

Gene Cloning and Molecular Characterization of Lysine Decarboxylase from *Selenomonas ruminantium* Delineate Its Evolutionary Relationship to Ornithine Decarboxylases from Eukaryotes

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Lysine decarboxylase (LDC; EC 4.1.1.18) from *Selenomonas ruminantium* comprises two identical monomeric subunits of 43 kDa and has decarboxylating activities toward both L-lysine and L-ornithine with similar K_m and V_{max} values (Y. Takatsuka, M. Onoda, T. Sugiyama, K. Muramoto, T. Tomita, and Y. Kamio, *Biosci. Biotechnol. Biochem.* 62:1063–1069, 1999). Here, the LDC-encoding gene (*ldc*) of this bacterium was cloned and characterized. DNA sequencing analysis revealed that the amino acid sequence of *S. ruminantium* LDC is 35% identical to those of eukaryotic ornithine decarboxylases (ODCs; EC 4.1.1.17), including the mouse, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Trypanosoma brucei*, and *Caenorhabditis elegans* enzymes. In addition, 26 amino acid residues, K69, D88, E94, D134, R154, K169, H197, D233, G235, G236, G237, F238, E274, G276, R277, Y278, K294, Y323, Y331, D332, C360, D361, D364, G387, Y389, and F397 (mouse ODC numbering), all of which are implicated in the formation of the pyridoxal phosphate-binding domain and the substrate-binding domain and in dimer stabilization with the eukaryotic ODCs, were also conserved in *S. ruminantium* LDC. Computer analysis of the putative secondary structure of *S. ruminantium* LDC showed that it is approximately 70% identical to that of mouse ODC. We identified five amino acid residues, A44, G45, V46, P54, and S322, within the LDC catalytic domain that confer decarboxylase activities toward both L-lysine and L-ornithine with a substrate specificity ratio of 0.83 (defined as the k_{cat}/K_m ratio obtained with L-ornithine relative to that obtained with L-lysine). We have succeeded in converting *S. ruminantium* LDC to form with a substrate specificity ratio of 58 (70 times that of wild-type LDC) by constructing a mutant protein, A44V/G45T/V46P/P54D/S322A. In this study, we also showed that G350 is a crucial residue for stabilization of the dimer in *S. ruminantium* LDC.

Ornithine decarboxylase (ODC) (EC 4.1.1.17) is an important enzyme for the biosynthesis of putrescine, a precursor of polyamines which are implicated in a wide variety of biological processes that include the synthesis of DNA, RNA, and protein in all living cells (26, 30, 31). Lysine decarboxylase (LDC) (EC 4.1.1.18), which exists in most bacteria, is involved in the biosynthesis of cadaverine, a molecule that participates in the closing of the porin channels in the outer membrane of *Escherichia coli* (5) and is also an essential component of the peptidoglycan of *Selenomonas ruminantium*, *Veillonella alcalescens*, *V. parvula*, and *Anaerovibrio lipolytica*, which are strictly anaerobic gram-negative bacteria (9, 12, 14, 15). Previously, we reported that in these bacteria, cadaverine is transferred to the D-glutamic acid residue of a lipid intermediate for the synthesis of the cadaverine-containing peptidoglycan by cadaverine transferase (11, 12, 15, 17). In *S. ruminantium*, cadaverine is constitutively synthesized from L-lysine (16) and its synthesis was completely prevented by DL- α -difluoromethyllysine (DFML) and DL- α -difluoromethylornithine (DFMO), which are irreversible inhibitors of LDC from *Mycoplasma dispar* (27)

and eukaryotic ODC, respectively, resulting in growth inhibition due to the synthesis of the abortive peptidoglycan without cadaverine (11, 15). These observations suggested that *S. ruminantium* ODC could decarboxylate L-lysine, as well as L-ornithine. Accordingly, in our preceding study (32), we purified and characterized *S. ruminantium* LDC and found the following evidence. (i) *S. ruminantium* LDC comprises two identical monomeric subunits of 43 kDa. (ii) *S. ruminantium* LDC has decarboxylase activities toward both L-lysine and L-ornithine, with similar K_m values. (iii) The decarboxylating activities toward L-lysine and L-ornithine are inhibited by either DFML or DFMO competitively and the catalytic domains for the enzyme activities toward both substrates are identical. We also showed in the preceding study that a drastic decrease in LDC activity occurred on entry into the stationary phase of cell growth, which was due to the degradation of LDC (32). This degradation resembled that of mouse ODC by an antizyme-mediated mechanism (8). These results indicate that *S. ruminantium* LDC resembles the eukaryotic ODC in both physicochemical and biochemical features except for its dual preference as a decarboxylase with L-lysine as well as L-ornithine with similar K_m and V_{max} values. In this study, we cloned and characterized the LDC gene (*ldc*) and showed that the amino acid sequence of *S. ruminantium* LDC is 35% identical and 53 to 60% similar to those of eukaryotic ODCs and that 26 amino acid residues, all of which are implicated in either contributing to pyridoxal phosphate (PLP)- and substrate-binding domains or in forma-

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TABLE 1. Oligonucleotides used for site-directed mutagenesis of the *ldc* gene

Mutant no. ^a	Primer sequence ^b
1	TCA TTT <u>GCC GCG</u> <u>GGC GGG ACC GTT</u> TTA TGC
2	TCA TTT <u>GCC GCG</u> <u>GGT GAC ACC GTT</u> TTA TGC CAT
3	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC <u>CGT GAA</u> GGC GAA TCC TAC GC
4	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC CAT GAA GGC GAA TCC <u>TTC GCC</u> AGA AAT ACT G
5	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC CAT GAA <u>GTG CAA</u> TCC TAC GC
6	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC CAT GAA GGC GAA <u>TGA TAC</u> GCC AGA AAT ACT G
7	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC <u>CGT GAA GTG CAA</u> TCC TAC GC
8	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC CAT GAA GGC GAA <u>TGA TTC</u> GCC AGA AAT ACT G
9	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC CAT GAA <u>GTG CAA TGA TAC</u> GC
10	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC <u>CGT GAA</u> GGC GAA <u>TGA TTC</u> GCC AGA AAT ACT G
11	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC <u>CGT GAA GTG CAA TGA TAC</u> GC
12	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC CAT GAA <u>GTG CAA TGA TTC</u> GCC AGA AAT ACT G
13	TCA TTT <u>GCC GCG</u> <u>GGC GGG AGT GTT</u> TTA TGC <u>CGT GAA GTG CAA TGA TTC</u> GCC AGA AAT ACT G
14	TCA TTT <u>GCC GCG</u> <u>GGT GAC ACC GTT</u> TTA TGC CAT GAA <u>GTG CAA</u> TCC TAC GC
15	TCA TTT <u>GCC GCG</u> <u>GGT GAC ACC GTT</u> TTA TGC CAT GAA GGC GAA <u>TGA TAC</u> GC
16	TCA TTT <u>GCC GCG</u> <u>GGT GAC ACC GTT</u> TTA TGC <u>CGT GAA GTG CAA</u> TCC TAC GC
17	TCA TTT <u>GCC GCG</u> <u>GGT GAC ACC GTT</u> TTA TGC CAT GAA <u>GTG CAA TGA TAC</u> GC
18	TCA TTT <u>GCC GCG</u> <u>GGT GAC ACC GTT</u> TTA TGC <u>CGT GAA GTG CAA TGA TTC</u> GCC AGA AAT ACT G
19	GAG CAC <u>ATC GAT</u> <u>GCC ATC GCA GCT</u> GGG GCC <u>CCA</u> GAA AGT CG
20	GAG CAC <u>ATC GAT</u> <u>GCC ATC GCA GCT</u> GGG GCC <u>CCA GAT</u> AGT CGA AG
21	GAG CAC <u>ATC GAT</u> <u>GCC ATC GCA GGT</u> GGG GCC GC
22	CAT GAA <u>GTC GCG ATA</u> GAG CAC ATC <u>GAG</u> GCC ATC GC
23	CAT GAA <u>GTC GCG ATA</u> GAG CAC ATC <u>GAG</u> GCC ATC GCA <u>GGT</u> GGG GCC GC
24	GAG CAC <u>ATC GAT</u> <u>GCC ATC GCA GGC</u> GGG GCC GC

