Cloning and Characterization of the ddc Homolog Encoding 1,2,4-Diaminobutyrate Decarboxylase in Enterobacter aerogenes

Shigeo YAMAMOTO, Nobuo MUTOH, Daisuke TSUZUKI, Hisato IKAI, Hiroshi NAKAO.
Sumio SHINODA, Shizuo NARIMATSU, and Shin-ichi MIYOSHI

Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan and
Department of Infectious Diseases Research, National Children’s Medical Research Centre, 3-33-31 Taishido, Tokyo, Japan.
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1,2,4-Diaminobutyrate decarboxylase (DABA DC) catalyzes the formation of 1,3-diaminopropane (DAP) from DABA. In the present study, the ddc gene encoding DABA DC from Enterobacter aerogenes ATCC 13048 was cloned and characterized. Determination of the nucleotide sequence revealed an open reading frame of 1470 bp encoding a 5365-Da protein of 490 amino acids, whose deduced NH2-terminal sequence was identical to that of purified DABA DC from E. aerogenes. The deduced amino acid sequence was highly similar to those of Actinetobacter baumannii and Haemophilus influenzae DABA DCs encoded by the ddc genes. The lysine-307 of the E. aerogenes DABA DC was identified as the pyridoxal 5’-phosphate binding residue by site-directed mutagenesis. Furthermore, PCR analysis revealed the distribution of E. aerogenes ddc homologs in some other species of Enterobacteriaceae. Such a relatively wide occurrence of the ddc homologs implies biological significance of DABA DC and its product DAP.

Key words: diaminobutyrate decarboxylase; E. aerogenes; gene cloning; Enterobacteriaceae

The only known biosynthetic pathway for 1,3-diaminopropane (DAP) in bacteria was the oxidation of spermidine by a spermidine dehydrogenase, until we demonstrated that a novel enzyme 1,2,4-diaminobutyrate decarboxylase (DABA DC) exists in Vibrio species to yield DAP, which in turn is utilized to form a higher polyamine norspermidine ubiquitously present in this genus. Later, the DABA DC enzymes were also purified from Actinetobacter baumannii and Serratia marcescens, all of which were, however, clearly distinct from V. alginolyticus DABA DC in some properties including molecular subunit composition, and effect of divalent metal cations. On the other hand, amino acid decarboxylases form a large group among the pyridoxal 5’-phosphate (PLP)-dependent enzymes, and, on the basis of the amino acid sequence similarity, they are subdivided into four different groups that seem to be evolutionarily unrelated to each other. In this respect, it is of great interest to elucidate and compare the structures and organizations of the genes encoding these enzymes. Recently, we cloned and characterized the genes termed ddc and dat involved in the DAP production pathway from A. baumannii. The ddc gene encodes 1-DABA: 2-ketoglutarate 4-amino transferase that catalyzes the formation of 1-DABA, a substrate of DABA DC, from 2-ketoglutaric acid and L-aspartic β-semialdehyde. In addition, a gene cluster with strong similarity to ddc and dat was found in Haemophilus influenzae, which was proved to be responsible for DAP production.

In this paper, we describe the cloning and characterization of the ddc homolog encoding E. aerogenes DABA DC and the identification of its active-site lysine residue by site-directed mutagenesis. We also report the distribution of the E. aerogenes ddc homologs in other enterobacterial species.

MATERIALS AND METHODS

Bacterial Strains and Plasmids All type strains of enteric bacteria were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Yersinia enterocolitica TB-1 and Y. pseudotuberculosis TB-2 were kindly supplied by K. Okamoto, Tokushima Bunri University. They were all grown in Luria-Bertani (LB) broth at 37 °C for 12 h. Transformation of Escherichia coli HB101 with plasmid DNA by electroporation was performed under standard conditions by using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.). DNA inserts of the recombinant plasmids were contained either in pUC18 or in pUC19. E. coli strains carrying a plasmid were grown at 37 °C in LB broth or LB agar containing 100 µg of ampicillin per ml, and the plasmid was isolated by the alkaline extraction method.

Manipulation of DNA Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and used according to the manufacturer’s protocols. Isolation of genomic DNA for PCR was performed with InstaGene matrix (Bio-Rad). Other general procedures were performed as described by Sambrook et al.

