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The *Escherichia coli* NadR Regulator Is Endowed with Nicotinamide Mononucleotide Adenylyltransferase Activity

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The first identification and characterization of a catalytic activity associated with NadR protein is reported. A computer-aided search for sequence similarity revealed the presence in NadR of a 29-residue region highly conserved among known nicotinamide mononucleotide adenylyltransferases. The *Escherichia coli nadR* gene was cloned into a T7-based vector and overexpressed. In addition to functionally specific DNA binding properties, the homogeneous recombinant protein catalyzes NAD synthesis from nicotinamide mononucleotide and ATP.

In the *Escherichia coli* and *Salmonella typhimurium* NAD biosynthetic pathways, the genes involved in both the de novo synthesis (*nadA* and *nadB*) and salvage routes (*pncB*) are under negative transcriptional control by the product of the *nadR* locus, also referred to as *nadI* (6, 15, 19). It has been demonstrated that the *nadR* gene product is a bifunctional protein that can both act as a repressor and control the transport of exogenous nicotinamide mononucleotide (NMN), the immediate NAD precursor, across the cytoplasmic membrane (5, 17). The latter function would be exerted through a regulatory modulation of the activity of the integral membrane protein PnuC, which is responsible for NMN transport (5, 18). While the repression function resides in the NadR N-terminal domain, the C-terminal domain is involved in the transport function (4). Both functions appear to exert their control in response to intracellular NAD (or NADP) levels (5, 18). In this regard, it has been proposed that NadR behaves as an allosteric protein: in the presence of high NAD levels it might assume a conformation which allows both repression of NAD biosynthetic genes transcription and inhibition of NMN transport system; conversely, when NAD levels are low, NadR might associate with the membrane, allowing full expression of biosynthetic genes and stimulating NMN uptake (5, 18).

Several eubacterial transcriptional factors are known to possess enzymatic activity (9, 16). However, in a recent study aimed at elucidation of the DNA binding properties of NadR, the possibility of the existence of a catalytic activity associated with the protein was ruled out (10). In the present note, we show that NadR is endowed with NMN adenylyltransferase activity catalyzing the transfer of the adenylyl moiety from ATP to NMN, thus forming NAD. This finding emphasizes the pivotal role of NadR, which may be able to ensure an efficient NAD production when the pyridine nucleotides pool is depleted, by both derepressing transcription of the NAD biosynthetic genes and directly converting the imported NMN to NAD. It may represent a sophisticated mechanism to quickly respond to NAD depletion, at the same time preventing the excessive increase of the intracellular NMN pool, which would be harmful for the cell (8).

Cloning and expression of *nadR* gene. When the archaeal *Methanococcus jannaschii* NMN adenylyltransferase sequence (12) was used as a probe in a BLAST search for homologous sequences, a region spanning 29 residues at the N terminus of the protein was found to significantly align with an internal region of both *S. typhimurium* and *E. coli* NadR (Fig. 1). Due to the strict conservation of most of the residues present in this region both in the known archaeal NMN adenylyltransferases and in the slr0787 NMN adenylyltransferase from *Synechocystis* sp. (11), we expected this region to be involved in catalysis (Fig. 1). Therefore, its presence in NadR prompted us to clone and express the protein to investigate its possible NMN adenylyltransferase activity.

The *nadR* gene was amplified from *E. coli* MG1655 chromosomal DNA by PCR using oligonucleotide primers d(CTA GAATTCGCATGAGATATACGGAGGGAGAT) and d(C GGCTGCAGTTATCTCTGCTCCCCCATCAT), designated to incorporate an *Eco*RI site at the start of the gene and a *Pst*I site at its end. The 1,274-bp product was digested with *Eco*RI and *Pst*I, purified, and ligated into *Eco*RI-*Pst*I-digested pT7-7 plasmid (14) under control of a T7 promoter. The resulting plasmid, pT7-7-*nadR*, was transformed into *E. coli* BL21(DE3) cells for protein expression. Even without isopropyl-1-thio-β-galactopyranoside induction, a high level of protein expression was achieved following aerobic growth at 37°C in Luria-Bertani medium (supplemented with ampicillin [100 μg/ml]), as revealed by the appearance of a major band of the expected size of 45 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell extracts (Fig. 2, lane c). The same extracts exhibited high levels of NMN adenylyltransferase activity (0.002 U/mg) [1 U is defined as the amount of NadR catalyzing the synthesis of 1 μmol of NAD per min at 37°C]) compared with control extracts prepared from *E. coli* cells transformed with the nonrecombinant plasmid (less than 4×10^{-5} U/mg). The enzymatic activity was assayed by incubating extract samples in 50 mM HEPES (pH 8.6)–13 mM $MgCl_2$–1 mM ATP–1 mM NMN. After 20 min of incubation at 37°C, NAD formed was measured as described previously (11).