^a See Fig. 5.

^b The boxed sequences are *Sac*II, *Cla*I, and *Nru*I restriction sites. The underlined boldface sequences are those that were replaced.

tion of the homodimeric forms of eukaryotic ODCs, are conserved in *S. ruminantium* LDC. From this information, we have now succeeded in converting *S. ruminantium* LDC to an enzyme with a preference for decarboxylating L-ornithine when five amino acid residues from the active site of *S. ruminantium* LDC were replaced with the corresponding residues found in mouse ODC.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains used in the present study included *S. ruminantium* subsp. *lactilytica* (18) and *E. coli* DH5 α , which was used as the host strain in cloning experiments. Plasmids pUC119, Charomid 9-36 (Nippon Gene Co., Tokyo, Japan), and pTrc99A (Pharmacia) were used as the vector plasmids for subcloning and gene expression, respectively. Culture media included a tryptone-yeast extract-glucose medium (18) and a tryptone-yeast extract-lactate medium (18) and L broth for the growth of *S. ruminantium* and *E. coli*, respectively. *S. ruminantium* was grown under the anaerobic conditions described previously (18).

Purification of LDC from *S. ruminantium*. The cells (125.7 g) derived from a 60-liter culture were suspended in 50 mM potassium phosphate buffer (pH 6.7; buffer A) containing 10 mM 2-mercaptoethanol and 0.1 mM PLP and disrupted in a French pressure cell. After centrifugation at 20,000 \times g for 20 min, the supernatant was collected. Ammonium sulfate (3 M) in buffer A was added to the enzyme fraction at a final concentration of 1 M with stirring. After 2 h, the supernatant was collected by centrifugation and put on a TSKgel butyl-Toyopearl 650M column (4.0 by 24 cm). The column was washed with 3.5 liters of buffer A containing 1 M ammonium sulfate and 50 μ M PLP. The enzyme was then eluted with a linear gradient created by mixing 750 ml of 1 M ammonium sulfate in buffer A with 750 ml of buffer A. The fractions which were eluted from 0.80 to 0.85 M ammonium sulfate were pooled. After 3 M ammonium sulfate in buffer A was added at a final concentration of 0.8 M, the enzyme preparation was put on a TSKgel phenyl-5PW column (21.5 by 150 mm; Tosoh, Tokyo, Japan). The column was washed with 450 ml of buffer A containing 0.8 M ammonium sulfate and then eluted with a linear gradient of ammonium sulfate in buffer A (0.8 to 0 M). The fractions eluted from 0.54 to 0.56 M ammonium sulfate were pooled. After being dialyzed against 20 mM potassium phosphate buffer containing 50 μ M PLP (pH 6.5; buffer B), the enzyme preparation was put on a TSKgel DEAE-5PW column (7.5 by 75 mm; Tosoh) equilibrated with 20 mM potassium

phosphate buffer (pH 6.5; buffer C). The column was washed with buffer C and then eluted with a linear gradient of NaCl in buffer C (0 to 0.4 M). The fractions eluted from 0.18 to 0.20 M NaCl were pooled and dialyzed against buffer B containing 20% glycerol.

Determination of the N-terminal and internal amino acid sequences of *S. ruminantium* LDC. The purified LDC preparation (350 μ g) was digested with 2.5 μ g of lysyl endopeptidase (Wako Chemicals, Osaka, Japan) at 37°C for 12 h in 0.4 ml of 100 mM Tris-HCl buffer (pH 9.0). The digest was subjected to high-pressure liquid chromatography (HPLC) with a TSKgel ODS-120T column (4.6 by 250 mm; Tosoh) at 40°C using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0 to 80%). Twenty-four fragments were obtained. Six fragments were analyzed for N-terminal amino acid sequencing by a gas-phase protein sequencer (model PSQ; Shimadzu, Kyoto, Japan) equipped with an on-line amino acid analyzer (model RF-550; Shimadzu).

Preparation of oligonucleotide primers for cloning of the *ldc* gene. Oligonucleotide primers corresponding to the N-terminal (Glu⁸-Lys-Glu-Val-Lys-Thr-Leu-Ala¹⁵) amino acid sequence and three internal amino acid sequences of LDC (Glu-Glu-Asn-Tyr-Gln-Phe-Met, Ala-Asn-Pro-Thr-Pro-Glu-Ile, and Glu-Met-Gly-Ser-Tyr) were designed and synthesized as primers I [5'-GA(A,G)AA(A,G)GA(A,G)GTI AA(A,G)ACI (T,C)TI GC-3'], II [5'-GAG GAA AAC TAC CAG TTT ATG-3'], III [5'-AT (T,C)TC IGG IGT (G,A,T,C)GG (A,G)TT (G,A,T,C)GC-3'], and IV [5'-GT (A,G)TA (G,A,T,C)GA (G,A,T,C)CC CAT (T,C)TC-3'], respectively (in the sequences, I represents inosine). In combination with primers I and III and primers II and IV, 152- and 967-bp fragments, respectively, were amplified from the chromosomal DNA of *S. ruminantium* by PCR. By DNA sequencing of the amplified fragments, it was confirmed that the peptides described above correspond to the amino acid sequences of the 21-, 8-, 13-, 11-, 13-, and 9-residue segments M1-D21, V30-M37, A52-G64, G153-L163, T276-G288, and V344-Y352, respectively, of intact LDC. Therefore, we used these 152- and 967-bp fragments as DNA probes for genomic cloning of *ldc* from the *S. ruminantium* chromosomal DNA.