Cloning and Sequencing of the Gene Encoding DABA DC from E. aerogenes Genomic DNA from E. aerogenes ATCC 13048 was partially digested with Sau3AI, and the fragments (4—6 kb) collected from an agarose gel were ligated into BamHI-digested pUC19 previously treated with calf intestine alkaline phosphatase. After transformation of E. coli HB101 competent cells with the ligation mixture, the ampicillin-resistant transformants were immobilized on a nitrocellulose membrane and immuno-screened with rabbit antiserum against purified E. aerogenes DABA DC followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (Boehringer GmbH, Mannheim, Germany) according to the procedure of Helfman et al.

The plasmid recovered from a positive clone was called pEDD30, contained a 4.4-kbp insert, and was used for further subcloning.

Nucleotide sequence of the insert of a subclone pEDD30.1 was determined in both directions by primer walking, where a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit with 7-deaza-dGTP (Amersham Pharmacia
Biotech, Buckinghamshire, England) and several 5'-Cy5-labeled primers were used. Nucleotide and deduced amino acid sequences were analyzed by GENETYX-MAC software (Software Development Co., Tokyo, Japan). Alignment of the DABA DC proteins was performed with the same software.

Site-Directed Mutagenesis To identify the PLP-binding K residue in the E. aerogenes DABA DC, two mutant proteins K198R and K307R were created by pEED30.1 with a Quiik Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications. Synthetic oligonucleotides (underlining indicates the mutated nucleotides) that served successfully as the mutagenesis primers were: 5'-CCACTTCTCGGTGACAGGGAA-CATGGCGCTGTGG-3' and its opposite strand counterpart for K198R; 5'-CAGTTACCTGGACTTCCATAG-GGAGGTCTTCCAGACCATAG-3' and its opposite strand counterpart for K307R. Some of the clones obtained in mutagenesis were subjected to DNA sequencing of the entire coding frame to confirm that the desired but not additional mutation was present.

PCR of Genomic DNA Genomic DNA samples from a number of species of Enterobacteriaceae were amplified with a set of primers prepared according to the known sequence of the E. aerogenes ddc gene. The following primers were designed (see Fig. 3): 5'-AGTTTGCTGGCG-CACCCTGTGCCC-3' (position 405 to 429) (primer 1) and 5'-GCAAGGCTGCTTCCCTGCTACGT-3' (position 1382 to 1406) (primer 2). The reactions were performed for 30 cycles with a Takara PCR Thermal Cycler (Takara Shuzo) with a 1-min denaturation at 95°C, a 1-min annealing at 60°C, and a 1-min extension at 72°C. The PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide.

Other Methods The DABA DC activity was assayed with dialyzed cell extracts as previously described. Protein concentrations were determined by the method of Lowry et al.

Nucleotide Sequence Accession Number The nucleotide sequence reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB032468.

RESULTS AND DISCUSSION

Cloning of the ddc Gene Of several thousand ampicillin-resistant colonies tested, one was found to strongly react with the antiserum against E. aerogenes DABA DC. Western blot analysis of the cell extract prepared from this transformant revealed a band of 51 kDa reacted with the antibody DABA DC antiserum (data not shown). A corresponding band was found in a crude extract of E. aerogenes, but not in that of the host strain HB101 harboring the parental plasmid pUC19. The DABA DC activity of this clone was 0.32 μmol min⁻¹ mg⁻¹, being approximately 65-fold higher than that of E. aerogenes. The recombinant enzyme in E. coli had the same distinctive feature as did the native enzyme; it was greatly activated by the addition of 10 mM Mg²⁺ to the reaction mixture. The plasmid recovered from this clone was termed pEED30, and contained a 4.4-kbp insert. The physical map of the insert of pEED30 was generated with several restriction enzymes (Fig. 1). Whereas a subclone, pEED30.1, constructed by introducing the 2.9-kbp BamHI-SacI fragment of the pEED30 into pUC19 showed a specific activity nearly equal to pEED30, pEED30.2 containing a 2.4-kbp EcoRI-BamHI fragment showed no enzyme activity, suggesting that part of the structural gene and/or a motif essential for gene expression are located in the upstream BamHI-EcoRI region. Moreover, pEED30.3 with the same fragment as pEED30.1, which was inserted into pUC19 in an inverse orientation with relation to the lac promoter, showed no enzyme activity, indicating that the vector-located promoter is essential for expression of the gene. Consistent with this, growth of E. coli HB101 carrying pEED30.1 for 6 h in the presence of 0.2 mM L-threo-β-D-galactoside caused about 2.5-fold increase in the specific activity.

