Cloning and Nucleic Acid Sequence of the *Salmonella typhimurium* pncB Gene and Structure of Nicotinate Phosphoribosyltransferase

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The pncB gene of *Salmonella typhimurium*, encoding nicotinate phosphoribosyltransferase (NAPRTase), was cloned on a 4.7-kb Sau3A fragment. The gene contains a 1,200-bp open reading frame coding for a 400-residue protein. Amino acid sequencing of the amino-terminal and two interior peptides of the purified protein confirmed the deduced sequence and revealed that the amino-terminal methionine residue was removed, giving a 399-residue mature protein of M_r 45,512. No signal sequence was observed in the predicted NAPRTase primary structure, suggesting that the enzyme is not periplasmic. The protein does not demonstrate clear sequence similarity to the other seven phosphoribosyltransferases of known primary structure and frustrates attempts to define a conserved 5-phosphoribosyl-1-pyrophosphate-binding region. The NAPRTase reaction is ATP-stimulated, and the protein contains a carboxy-terminal sequence diagnostic of an ATP-binding site. An inverted repeat of the sequence TAAACAAA observed in the proposed promoter region of pncB is also present in the promoter of *nadA*, which, like pncB, is also regulated by the NadR (NadI) repressor. The sequence may thus define an NadR repressor-binding site.

The pncB gene of *Escherichia coli* and *Salmonella typhimurium* encodes the enzyme nicotinate phosphoribosyltransferase (NAPRTase; EC 2.4.2.11), which catalyzes the formation of nicotinate mononucleotide (NAMN) (18, 31). The reaction thus allows use of external nicotinate and serves to salvage nicotinate formed in NAD breakdown (31). Although the metabolic origin of this cycling remains obscure, in enteric bacteria the NAD pool turns over with a half-time of about 90 min under aerobic conditions (27). The pncB locus maps at 25 min on the *S. typhimurium* chromosome (7, 9) and is under the control of a repressor, the product of the *nadR* gene (8); also called *nadI* (32), which also serves to regulate the expression of the *nadA* and *nadB* genes.

NAPRTase has been isolated from many sources, including yeast cells, *Bacillus* species, mammalian erythrocytes, and liver (24). The protein from yeast cells is a monomer of M_r 43,000 and has been well characterized (13, 21).

NAPRTase provides a unique paradigm for energy coupling in enzyme mechanisms. Like the other nine phosphoribosyltransferases (PRTases; see reference 24 for a review), NAPRTase catalyzes the formation of a mononucleotide and PP from a nitrogenous base and 5-phosphoribosyl-1-pyrophosphate (PRPP). Unlike the other PRTases, however, NAPRTase from many sources catalyzes an ATP phosphohydrolase reaction stoichiometrically coupled to the NAMN synthetic reaction (15, 20). The ATP hydrolysis reaction is not compulsory for the NAMN synthesis activity in all cases however, being unobserved for a protozoal NAPRTase (19), stimulatory but not required for the mammalian enzyme (25, 30), and apparently required for the well-studied yeast enzyme (20). Our own studies on the *S. typhimurium* NAPRTase have shown that it catalyzes a slow NAMN synthesis reaction in the absence of ATP. When ATP is present, it is hydrolyzed, and the ATP phosphohydrolase reaction increases the equilibrium constant for NAMN synthesis by about 4,000-fold (unpublished data).

In the current work, we have cloned a 4.7-kb Sau3A fragment containing the *S. typhimurium* pncB gene and sequenced a 1.84-kb *Sal-SphI* region containing the gene. The sequence reveals an open reading frame of 1,200 bp that produces a 399-residue mature protein. The deduced amino acid sequence was confirmed at several locations by protein sequencing. The primary structure of NAPRTase is not similar to those of other PRTases, but the enzyme does contain a consensus ATP-binding site at its carboxyl terminal.