Cloning of *ldc* from chromosomal DNA of *S. ruminantium*. The chromosomal DNA of *S. ruminantium*, which was isolated and purified by the standard method, was digested with *Eco*RI and resolved by 0.7% agarose gel electrophoresis. For Southern blot hybridization with the fluorescein-labeled 152- and 967-bp DNA fragments, the procedure was carried out using the ECL random prime labeling and detection systems (Amersham Life Science, Inc., Cleveland, Ohio). Only a single 4-kbp fragment was hybridized; this fragment was extracted from the gel and ligated into the *Eco*RI site of Charomid 9-36 vector DNA. After transduction into *E. coli* DH5 α , a recombinant *E. coli* strain containing *ldc* was selected

1 GAATCAAATAAGCGAAATAATAAGGGAATCCTGTTGGTGGAGATTCCCTTTTGTTCCTCATTATTATTGTTGATACGCGTT 90
 ◆ K N Y M R N

91 CAGGGCATTGTCTTTGAAGATATTGCCTTGTTCGATATAGCGGTGGCCATCTTTTCGGATTGTGCCGTGCTGCCGATGATGGTCAG 180
 L A N D K F I N Q E I Y R H K V M K E S K H R T Q R M I T L

181 GGCCTGATATCGTGTGGCGGCTGGTGGCAAGCCGCGCGGCTGTGGCGGCAAGAGGTGTTTCATCGAGGCTGCCAGCCC 270
 A D I D H Q A A S T A F G R R L S H G A F L H E D L G A L G

271 CACGATTTCTTACGATCAGGGCCAGGATTTATCGGAGAGCTGGTATTGCGCAGGCTCTGACCCCTGGTAAATGGGCGGAAGATTGG 360
 V Y K K V I L A V S K D S L Q T N R L S Q V R T I P R F I P

361 TCCCCGGTGGATCTGGGCGATCCAGCCATTTTTCAGGGCCGTACGGCGCAGATTCAGCTCGGGGCATAGGGGATGGCAATCTG 450
 G R H I Q A T D L W K K L A R V A C I E A Q P A Y P I A I Q

451 TTACCATGGCCAGCTGGTCCCTTTGGAATGGCCATAAAGATGATGACGCCCTGGGGCGTGAATGTCAGGCTTCATATTGGATGG 540
 E G H G L Q D G K S H A M F I I V G Q P T F T L D E Y Q I H

541 CACGAGTCCGAGCGGGAAGGCCCGGCAAGCCAGGTAGATCAGGGCTTGTACCGCAGGCCACTCAATTTTCTCATCGCAACAG 630
 V L E S R R F A G A F G L Y I L A K D R L G S L K E E D F L

631 GTCAGCCAGCAGATAGAGGGTTTCCATCAGGATGGGGATTGGCTTCGTTGGAAGGTGCTTTTCCCGCGGATGGCGGACATGGCTGC 720
 D A L L Y L T E M L I P S K G R Q F T G K E R R I A S M A A

721 CCGGACATGCCCTTGGCGGGATTATGACGGCCGTGAAGCCGCGCGATATGGTTTCGGAGATGGCGGTACCAGCGGGGAGAG 810
 R V M G D K A P N H R G S F G A A I H N E S I A T V R R S V

811 GGTATTGGCTTGGCATCATCGGCCAGGTCATTGATGATGACGATGGTTCCGGCAGGCGGCAAGTCCGTACGCCATGGTATTG 900
 T N A K A D D A L D N I Y N V I T E P S A P L D T V G H Y Q

901 ACACCGTGGAAAAATCGCTCAGTCACAGGCATAGGCCCTTGGGGTATTGTCGGCCTGGATTTTCCAGACGGCCTTGGCCTTCAG 990
 S W D S F D C A Y A K L T N D A K S K E L R R K A C L

991 GGAAGCGCTTCAATTTTCAATCGGGCATTTTTATCAGCATAAAATATCTTTGATTTTCATGATGAACCTGCTTCAATAAAAG 1080
 S L R E N N E I R A N K I L M F N D K I K M

1081 GATATATAATAACATCTTACAACATTTTGGCAGAAATAAAATAACCTACCGCAACCGCATAGGGTTATTTATGATATATCAGCTTT 1170
 -10 -35

1171 TGGTGATATTATGCGCAAATAAAGGGCACGCTGAACCTTACCAGAACCGAGCAACCGCTGCGACGTTGACACGCTTAACTTCGCTT 1260
 ◆ L A R Q V K G S R L C R T C V N V R K V E G

1261 CAACGACAGCGCTACAGCTGGATGTTCCGCTTCCAAGTCCGCTCTGCTTTCAGATGGGAATGGCTGACATGTTGCCGGACAGTTCCG 1350
 E V V A R V R Q I N P K W T R R T K L H S H S V N N G S L E

1351 CTTTCGGCAGATTTCCGCAACACTTGCATGTTGACCTCTTAACCTGTGGGAGCAACTGCTCCCAACACACAATAATGGTATTG 1440
 G K A C I E C V S A M

1441 TAGCACAAAAGCAGGGGGTGAAGTATTTTACCTTGGACAGCCTGAAAAAATTTTATTTTCGATTTTATAATTTGTTAATTGCCCTT 1530
 P2(-35) P2(-10) P1(-35)

1531 TTATAATACAAATGAAGCCTATGAGATGAACAGTTGTCAATTCATCTTAAATTTATTATCGGAATGGGTTGTGTTATGAAAAATTCAGAC 1620
 P1(-10) M K N F R L

1621 TTAGCGAAAAAGAAATAAAACGCTTGGCAAGCGTATCCCGACGCCCTTTTGGTGGCTTCACTGGCAAGGTTGAGGAAACTACCAGT 1710
 S E K E V K T L A K R I P T P F L V A S L D K V E E N Y Q F

1711 TTATGCGCCCTCATTTCGCCGGCGGGAGTGTTTTATGCCATGAAGGCGAATCTACGCCAGAAATACTGCTCCCTGCTGGCTGGCCCTTG 1800
 M R R H L P R A G V F Y A M K A N P T P E I L S L L A G L G

1801 GTTCTCACCTTGATGGCTCTGCGGGGAGATGGAGCTCCTCATGAATGGCGTAGATGGTTCCAGATGATATGCCAATCCGG 1890
 S H F D V A S A G E M E I L H E L G V D G S Q M I Y A N P V

1891 TAAAGGATGCCGCTGGCTCAAGGCTGCGCTGACTACAATGTCGCGGTTTACTTTTCAGCATCCGTCGGAATCGACAAGATGGCCA 1980
 K D A R G L K A A A D Y N V R R F T F D D P S E I D K M A K