Sequencing of the ddc Gene Determination of the nucleotide sequence of the insert of pEED30.1 revealed an open reading frame (ORF) consisting of 1470 bp starting with an initiation codon ATG and ending with a termination codon TAA. The deduced amino acid sequence of residues 2 to 11 was identical to the NH₂-terminal amino acid sequence of purified DABA DC (Fig. 2), confirming that the coding region represents the E. aerogenes ddc gene. A 12-base inverted repeat resembling a β-independent transcription terminator is located 27 bp downstream of the translation stop codon. The ATG initiation codon is preceded by a putative ribosome-binding site GGAG, although the spacing of 12 bp from the initiation codon is not consistent with the optimal distance of 7±2 bp in E. coli. However, no putative promoter similar to the E. coli consensus -35 (TTGACA) and
-10 (TATAAT) sequences was found within the sequenced 5'-upstream region, suggesting co-transcription with the upstream ORF. This notion may also be supported by a vector-located promoter-dependent expression of ddc as described above as well as a relatively narrow spacing (18 bp) between the termination codon of the upstream ORF and the initiation codon of ddc (Fig. 2). The ddc gene encodes a protein of 490 amino acid residues with a calculated molecular mass of 53659 Da, which is similar to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) value of 51 kDa found for the subunit of the wild type enzyme. The G+C content of the gene is 59.4%, which falls within that of 52 to 60% determined for the genomic DNA of Enterobacter species. Interestingly, the E. aerogenes ddc preferentially uses GC-containing isocodon (data not shown), as compared with the A. baumannii and H. influenzae ddc genes, whose G+C contents are 41.9% and 42.1%, respectively. The amino acid sequence deduced from a partial ORF (orf) in Fig. 2) with a TGA termination codon 18 bp upstream of the ddc gene, on the other hand, showed 40% identity and 83% similarity to that of the C-terminal region of the A. baumannii aminotransferase (encoded by the dat gene) responsible for the formation of i-DABA. In addition, the lack of a putative terminator signal in orf suggests that, as in A. baumannii, both dat and ddc genes in E. aerogenes are co-transcribed from a common promoter upstream of the dat gene to the transcriptional terminator located downstream of the ddc gene.

Homology to Other Proteins A database search using the FASTA and BLAST search programs revealed that the amino acid sequence deduced from the E. aerogenes ddc shares high similarities with and is similar in size to those from the ddc genes of Acinetobacter baumannii ATCC 19606 (GenBank accession no. D55724) (56% identity and 88% similarity), Haemophilus influenzae Rd (GenBank accession no. U32776) (55% identity and 87% similarity), and to a much lesser extent (33% identity or lower) with members of group II amino acid decarboxylase. Alignment of these DABA DC proteins revealed several domains with extensive identities (Fig. 3), some of which may be implicated in the catalytic properties of substrate specificity and activation by Mg$^{2+}$. Moreover, all of the 10 invariant residues in the core region that have been proposed to be functionally important in the group II amino acid decarboxylases by Ishii et al.31 are conserved in such domains, supporting the classification of DABA DCs into group II amino acid decarboxylase.