Purification of the recombinant NadR protein. Purification of the recombinant protein was monitored by assaying the NMN adenylyltransferase activity. The cell extract was pre-

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ENadR   61 FLGLEFPRQKKTIGVVFGKFYPLHTGHIYLIQRACSQVDELHIIMGFDDTRDRALFED 118
SNadR   53 FLGLEFPRRQKNIGVVFGKFYPLHTGHIYLIQRACSQVDELHIIMGYDDTRDRGLFED 110
MjAT     1 -----------LRGFIIGRFQPFHKGHLEVIKKIAEEVDEIIIGIGSAQKS------- 40
MtAT     1 --------VMTMRGLLVGRMQPFHRGHLQVIKSILEEVDELIICIGSAQLS------- 43
AfAT     1 -----------MRAFFVGRFQPYHLGHHEVVKNVLQKVDELIIGIGSAQES------- 40
PhAT     1 ----------MIRGLFVGRFQPVHKGHIKALEFVFSQVDEVIIGIGSAQAS------- 41
SynAT    1 ------MQTKYQYGIYIGRFQPFHLGHLRTLNLALEKAEQVIIILGSHRVA------- 45
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FIG. 1. Sequence alignment of homologous regions of NadR proteins from *E. coli* (*E*NadR) and *S. typhimurium* (*S*NadR), NMN adenylyltransferase from *M. jannaschii* (*Mj*AT), putative NMN adenylyltransferases from *Methanobacterium thermoautotrophicum* (*Mt*AT), *Archaeoglobus fulgidus* (*Af*AT), and *Pyrococcus horikoshii* (*Ph*AT), and NMN adenylyltransferase from *Synechocystis* sp. (*Syn*AT). Identical and similar residues are in boldface. The alignment was generated by using the CLUSTAL W program.

pared from 1 liter of saturated culture of *E. coli* cells harboring the pT7-7-*nadR* construct as previously reported (11). Crude extract (12 ml) was diluted fourfold with buffer A (11) and mixed with 50 ml of DNA-agarose suspension prepared as described previously (13), equilibrated with buffer A. After stirring overnight at 4°C, the resin was washed with buffer A plus 0.1 M NaCl, and NadR was eluted by incubating the resin with 100 ml of buffer A plus 1 M NaCl for 20 min at 4°C. The DNA-agarose fraction (100 ml) was loaded onto a 1.3- by 3.5-cm hydroxyapatite (Bio-Rad) column equilibrated with 10 mM buffer B (potassium phosphate, 1 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 5% glycerol, [pH 7.0]). After a wash with 150 mM buffer B, a linear 150 to 250 mM buffer B gradient (25 ml plus 25 ml) was applied. Fractions containing NadR were pooled and concentrated by ultrafiltration. Throughout the overall purification procedure, NMN adenylyltransferase activity copurified with NadR. The homogeneous final preparation (Fig. 2, lane a) showed a specific activity of 0.05 U/mg. N-terminal sequencing of the pure protein confirmed that it was the product of the *nadR* gene. Gel filtration experiments performed on a Superose 12HR 10/30 (Pharmacia) column, equilibrated with sodium phosphate buffer (pH 7.4)–1 mM $MgCl_2$–0.5 mM EDTA–1 mM DTT–0.5 M NaCl, both in the presence and in the absence of 1% dimethyl sulfoxide, gave a native molecular mass of about 180 kDa, suggesting a tetrameric form of the protein.