1981 AGGCTGTGCCGGGAGCGGATGCTGCTGCGCATCGCCGTGCGCAACAACAAGCTTTGGTGGATCTGAATACGAAGTTGGTGGCCGG 2070
 A V P R L A D V L V R I A V R N N K A L V D L N T K F G A C P V

2071 TGGAAGAAGCGTGATCTTTTAAAGGCTGCGCAGGATGCTGGCTGCATGCCATGGGGATTTGCTTCCATGTGGGCAGCCAGTCCCTGT 2160
 E E A L D L L K A A Q D A G L H A M G I C F H V G S Q S L S

2161 CTACGGCGCTTATGAGGAAGCCCTGCTGGTGGCTGTAGGCTTTTGTGAGGCGGAAGAAATGGGCATGCACCTGACTGACCTCGACA 2250
 T A A Y E E A L L V A R L F D E A E E M G M H L T D L D I

2251 TCGGCGGCGTTTCCCTGTTCCCGATGCCAAGGGCTCAATGTGGATCTGGCGCATGATGGAAGCCATCAACAAGCAGATCGACCGCC 2340
 G G G F P V P D A K G L N V D L A A M M E A I N K Q I D R L

2341 TGTTCCGGATACAGCTGTTGGACGGAACCGGCGCTTATGTGCGGTACGGCGGTGAACCTCGTCACATCGGTTATCGGCAGAAAA 2430
 F P D T A V W T E P G R Y M C G T A V N L V T S V I G T K I

2431 CCCGTGGTACAGCCTTGGTATATCTTAGATGAAGGCATCTAGCTGCTTCTCCGGCATCATGATGACCACTGGACGTACCCGCTTC 2520
 R G E Q P W Y I L D E G I Y G C F S G I M Y D H W T Y P L H

2521 ATTGCTCGCAAGGGGAATAAGAAACCTTGCATTTTCCGGCGCCAGCTGCGATGCGATCGATGTGCTCTATCGGACTTCAATGGCA 2610
 C F G K G L N K K P S T F G G P S C D G I D V L Y R D F M A P

2611 CGGAGCTCAAGATCGGGGACAAGGTGCTGGTACGGAATGGGTTCTATACCAGCGTCAGCGCTACGCGTTTCAACGGTTTCTACCTGG 2700
 E L K I G D K V L V T E M G S Y T S V S A T R F N G F Y L A

2701 CGCCACCATCATCTTTGAGGACAGCCGGAATATGACGCCGCTGACGGAAGATGATGATGGAAGAAAAAGCGGCTGTATAATGGA 2790
 P T I F E D Q P E Y A A R L T E D D D V K K K A A V *

2791 TATTGAGGAAATCAAGCATATGCTGTTCCACGCTTTGACGGAAGAATCTTGAAGCAAAGCTGGACGAAGCAAATCCAGCAGGAAGT 2880
 2881 CTACAGGATTTGACAGGAGCTGGATTACCTTACCCTGACCATGGAGGATTTGACGAGGATAGAAAGCAATGACAGAAAGAGCAGCAATA 2970

2971 AAATCCTCCCTCGGGGAGGCTTTTGTCTACAGACTCTTTTCAGATAGAGATGTTGTAGTATACTAGCAAAGTTACTATACTTAT 3060

3061 TCAATGATAATTTTAGGGAGAACGATGGAATCATTTATGGGGCGCTGGGGCTTTATGGCTCGGCACCTTGGCACTTGGCACTGGCCCA 3150
 3151 AAACGGCTGGAACCTGGCTGCCATATGATCCATGGGCTGGCGGCTGGCCAGCCTTATGGTGTGGCCCTGCTCCCTGCTGGAATG 3240
 3241 GGGCTGGGCACTGGCGGCTTTGGCAGCTGGGCGTATTTCATTGGTGGTGGATGGCTGGCTGACGCTTCTGCTGATCATCGGCTT 3330
 3331 TCGGGACGGCAGTGGACCTATGGTCTGGCTACGGCGGGGATACTTAGGTGCCCGCATCCGGCAGCTGGCAGGACTTTGGAACCTG 3420
 3421 TTCTTAGGCTCATGGTGTGGTACTGGCTGGCGATGCTTTACCTTATCTGGCTGGGAAGTCATGGCACTGGTATCATCTCTG 3510
 3511 CTGGTCAATCAGGAGAGTGAAGAAGCAGGTGGTGGTGGCTATCAGTATAGGTCATGACGCACTGGGCACTGGGCGGCGGCTTCTG 3600
 3601 GTGGCTTCTATATTTAGGACGCGGAGGAGGCTTTGCTTTGTGGATTTAGCGCATAGTGAACCTTCCGCACTGATGAAGCATATC 3690
 3691 GCGCTTGGCTGTGCTTACAGGCTTGGCTGAAATCCGGCTGATGCCGCTGATGCTGGTCCCAATGCCCTCCGCGGCGGCGCA 3780
 3781 TCCCATGTGTGGCGCTCATGAGCGGCGTATGCTGAAAGTGGCGGCTACGGTTTCGGCGGCTGTTTTGAAATC 3858

ORF3

ORF2

ORF1
(LDC)

FIG. 1. Nucleotide sequence of the *ldc* gene of *S. ruminantium* and the flanking region. The strand shown is in the 5'-to-3' direction. The deduced amino acid sequence is indicated by the single-letter code under the nucleotide sequence. For *ldc*, the amino acid sequence, putative ribosomal binding site, promoter sequences, translation termination codon, and transcription termination sequences are indicated by single underlining, double underlining, boxes (white boxes, promoter 1; black-shaded boxes, promoter 2), a single asterisk, and horizontal arrows, respectively. For ORF2 and ORF3, ribosome-binding sequences, promoter sequences, translation termination codons, and transcription termination sequences are indicated by double-dotted and triple underlining, double and single wavy lines, diamonds and triangles, and double- and single-dotted inverted arrows, respectively.