Identification of the PLP-Binding Lysine Residue PLP-dependent enzymes first bind to PLP to form PLP-lysine Schiff bases, which in turn undergo transaldimination reaction to form Schiff bases with added substrate amino acids. On the basis of chemical identification together with the primary structures around the proposed PLP-binding lysine residues, the partial structure, S-X-X-K(LP)-, was suggested to be a structural feature common to PLP-dependent enzymes as a whole.24,25 In particular, it was shown that the active site sequences for amino acid decarboxylases were well conserved and contained the sequence (S,N,T)-X-H-K(LP)-.24,25,26 On inspection of the alignment of these DABA DCs (Fig. 3), we found such PLP-binding motif proximate to the conserved K residues at positions 198 and 307. To clarify which K residue is responsible for PLP-binding, the AAG codons of K-198 and K-307 were each changed to the AGG (arginine) by site-directed mutagenesis. The mutant enzyme K307R was produced in E. coli XL1-Blue (Stratagene) in a slightly lower amount than was the wild-type enzyme or the mutant enzyme K198R, as judged by SDS-PAGE followed by immunoblotting of the cell extract (data not shown). This suggested that the mutant protein K307R unable to bind PLP might be more susceptible to proteolytic digestion. Comparison of the specific activities in the cell extracts of the mutant K198R and K307R enzymes with that of the wild type enzyme revealed that the former retained 96% of the wild-type specific activity and the latter lost all detectable enzyme activity. Furthermore, K-307 in the E. aerogenes DABA DC was aligned with K-303 in rat liver aromatic L-amino acid decarboxylase29 and with K-232 in Morganella morganii histidine decarboxylase27 (both enzymes have been classified into the group II amino acid decarboxylase28), both of which have been shown to be the PLP-binding ones by chemical and/or mutational analysis. K-307 is accompanied by the adjacent H-306 which is completely conserved for each of the amino acid decarboxylases for which the active-site peptide sequence had been determined,4 again supporting the validity of identification of K-307 as the PLP-binding residue in this protein. However, it is noteworthy that the consensus S(N,T) residue (all hydrogen bond donors) is substituted by the D residue with a negative charge at position 304 (E. aerogenes DABA DC numbering) in all DABA DCs, although its involvement in the structure and function of these enzymes is unclear.

Detection of ddc Homologs by PCR in Various Species of Enterobacteriaceae It was of interest to examine whether ddc homologs are distributed in other enteric bacteria. The genomic DNAs were amplified by PCR with a set of oligonucleotide primers designed on the basis of the sequence of E. aerogenes ddc gene (see Fig. 3). The expected PCR product of about 1 kbp was detected for E. cloacae, Serratia marcescens, Klebsiella pneumoniae, K. oxytoca, and
Citrobacter freundii as well as for _E. aerogenes_ (Fig. 4), being in agreement with the occurrence of the DABA DC activity in these strains. However, the expected PCR product was not detected in the following strains having no DABA DC activity: _E. coli_ HB101, _C. diversus_ ATCC 27156, _C. koseri_ ATCC 27028, _Hafnia alvei_ ATCC 9760, _Morganella morgani_ ATCC 23548, _Proteus vulgaris_ ATCC 13315, _Salmonella typhimurium_ ATCC 13311, _Yersinia enterocolitica_ TB-1 and _Y. pseudotuberculosis_ TB-2. These data indicated that the ddc gene is mainly distributed in enterocusters 1b and 2 of the dendrogram based on 16S rRNA oligonucleotide cataloging and biochemical character states. As Woese has noted that biochemical diversity in addition to sequencing of 16S rRNA can be used to generate a perfect phylogenetic dendrogram in closely related groupings, the distribution of the ddc gene in _Enterobacteriaceae_ may warrant its consideration as a taxonomic or diagnostic marker.

In conclusion, we demonstrated that _E. aerogenes_ possesses the ddc gene, whose deduced amino acid sequence showed a significant similarity to members of the group II amino acid decarboxylases, and that some other species of _Enterobacteriaceae_ also contain the ddc homologs. The finding that the ddc homologs exist in several bacteria belonging to taxonomically different genera imply the biological significance of the DABA-DAP production pathway.

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Fig. 3. Alignment of the Dced Amino Acid Sequence of _E. aerogenes_ DABA DC with Those of _A. baumannii_ and _H. influenzae_ DABA Dcs

Alignment was obtained using GENETYX-MAC software (Software Development, Tokyo, Japan). Amino acids which are conserved in three proteins are denoted by asterisks. The PLP-binding lysine residue identified in the _E. aerogenes_ DABA DC by site-directed mutagenesis (in this study) is marked by a closed circle. The amino acid residues which have been proposed to be conserved among the group II amino acid decarboxylases are boxed. The underlined amino acids correspond to the synthetic oligonucleotide primers (1 and 2) used for PCR.

Fig. 4. Detection of the Homologs to _E. aerogenes_ ddc in Various Species of _Enterobacteriaceae_ by PCR

A primer set prepared according to the known nucleotide sequence of the _E. aerogenes_ ddc gene was used for PCR amplification of the genomic DNAs from bacterial strains indicated. An arrowhead indicates expected amplicon bands (1002 bp for _E. aerogenes_).
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REFERENCES