DNA binding activity of the recombinant NadR protein. DNA fragments of 200 bp containing the predicted promoter regions of *nadA* and *nadB* genes were used as probes in gel mobility shift assays to verify the DNA binding activity of recombinant NadR. These probes were produced by PCR amplification from *E. coli* MG1655 genomic DNA by using primers designated to amplify regions corresponding to nucleotides −180 to +19 and −199 to −1 with respect to the predicted transcription start sites of *nadA* and *nadB*, respectively. Different concentrations of the pure protein were mixed either with the probe or with the probe in the presence of PCR markers (Promega) as competitor DNA. After 30 min at 25°C in 50 mM Tris-HCl (pH 7.5)–1 mM EDTA–2 mM $MgCl_2$–5% glycerol–2 mM DTT, samples were loaded on a 5% native polyacrylamide gel (30:0.8 [wt/wt] acrylamide to *N*,*N*′-methylenebisacrylamide). Electrophoresis was carried out in 89 mM Tris–89 mM borate–2.5 mM EDTA, at constant voltage (8 V/cm), at 4°C. Gels were stained with ethidium bromide. As shown in Fig. 3, the mobility of the specific 200-bp fragment containing the *nadB* operator region was shifted upon addition of NadR. The band shift was not affected by the presence of competitor DNA (unpublished results). Identical results were obtained with the *nadA* control region (not shown), suggesting that the recombinant NadR retained its ability to specifically bind to NAD biosynthetic genes operator regions.

NMN adenylyltransferase activity of the recombinant NadR protein. Unlike the NMN adenylyltransferases so far characterized (1, 3, 7, 11, 12), which show a broad pH optimum ranging from 6.0 to 9.0, the activity associated with NadR exhibits an alkaline pH optimum. In HEPES buffer, the enzyme was maximally active at pH 8.6, the activity at pH 7.0 being only about 5% of that measured at pH 8.6. NadR requires divalent metal cations for activity. All ion species tested,

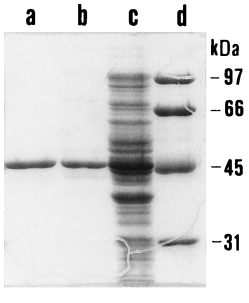

FIG. 2. Purification of recombinant NadR protein. A Coomassie blue-stained polyacrylamide gel (10%) shows the fractions from the purification: (3 μg of hydroxyapatite fraction [lane a], 5 μg of DNA agarose fraction [lane b], and 28 μg of crude extract [lane c]), and positions of molecular weight standards (2 μg; lane d).

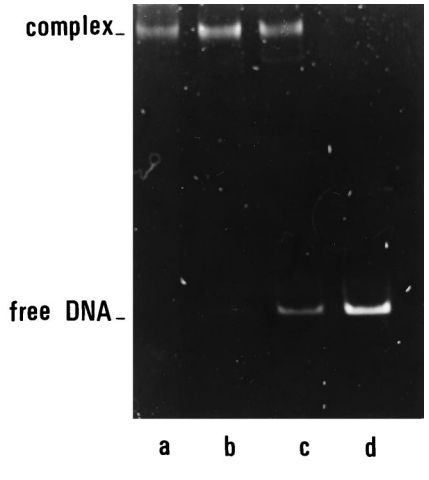

FIG. 3. Gel retardation of the *nadB* operator region by NadR protein. An ethidium bromide-stained polyacrylamide gel shows the migration of a 200-bp DNA fragment containing the *nadB* control region (60 nM) after incubation with increasing amounts of pure NadR. Concentrations of NadR were 211 nM (lane a), 114 nM (lane b), 55 nM (lane c), and 0 nM (lane d).

including Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}, stimulated the activity. Routine enzymatic assays were carried out in the presence of Mg^{2+}, which is the most effective for all known NMN adenylyltransferases. However, Ni^{2+} and Co^{2+} at 1 mM increased the reaction rate about fourfold compared to Mg^{2+} at the optimal concentration. NadR was highly specific for the amidated form of NMN, NAD synthesis occurring at a rate 170 times faster than nicotinic acid adenine dinucleotide synthesis. Such a strict specificity clearly differentiates the NMN adenylyltransferase activity associated with *E. coli* NadR from the *E. coli* NMN adenylyltransferase described by Dahmen et al. (2), which exhibits a remarkable preference for the NMN. Another distinctive feature of NadR activity is represented by the significantly different K_m values of 0.7 mM and 1.7 μM for NMN and ATP, respectively. The K_m for NMN is much higher than K_ms for other known NMN adenylyltransferases (1, 3, 7, 11) and may account for the conversion of NMN to NAD by NadR under conditions of NAD depletion, which stimulate NMN uptake.

Conclusions. Evidence for the existence of an NMN adenylyltransferase activity associated with NadR protein has been presented. Although the physiological role of this enzymatic function has not been fully elucidated, this finding represents a novel feature of NAD biosynthesis regulation system in eubacteria and may shed light on the understanding of the mechanisms underlying NMN uptake. In addition, the present work provides the basis for the identification of a novel putative consensus sequence representing an NMN adenylyltransferase catalytic motif.

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