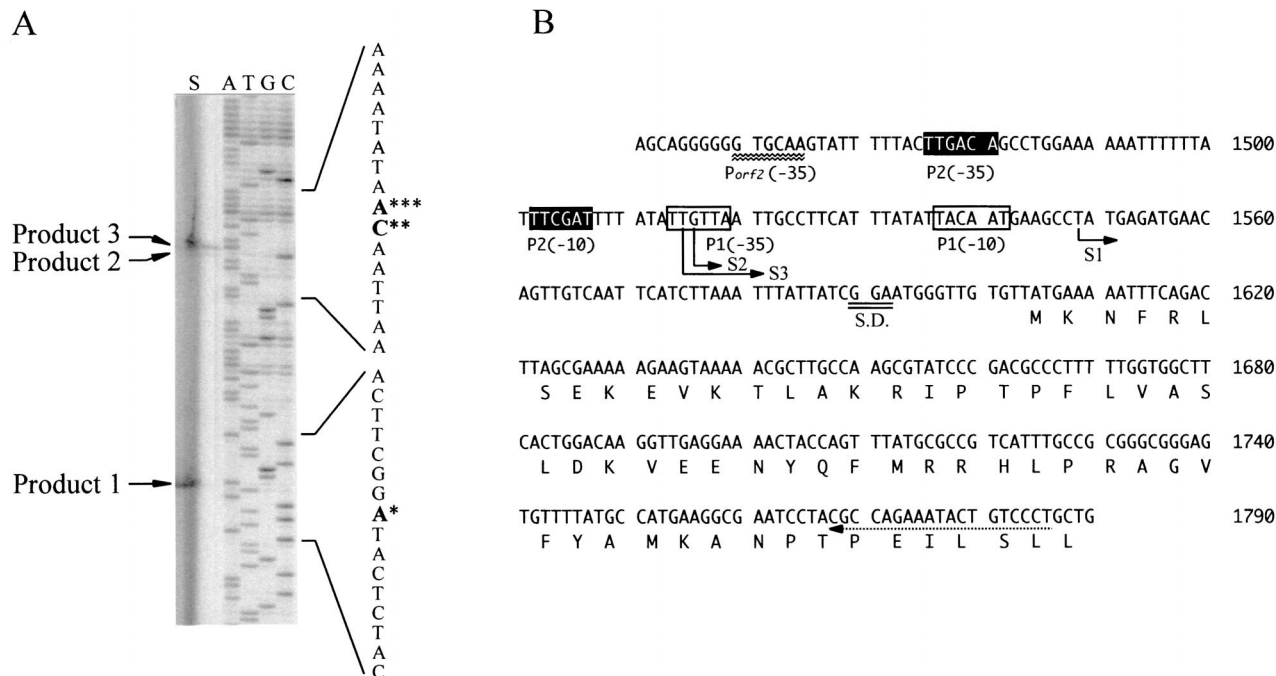


FIG. 2. Determination of the *ldc* transcription start site by primer extension using a 19-mer complementary to nt 1768 to 1786, counting from the *EcoRI* site of the *S. ruminantium* chromosomal DNA sequence. The RNA preparation (30 μ g) extracted from cells cultivated for 3 h was used for primer extension. Panel A (lane S) shows gel electrophoresis of primer extension products 1, 2, and 3. The sequence shown next to the sequencing gel is complementary to that displayed in panel B. The single, double, and triple asterisks indicate the bases complementary to the 5' end of the transcript for S1, S2, and S3, respectively. Panel B represents the nucleotide sequence (nt 1451 to 1790) containing the putative promoter site (-35 region) for ORF2 and the beginning of *ldc*. The dashed arrow indicates the region complementary to the primer used. The transcription start sites (boldface T, G, and T) determined here (nt 1558, 1516, and 1515) are indicated by S1, S2, and S3. S.D., Shine-Dalgarno sequence.

by colony hybridization using the labeled 152- and 967-bp DNA fragments. The plasmid containing *ldc* was designated pCLDC.

Nucleotide sequence determination. The 4-kbp DNA was fragmented by digestion with various restriction enzymes; the resulting fragments were subcloned into plasmid pUC119 and sequenced. Analysis was done with a Thermo Sequenase cycle sequencing kit (Amersham) with IRD-41 dye-labeled M13 forward and reverse primers (Aloka, Ltd., Tokyo, Japan) using a model 4000 DNA sequencing system (Li-Cor, Lincoln, Nebr.).

ORF identification, homology search, and alignment of multiple nucleotide and amino acid sequences. Protein and nucleotide sequences were compared with the sequence databases using the FASTA (version 3.0) and BLAST (version 1.49) programs implemented at the EMBL/GenBank/DBJ nucleotide sequence databases and the SWISSPROT/NBRF-PIR protein sequence databases. Open reading frame (ORF) identification and multiple-sequence alignment were performed using the GENETYX program (Software Development Co., Tokyo, Japan).

RNA analysis. For total cellular RNA isolation, *S. ruminantium* cells were grown in 50 ml of tryptone-yeast extract-glucose medium at 37°C under anaerobic conditions for various amounts of time. Total RNAs were extracted as described previously (1) and dissolved in 2 ml of distilled water and then stored at -80°C until used.

Primer extension analysis was done in accordance with the protocol of Aloka Ltd. using an IRD-41-labeled 19-mer complementary to nucleotides (nt) 1768 to 1786 (see Fig. 2B).

Assay for LDC and ODC activities and kinetic analysis. LDC and ODC activities were measured by the amount of cadaverine or putrescine formed from L-lysine or L-ornithine by HPLC using a TSK-gel Polyaminepak column (Tosoh) as described previously (13). The standard assay was done at saturating PLP (50 μ M) and lysine or ornithine concentrations ranging from 0.15 to 500 mM, depending on the K_m of the enzyme being tested. The incubation was done at 30°C, pH 6.0.

Construction of plasmid pTLDC for LDC expression in a *trc* promoter expression system. For expression of recombinant LDC (rLDC), a DNA fragment containing only the *ldc* structural gene was amplified from chromosomal DNA by PCR using primers 5'-ATGCATGAATTCTATCGGAAGGAGGTGTGTTATGAAAAATTTTCAG-3' (primer V) and 5'-ATGCATCTGCAGTAATCCAGCTCCTGCA-3' (primer VI), which are located upstream and downstream of *ldc*, respectively. In primer V, wavy underlining and double underlining represent the *EcoRI* site and the ribosomal binding site which replaced the original Shine-Dalgarno sequence of *ldc* (see Fig. 1), respectively. On the other hand, wavy underlining and single underlining in primer VI indicate the *PstI* site and the

complementary sequence at positions 2892 to 2909 (see dotted underlining in Fig. 1), respectively. The amplified fragment was digested with *EcoRI* and *PstI*, and the *EcoRI-PstI* fragment was inserted into the *EcoRI-PstI* site of expression vector pTrc99A to construct plasmid pTLDC, in which *ldc* was under the control of the *trc* promoter. pTLDC was then introduced into *E. coli* DH5 α . The *ldc* region of pTLDC was sequenced, and it was confirmed that there was no point mutation in the region.

Purification of rLDC. Cells of *E. coli* DH5 α (pTLDC) were grown in 2 \times TY (1.6% Bacto Tryptone-1.0% Bacto Yeast Extract-0.5% NaCl) medium containing ampicillin (100 μ g/ml) at 37°C for 20 h with shaking and were collected by centrifugation at 10,000 \times g for 5 min at 4°C. rLDC was purified to electrophoretic homogeneity from a sonicated extract of the cells by the method used for the purification of native LDC from *S. ruminantium*. This method was also applicable to the purification of mutant LDC obtained by site-directed mutagenesis of *ldc*.

