such a similarity.

ACKNOWLEDGMENTS

This work was partly supported by grant 95.02747.CT04 from the Consiglio Nazionale delle Ricerche (CNR) Italy.

We thank Nancy Denslow of the Interdisciplinary Center for Biotechnology Research, Protein Chemistry Core, at the University of Florida, Gainesville, Fla., for the NH_2-terminal sequence. We are grateful to Agata Gambacorta, Laboratorio di Molecole di Interesse Biologico, CNR, Naples, Italy) for kindly providing the *S. solfataricus* cells. We are also indebted to Mosè Rossi (Istituto di Biochimica delle Proteine ed Enzimologia, CNR, Naples, Italy) for valuable suggestions and continuous encouragement.

REFERENCES

1. **Allison, R. D., and D. L. Purich.** 1996. Practical considerations in the design of initial velocity enzyme rate assays, p. 37–56. *In* D. L. Purich (ed.), Contemporary enzyme kinetics and mechanism. Academic Press, New York, N.Y.
2. **Altschul, S. F., G. Warren, M. Webb, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Andrews, P.** 1965. The gel filtration behaviour of proteins related to their molecular weight over a wide range. Biochem. J. **96:**595–606.
4. **Balducci, E., G. Orsomando, V. Polzonetti, A. Vita, M. Emanuelli, N. Raffaelli, S. Ruggieri, G. Magni, and P. Natalini.** 1995. NMN adenylyltransferase from bull testis: purification and properties. Biochem. J. **310:**395–400.
5. **Collins, K. D., and W. Washabaugh.** 1985. The Hofmeister effect and the behaviour of water at interfaces. Q. Rev. Biophys. **18:**323–422.
6. **Dahmen, W., B. Webb, and J. Press.** 1967. The deamidodiphosphopyridine nucleotide and diphosphopyridine nucleotide pyrophosphorylases of *Escherichia coli* and yeast. Arch. Biochem. Biophys. **120:**440–450.
7. **Danson, M. J., D. W. Hough, and G. G. Lunt.** 1992. Archaebacteria: biochemistry and biotechnology. Biochem. Soc. Symp. **58:**47–48.
8. **D'Auria, S., F. La Cara, F. Nazzaro, N. Vespa, and M. Rossi.** 1996. A thermophilic alcohol dehydrogenase from *Bacillus acidocaldarius* not reactive towards ketones. J. Biochem. **120:**498–504.
9. **Dayhoff, M. O., R. M. Schwartz, and B. C. Ovcutt.** 1978. A model of evolutionary change in proteins, p. 345–352. *In* M. O. Dayhoff (ed.), Atlas of protein sequence and structure, vol. 5. National Biomedical Research Foundation. Silver Spring, Md.
10. **Denicola-Seoane, A., and B. M. Anderson.** 1990. Studies of NAD kinase and NMN:ATP adenylyltransferase in *Haemophilus influenzae*. J. Gen. Microbiol. **136:**425–430.
11. **Dubray, G., and G. Bezard.** 1982. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal. Biochem. **119:**325–329.
12. **Emanuelli, M., P. Natalini, N. Raffaelli, S. Ruggieri, A. Vita, and G. Magni.** 1992. NAD biosynthesis in human placenta: purification and characterization of homogeneous NMN adenylyltransferase. Arch. Biochem. Biophys. **298:**29–34.
13. **Foster, J. W., and A. G. Moat.** 1980. Nicotinamide adenine dinucleotide biosynthesis and pyridine nucleotide cycle metabolism in microbial systems. Microbiol. Rev. **44:**83–105.
14. **Greenbaum, A. L., and S. Pinder.** 1968. Changes in the activities of enzymes of the biosynthetic pathway of the nicotinamide nucleotides in rat mammary gland during the lactation cycle. Biochem. J. **107:**63–67.
15. **Hogeboom, G. H., and W. C. Schneider.** 1952. The synthesis of diphosphopyridine nucleotide by liver cell nuclei. J. Biol. Chem. **197:**611–620.
16. **Hughes, K. T., D. Ladika, J. R. Roth, and B. M. Olivera.** 1983. An indispensable gene for NAD biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **155:**213–221.
17. **Kaneko, T., A. Tanaka, S. Sato, H. Kotani, T. Sazuka, N. Miyajima, M. Sugiura, and S. Tabata.** 1995. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. I. Sequence features in the 1Mb region from map position 64% to 92% of the genome. DNA Res. **2:**153–166.
18. **Kristjansson M. M., and J. E. Kinsella.** 1990. Alkaline serine proteinase from *Thermomonospora fusca* YX. Biochem. J. **270:**51–55.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277:**680–685.
20. **Ma, K., and R. K. Thauer.** 1991. N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase (H_2-forming) from the extreme thermophile *Methanopyrus kandleri*. Arch. Microbiol. **156:**43–48.
21. **Ma, K., and R. K. Thauer.** 1991. Purification and properties of N^5,N^{10}-methylenetetrahydromethanopterin reductase (coenzyme F_{420}-dependent) from the extreme thermophile *Methanopyrus kandleri*. Arch. Microbiol. **155:** 593–600.
22. **Moore, S.** 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. **238:**235–237.
23. **Morton, R. K.** 1958. Enzymic synthesis of coenzyme I in relation to chemical control of cell growth. Nature **181:**540–543.
24. **Natalini, P., S. Ruggieri, N. Raffaelli, and G. Magni.** 1986. Nicotinamide

mononucleotide adenylyltransferase. Molecular and enzymatic properties of the homogeneous enzyme from baker's yeast. Biochemistry **25:**3725–3729.
25. **Oakley, B. R., D. R. Kirch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105:**361–363.
26. **Ohtsu, E., and Y. Nishizuta.** 1971. Nicotinamide phosphoribosyltransferase and NAD pyrophosphorylase from *Lactobacillus fructosus*. Methods Enzymol. **188:**127–132.
27. **Pisani, F. M., R. Rella, C. Rozzo, C. A. Raia, R. Nucci, A. Gambacorta, M. De Rosa, and M. Rossi.** 1990. Thermostable β-galactosidase from the archaebacterium *Sulfolobus solfataricus*. Purification and properties. Eur. J. Biochem. **187:**321–328.
28. **Raffaelli, N., A. Amici, M. Emanuelli, S. Ruggieri, and G. Magni.** 1994. Pyridine nucleotide biosynthesis in archaebacteria: presence of NMN adenylyltransferase in *Sulfolobus solfataricus*. FEBS Lett. **355:**233–236.
29. **Ruggieri, S., L. Gregori, P. Natalini, A. Vita, M. Emanuelli, N. Raffaelli, and G. Magni.** 1990. Evidence for an inhibitory effect exerted by yeast NMN adenylyltransferase on poly(ADP-ribose)polymerase activity. Biochemistry **29:**2501–2506.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Schagger, H., and G. Von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
32. **Solao, P. B., and S. Shall.** 1971. Control of DNA replication in *Physarum polycephalum*. Exp. Cell Res. **69:**295–300.
33. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
34. **Uhr, M. L., and M. Smulson.** 1982. NMN adenylyltransferase: its association with chromatin and with poly(ADP-ribose)polymerase. Eur. J. Biochem. **128:**435–443.
35. **Villa, A., L. Zecca, P. Fusi, S. Colombo, G. Tedeschi, and P. Tortora.** 1993. Structural features responsible for kinetic thermal stability of a carboxypeptidase from the archaebacterium *Sulfolobus solfataricus*. Biochem. J. **295:** 827–831.