MATERIALS AND METHODS

Materials. Bacterial strains used in this work are listed in Table 1. ['^14^C]nicotinic acid was from New England Nuclear. GeneClean was obtained from Bio101, La Jolla, Calif. High-pressure liquid chromatography and sequencer solvents were Fisher Optima grade; trifluoroacetic acid and phenylisothiocyanate were from Pierce. Bacterial growth media were obtained from Difco, restriction enzymes and DNA sequencing supplies were from U.S. Biochemicals, and all other biochemicals were products of Sigma.

Protein sequencing. For amino-terminal sequencing, 0.7 mg of purified NAPRTase was passed through a Sephadex G-50 centrifuge column (29) equilibrated with 50 mM NaCl–20 mM sodium phosphate, pH 8.0. The eluate was applied to the sample disk of a Porton Sequencer and subjected to automated Edman degradation. For preparation of tryptic peptides, the protein was applied to a column of Sephadex G-50 (10 by 0.5 cm) equilibrated with 10 mM NH_4HCO_3–0.1% sodium dodecyl sulfate. The pooled protein was lyophilized, suspended in water, and precipitated with 9 volumes of acetone–1 mM HCl at −20°C for 3 h. The protein pellet was washed with ice-cold acetone and suspended in 8 M urea–0.4 M NH_4HCO_3–4 mM diethiothreitol. After 15 min at 50°C, iodoacetamide was added to 10 mM Water (3 volumes) was added, and the protein was digested with 1:15 (wt/wt) trypsin (treated with tolylsulfonyl phe-
nylanyl chloromethyl ketone [ Worthington ]) at 37°C overnight. Samples of the digest were resolved by reverse-phase liquid chromatography using a Waters 600 high-pressure liquid chromatograph equipped with a Vydac 218TP54 C18 column. A linear gradient of 0 to 80% B was used, where solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. Elution was monitored at λ248 with a Waters 481 spectrophotometer. Tryptic peptides were dried under vacuum, dissolved in 30% acetonitrile, and sequenced on the Porton sequencer with Porton peptide samples supports.

DNA sequencing. The protocols of Ausubel et al. (3) were used for preparing single-stranded DNA from M13 clones. DNA was sequenced with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio) by following the protocols for manual sequencing suggested by the manufacturer. Enough distinct clones were employed (Fig. 1) that each strand of DNA was sequenced at least once over the entire sequence presented. In several cases sequence discrepancies between the two strands occurred and were readily resolved by sequencing with 7-deaza-dGTP or dTTP replacing dGTP.

Nucleotide sequence accession number. The GenBank accession number for the sequence shown in Fig. 2 is M55986.

RESULTS

Cloning of pncB. The pncB gene was cloned by using a S. typhimurium genomic library prepared in lambda 1059 that was donated by R. Maurer. The library was screened using JF1483 (a nadA pncB mutant), an “ Eschinella” (lambda-sensitive S. typhimurium) (14) derivative which is blocked in the de novo NAD biosynthetic pathway as well as being a pncB deletion. This strain was constructed for this work by John Foster, University of South Alabama. Screening was carried out on plates of M9 supplemented with 0.5 μg of nicotinic acid per ml. From one of these colonies, plasmid pC18, was isolated from one of these clones and used to transform JF1483 again to Amp” pncB*.

A map of the 4.7-kb Sau3A-Sau3A insert in pC18 (Fig. 1) shows 6-base restriction sites that were located or confirmed by digestions with the appropriate enzymes. A pUC19 subclone of the restriction fragment proceeding from SalI to the right-hand Sau3A site, pSC186, also complemented JF1483 and therefore also contains the pncB gene. pSC186 was used to develop a purification process for the NAPRTase protein, employing polyethyleneimine and (NH₄)₂SO₄ precipitations, anion-exchange chromatography, and final purification on a TSK-Cibacron Blue dye affinity column with salt elution (details of this procedure will be presented elsewhere). The homogeneous protein thus produced showed an apparent Mr of 45,000 on a sodium dodecyl sulfate-polyacrylamide gel and catalyzed the NAPRTase reaction with a velocity of 3.6 U/mg, a value in good agreement with values for the yeast enzyme (13, 20).