Site-directed mutagenesis. Site-directed mutagenesis was done by PCR using the oligonucleotide primer shown in Table 1 together with either primer VI or V, used to create plasmid pTLDC. The PCR products were digested with both *SacII* (Table 1) and *PstI*, both *EcoRI* and *ClaI*, or both *EcoRI* and *NruI*, and the mutant *SacII-PstI*, *EcoRI-ClaI*, or *EcoRI-NruI* fragments were used to replace the *SacII-PstI*, *EcoRI-ClaI*, or *EcoRI-NruI* fragments from pTLDC which had been digested with *SacII* and *PstI*, *EcoRI* and *ClaI*, or *EcoRI* and *NruI*. Mutations were confirmed by DNA sequencing.

Other analytical procedures. Protein was measured by the method of Bradford (3), with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described previously (20). Circular dichroism (CD) analysis of the wild-type and mutant LDCs was done with a Jasco J-720 spectropolarimeter at room temperature in a 1-mm path length cell containing 5 μ M enzyme in 5 mM potassium phosphate buffer (pH 6.5) containing 50 μ M PLP. Secondary-structure computer analysis of *S. ruminantium* LDC was done by the new-joint method (10, 23).

Nucleotide sequence accession number. The nucleotide sequence of *ldc* has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB011029.

RESULTS AND DISCUSSION

Cloning and nucleotide sequencing of *ldc*. We cloned *ldc* by obtaining N-terminal and internal amino acid sequence information from purified *S. ruminantium* LDC, designing appro-

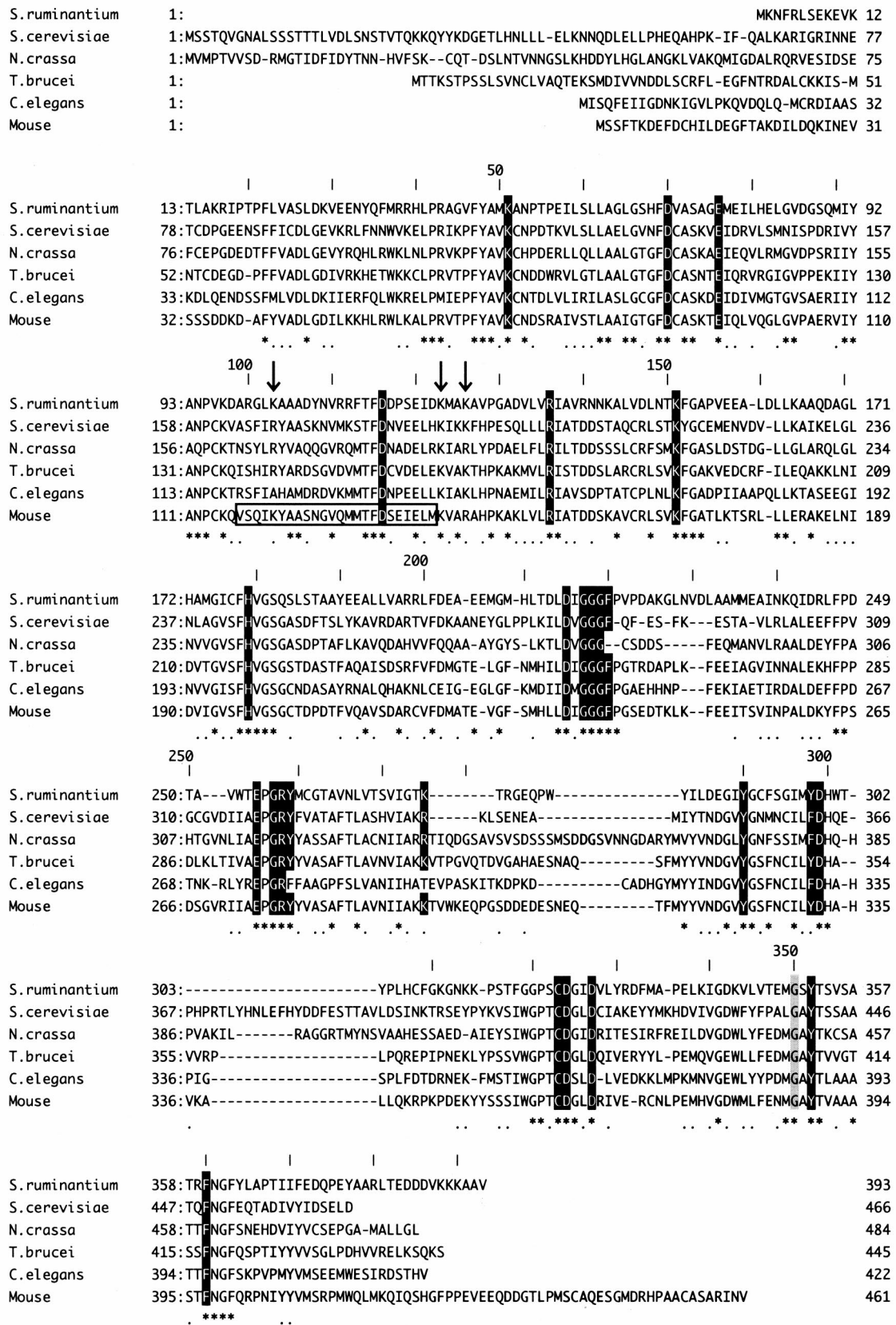


FIG. 3. Amino acid sequence alignment of *S. ruminantium* LDC with ODCs of various eukaryotes. *S. ruminantium* LDC exhibits 60% similarity to eukaryotic ODCs, including the *S. cerevisiae*, *N. crassa*, *T. brucei*, *C. elegans*, and mouse ODCs. On the consensus line below the aligned sequences, identical amino acids are indicated by asterisks and conserved residues are indicated by dots. Amino acids required for formation of the active sites of the ODCs are boxed. The residues marked in black and gray and vertical arrows are explained in the text. A large box in the sequence of mouse ODC represents the region to which antizyme is bound.