DNA sequencing of pncB and deduced amino acid sequence of NAPRTase. The DNA sequencing strategy employed is shown in Fig. 1. The nucleotide sequence of the SalI-Sphi fragment revealed an open reading frame of 1,200 bp beginning at nucleotide 238 (Fig. 2). The coding sequence is preceded by a poor ribosome-binding site, AACAGGG, with an alternative ribosome-binding site, GAGGA, at nucleotides 217 to 221. Upstream 72 bp from the ATG is a potential promoter with TTTAATA (TGACAT) at -35 and TATACT (TATAAT) at -10 (consensus sequences from reference 23) separated by 15 nucleotides. At 14 nucleotides past the UAA terminator codon, nucleotides 1452 to 1478 can form a stem-loop with 11 nucleotides in each half of the stem (including two GU base pairs) and a 5-residue loop. This structure, which at its 3’ end has the T-rich sequence TTGTGTCGAT, may act as a [rho]-independent transcriptional terminator (5).

The deduced primary structure of NAPRTase predicts a peptide of 400 amino acids. If the amino-terminal methionine is removed, the calculated molecular weight of the 399-residue protein is 45,512. The molecular weight is in good agreement with the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the purified protein. We have also sequenced the amino terminus of the purified NAPRTase and portions of two tryptic peptides (underlined in Fig. 2) and observed excellent agreement with the sequence deduced from the DNA. The amino-terminal sequence analysis revealed that the terminal methionine is removed in the wild-type protein. Further analysis of the sequence is presented below in Discussion.
Previous work (4) led to the conclusion that the NAPRTase protein of E. coli was localized in the periplasmic space. Such proteins are synthesized as preproteins with a consensus signal sequence that is removed during processing. It is thus possible that our purified protein has not been processed and contains the signal peptide or that it is the processed product of a longer protein. A search for an amino-terminal signal sequence within the protein as iso-
NAPRTase 368  L S D S P G R T I C H D K A F V R 384
adenylate kinase 15  G G P G S G R G T O C E K I V H K 31
recA  66  G P E S S G K T T L T L Q V I A  81

FIG. 3. Sequence comparisons among ATP-binding regions. Sequence sources were reference 12 for adenylate kinase and reference 2 for RecA.

lated, using the criteria of an amino-terminal positive charge followed by 14 to 20 neutral, primarily hydrophobic residues, as presented by Oliver (26), was negative. Alternative upstream initiation codons at bases 106, 131, and 134 are not in frame and are closely followed by translational terminator codons. An in-frame GTG initiator at nucleotide 214 would produce the additional amino-terminal sequence Met-Arg-Gly-Pro-Asn-Ser-Ala-Pro-Met. This sequence has one positive charge followed by seven neutral residues and is not a compelling candidate for a signal sequence.

Expression of the pncB gene, like that of nadA, is under the control of the repressor product of the nadR gene (10). Comparison of the sequence of the putative pncB promoter region with that of nadA, published recently (11), reveals that the sequence TAAACAA and its inverted repeat TTGTTTA are present in both genes, in each case with one occurrence between the −10 and −35 regions of the promoter.

DISCUSSION

The sequence of the S. typhimurium pncB gene presented here allowed the first NAPRTase sequence to be deduced. The 10 known PRTases employ purine, pyrimidine, and pyridine bases, except for anthranilate PRTase and glutamine PRPP amidotransferase, which employs ammonia produced in a tightly coupled glutaminase reaction. None of the pyridine-linked enzymes (nicotinate, nicotinamide, and quinolinate PRTases) has previously been sequenced.