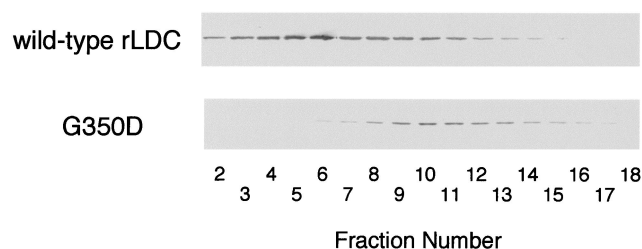


FIG. 4. Analysis of wild-type rLDC and G350D mutant rLDC by gel filtration. Cells of *E. coli* DH5 α harboring plasmid pTLDC or pG350D were grown in 4 ml of 2 \times TY medium containing ampicillin (100 μ g/ml) at 37°C for 18 h and collected by centrifugation at 10,000 \times g for 5 min at 4°C. The cells were suspended in 2 ml of dimer buffer (20 mM potassium phosphate [pH 6.7] containing 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 M NaCl, 50 μ M PLP, 0.5 mM ornithine) (33) and sonicated. The sonicated extracts were filtrated by column chromatography with a Superdex 200 HR 10/30 column (Pharmacia) equilibrated with dimer buffer. Proteins in the fractions were analyzed by SDS-PAGE and then transferred to nitrocellulose membrane (Hybond-C pure; Amersham), and the LDC protein was visualized immunologically using anti-LDC antibodies and anti-rabbit immunoglobulin G(Fc)-alkaline phosphatase conjugate (Seikagaku Kogyo Co., Tokyo, Japan).

priate oligonucleotide primers, and preparing labeled DNA fragments obtained by subsequent PCR. The screening of an *Eco*RI library gave a single positive clone from approximately 100,000 colonies. The positive clone contained an insert of 3,858 bp whose sequence included three ORFs. The first ORF encoded a 393-amino-acid protein of 43,213 Da whose N-terminal sequence matched that determined for purified LDC and its fragments (Fig. 1, underlined); later functional tests (described below) verified the assignment of this ORF as *ldc*. The *ldc* gene, spanning positions 1605 to 2786 within the genomic clone (pCLDC), started with an ATG codon and ended with a TAA stop codon. Thirteen base pairs upstream from the ATG codon, a ribosome-binding site consensus sequence (GGA) was found at positions 1590 to 1592. After analysis of the transcription start sites as determined by primer extension (described below), two putative promoter regions of *ldc* were identified, one at positions 1537 to 1542 (TACAAT) and 1514 to 1519 (TTGTTA) for the respective -10 and -35 sequences (Fig. 1, white boxes) and the second at positions 1502 to 1507 (TTCGAT) and 1476 to 1481 (TTGACA) for the -10 and -35 sequences (Fig. 1, black-shaded boxes). An inverted-repeat sequence consisting of a stem-loop with an 11-bp arm appeared at positions 2970 to 2997, as indicated by the GENETYX program. It is probably used as a transcription terminator for mRNA of *ldc*.

The second ORF (ORF2 in Fig. 1), consisting of 192 nt, was found at positions 1381 to 1190 in the antisense DNA strand. ORF2 encoded a 63-amino-acid protein which is tentatively called protein 2. From the primary structure of protein 2, 44% identity was observed in an alignment with the sequence of ribosomal protein L28 from *E. coli* (21).

The third ORF (ORF3 in Fig. 1), consisting of 987 nt, extended from position 1056 to position 70 in the antisense DNA strand. ORF3 encoded a 328-amino-acid protein which is tentatively called protein 3. The amino acid sequence of protein 3 exhibited 34% overall identity with that of *E. coli* recombinase XerD (2).

Functional analysis of the *ldc* gene product. To prove that *ldc* is the gene encoding *S. ruminantium* LDC, the gene was expressed in *E. coli*; rLDC was purified, and its characteristics were compared with that of the earlier *S. ruminantium* LDC preparation (32). *E. coli* DH5 α (pTLDC) expressed *ldc* at a final concentration of approximately 2% of its total protein.

The purified rLDC preparation had characteristics identical to those of the earlier *S. ruminantium* LDC preparation, as judged by the molecular mass of the native enzyme (88 kDa), its subunit mass (43 kDa) determined by SDS-PAGE, and the N-terminal 25-residue and internal amino acid sequences underlined in Fig. 1. When the decarboxylase activities of rLDC toward L-lysine and L-ornithine were studied, the following became evident: (i) rLDC decarboxylated both substrates with K_m values identical to those obtained with the original LDC preparation from *S. ruminantium*, and (ii) the decarboxylase activities toward both substrates were inhibited competitively by both DFML and DFMO with a K_i identical to that observed for the *S. ruminantium* LDC preparation (32). Thus, we concluded that ORF1 is the LDC-encoding gene (*ldc*) of *S. ruminantium*.

Transcriptional start site of *ldc*. The transcriptional start site of *ldc* was determined by primer extension analysis of mRNA isolated from *S. ruminantium* (Fig. 2A). The *ldc* transcriptional start sites were identified as T at position 1549 (S1), G at position 1516 (S2), and T at position 1515 (S3) (Fig. 2B). Looking further upstream of these start sites, putative promoter structures 5'-TTGTTA(17 bp)TACAAT-3' for S1 and 5'-TTGACA(20 bp)TTCGAT-3' for S2 and S3 were identified with 7, 9, and 8 bp separating the -10 region of the promoter structure and the respective transcriptional start sites.

Amino acid sequence homologies of LDC with eukaryote ODCs. The amino acid sequence of *S. ruminantium* LDC had no similarity to that of the other reported bacterial LDCs and ODCs but showed 35% identity and approximately 60% similarity to eukaryotic ODCs, including the mouse, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Trypanosoma brucei*, and *Caenorhabditis elegans* ODCs (Fig. 3). In addition, absolute conservation among those amino acid residues implicated in homodimer stabilization, in the PLP- and substrate-binding domains, were found for *S. ruminantium* LDC and mouse ODC as follows. (i) From the crystal structure (19), mouse ODC was found to consist of a symmetrical homodimer which is formed by a head-to-tail interaction between the barrel domain of one monomer and the sheet domain of the second. The two salt bridges (K169-D364' and D134-K294' [the primed residue belongs to the second subunit in the dimer]) and the stack of aromatic residues (F397'/Y323'/Y331), which are involved in stabilization of the dimer by formation of the primary interaction between monomers of mouse ODC, were conserved in *S. ruminantium* LDC (K151-D327' and D116-K275' and F360'/Y290'/Y298) (Fig. 3). (ii) G387 in mouse ODC, which is located between the barrel and sheet domains and is crucial for stabilization of the homodimer (33), was also conserved in *S. ruminantium* LDC (G350; shaded gray in Fig. 3). Mutation of G350 to D350 in *S. ruminantium* LDC caused the loss of both dimer formation (Fig. 4) and decarboxylase activity (data not shown). (iii) Eighteen amino acid residues, K69, D88, E94, R154, H197, D233, G235, G236, G237, F238, E274, G276, R277, Y278, D332, C360, D361, and Y389 (mouse ODC numbering) (shaded black in Fig. 3), were identified as the crucial active-site residues, especially residues K69 and E274, which interact with PLP by forming an internal Schiff base and by stabilizing the protonated pyridine nitrogen of PLP (19), respectively, in mouse ODC. Residues C360 and D361 function in the binding of ornithine as a substrate in the active center in eukaryotic ODCs (19). All of these residues were conserved in *S. ruminantium* LDC (Fig. 3). (iv) In *S. ruminantium* LDC, a unique five-residue sequence (T276-R277-G278-E279-Q280) corresponding to a five-residue sequence (S303-D304-D305-E306-D307) which had been reported to be phosphorylated [consensus sequence, S(or T)