The NAPRTase sequence has been examined for similarity to nucleotide-binding proteins. For many of the NAD-binding proteins, a consensus motif of sequence Gly-X-Gly-X-X-Gly has been observed and is involved in a tight turn between a beta strand and an alpha helix forming part of the nucleotide-binding structure (6). No good fit to this sequence is observed in the primary structure of NAPRTase. For some ATP-utilizing enzymes, a consensus motif (the “flexible loop” [2, 12]) that contains the sequence GKT preceded by several small residues was found. Examples from adenylate kinase and RecA are shown in Fig. 3. This theme was also found in NAPRTase, extending from residues 368 to 384. In studies of the ATP-binding site of adenylate kinase, Fry et al. (12) showed that the flexible loop (residues 15 to 23) is near the triphosphate portion of the ATP-binding site. In NAPRTase, replacements in this region mainly substitute other small polar residues (Ser and Asp) for Gly and Pro residues. The GKT which is present in most of the ATP-binding proteins is completely conserved. Immediately following this structure is the polar sequence Cys-Glu-Lys in adenylate kinase, which is replaced by His-Asp-Lys in NAPRTase. In adenylate kinase this lysine makes an important interaction with the gamma phosphate group of ATP (12). The NAPRTase lacks a second portion of the nucleotide-binding site, about 100 residues downstream in adenylate kinase, that is involved in base binding. We have noted that S. typhimurium NAPRTase, like its homolog from Bacillus subtilis (17), is extremely nonspecific for nucleoside triphosphate and will perform its reaction well with tripolyphosphate replacing ATP. Further mechanistic and structural work on NAPRTase may reveal the relationship of nucleotide-binding sites in this protein with those identified elsewhere. A naive hypothesis for NAPRTase structure is that the ATP-binding site has been added to an otherwise competent ATP-independent NAPRTase functional unit and now affects the behavior of the latter. This naive view would be in good accord with our observation that the enzyme can function in either an ATP-coupled or an independent fashion.

NAPRTase was compared with other proteins in several databases by using the program FASTA (28). No convincing similarities were found. In addition, when NAPRTase was directly compared with orotate PRTase by using multiple sequence alignment (22), no convincing similarity was observed. When primary structures of PRTases are compared, in general they reveal little apparent similarity (1). However, Hove-Jensen et al. (16) have identified a region of weak sequence similarity which may define a PRPP-binding site. This consensus contains three major elements: a central pair of carboxylic acid residues flanked by hydrophobic residues, an amino terminal glycine, and the carboxy-terminal sequence Thr-Gly-Gly-Thr. When the primary structure of S. typhimurium NAPRTase was compared with this consensus PRPP-binding sequence, no strong similarity was observed. Although the primary structure does not reveal significant similarity, the higher-level structure may reveal a common folding pattern constituting a PRPP-binding site. The sequences of the two other pyridine-based PRTases, quinolinate PRTase and nicotinamide PRTase, remain to be determined and may assist in structure comparisons.

It has been shown that both pncB and nadA are under the control of NAD levels and that this control is mediated through the action of the product of the nadR gene (8, 32). The putative promoter regions of pncB and nadA (11) share the sequence TAAACAA and its inverted repeat TTGTTTA. In both cases they occur near the −10 region of the proposed promoter. The order of the element and its repeat is reversed in the two cases, and in nadA, one of the sequences contains an additional A residue, TAAAACAA. The center-to-center distances of these two elements are 12 residues in nadA and 32 residues in pncB. This separation suggests that the NadR repressor-effector complex may bind to its recognition element and dimerize, causing a loop or kink in the DNA and also preventing the action of RNA polymerase. The NadR protein would respond to lower levels of its as-yet-unknown effector by depolymerizing and/or dissociating from the DNA, allowing transcription to proceed.

Previous work which compared yields of NAPRTase activity following osmotic shock, spheroplast production, and other cell disintegration methods suggested that the NAPRTase of E. coli was localized to the periplasmic space (4). The metabolic sense of this localization is not clear, since ATP, PRPP, and nicotinate arising from NAD breakdown would normally be found in the cytoplasm, although periplasmic nicotinic acid might also arise from the breakdown of external NAD or metabolites. Our failure to find a signal sequence in the pncB gene appears to rule out a periplasmic location for the enzyme.

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ADDITIONAL IN PROOF

The sequence of the E. coli pncB gene was recently reported (M. G. Wubbolts, P. Terpstra, J. B. van Beilen, J. Kingma, H. A. R. Meesters, and B. Witholt, J. Biol. Chem. 265:17665-17672, 1990); it is highly similar to the S. typhimurium sequence reported here.

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