Mutant No.	Enzyme	Residue number				Ornithine preference
		41	46	51	55	
			Box A		Box B	
Mouse ODC		L P R	V62 T63 P64	F Y A	V68 K69 C70 N71 D72 S73	
<i>S. ruminantium</i> LDC wild type		L P R	A44 G45 V46	F Y A	M50 K51 A52 N53 P54 T55	0.69 ± 0.07 (n=6)
<i>S. ruminantium</i> LDC mutant						
1 V46P		L P R	A G P F	Y A M K A N P T		0.43 ± 0.00 (n=2)
2 A44V/G45T/V46P		L P R	V T P F	Y A M K A N P T		1.1 ± 0.1 (n=2)
3 M50V		L P R	A G V F Y A	V K A N P T		0.64 ± 0.00 (n=2)
4 T55S		L P R	A G V F Y A	M K A N S		0.66 ± 0.01 (n=2)
5 A52C		L P R	A G V F Y A	M K C N P T		1.2 ± 0.3 (n=4)
6 P54D		L P R	A G V F Y A	M K A N D T		1.8 ± 0.1 (n=2)
7 M50V/A52C		L P R	A G V F Y A	V K C N P T		1.9 ± 0.1 (n=2)*
8 P54D/T55S		L P R	A G V F Y A	M K A N D S		1.8 ± 0.1 (n=2)
9 A52C/P54D		L P R	A G V F Y A	M K C N D T		2.6 ± 0.2 (n=2)
10 M50V/P54D/T55S		L P R	A G V F Y A	V K A N D S		1.4 ± 0.1 (n=2)
11 M50V/A52C/P54D		L P R	A G V F Y A	V K C N D T		2.4 ± 0.3 (n=2)*
12 A52C/P54D/T55S		L P R	A G V F Y A	M K C N D S		2.7 ± 0.6 (n=4)**
13 M50V/A52C/P54D/T55S		L P R	A G V F Y A	V K C N D S		4.0 ± 0.1 (n=2)**
14 A44V/G45T/V46P/A52C		L P R	V T P F	Y A M K C N P T		N.D.
15 A44V/G45T/V46P/P54D		L P R	V T P F	Y A M K A N D T		2.6 ± 0.2 (n=2)
16 BoxA/M50V/A52C		L P R	V T P F	Y A V K C N P T		N.D.
17 BoxA/A52C/P54D		L P R	V T P F	Y A M K C N D T		N.D.
18 BoxA/BoxB		L P R	V T P F	Y A V K C N D S		N.D.

Mutant No.	Enzyme	Residue number			Ornithine preference
		316	321	326	
			Box C		
Mouse ODC		S S	I355 W356 G P T359 C360 D361 G L363 D364		
<i>S. ruminantium</i> LDC wild type		S T	F318 G319 G P S322 C323 D324 G I326 D327		0.69 ± 0.07 (n=6)
<i>S. ruminantium</i> LDC mutant					
19 G319W		S T F	W G P S C D G I D		1.1 ± 0.1 (n=2)
20 F318I/G319W		S T I W	G P S C D G I D		1.1 ± 0.0 (n=2)
21 S322T		S T F G G P	T C D G I D		1.2 ± 0.1 (n=2)
22 I326L		S T F G G P S C D G	L D		1.0 ± 0.1 (n=2)
23 S322T/I326L		S T F G G P	T C D G L D		1.3 ± 0.2 (n=2)
24 S322A		S T F G G P	A C D G I D		3.9 ± 0.4 (n=2)

Mutant No.	Enzyme	Residue number		Ornithine preference
		41	55	
25 BoxA/P54D /F318I/G319W		L41 P R V T P F Y A M K A N D T55 S316 T W G P S C D G I D330	4.1 ± 0.2**
26 BoxA/P54D /S322T/I326L		L41 P R V T P F Y A M K A N D T55 S316 T F G G P T C D G L D330	4.4 ± 0.2 (n=2)
27 BoxA/BoxB /F318I/G319W		L41 P R V T P F Y A V K C N D S 55 S316 T I W G P S C D G I D330	N.D. weak activity
28 BoxA/BoxB /S322T/I326L		L41 P R V T P F Y A V K C N D S 55 S316 T F G G P T C D G L D330	N.D.
29 BoxA/P54D /S322A		L41 P R V T P F Y A M K A N D T55 S316 T F G G P A C D G I D330	34 ± 0**

FIG. 5. Comparison of amino acid sequences conferring substrate specificity upon eukaryote ODCs or *S. ruminantium* LDC. *S. ruminantium* LDC numbering is used to define residue position numbers. Residues K51 and C323 match K69 and C360 in mouse ODC, respectively. The mutated positions are in boldface and circled. Ornithine preference is expressed as the ODC/LDC activity ratio. Cells of *E. coli* DH5 α harboring each plasmid were grown in 2 ml of LB medium containing ampicillin (100 μ g/ml) at 37°C for 18 h and collected by centrifugation at 4°C. Cells suspended in 1 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM PLP and 5 mM dithiothreitol were sonicated. The LDC or ODC activity in cell extracts was measured by the amount of cadaverine or putrescine formed from lysine or ornithine by HPLC using a TSK-gel Polyaminepak column as described in Materials and Methods. The incubation mixture contained 0.5 M sodium acetate buffer (pH 6.0), 5 mM L-lysine or L-ornithine, 50 μ M PLP, and the cell extracts in a total volume of 50 μ l. The reaction mixture was incubated at 37°C for 10 min. Under these conditions, the

XXE(or D)X] by casein kinase II in mouse ODC (29) was also conserved. (v) Residues 117 to 140 in mouse ODC were identified as the antizyme-binding region (Fig. 3, large box), in which basic residues K121, K141, and R144 have been suggested to be pivotal in antizyme binding (19). In *S. ruminantium* LDC, basic residues K103, K123, and K126 correspond to these amino acids in mouse ODC (Fig. 3, residues marked with vertical arrows). In the preceding paper (32), we observed a dramatic decrease in the LDC protein level in early stationary phase due to rapid degradation. The presence of the proposed antizyme recognition site in *S. ruminantium* LDC suggests the presence of a similar mechanism for its degradation during entry into the stationary phase of *S. ruminantium* cells.

Comparison of the secondary-structure assignments of *S. ruminantium* LDC and a truncated form of mouse ODC in which the C-terminal 37 residues are missing revealed approximately 70% secondary-structure identity overall (data not shown). This suggests a strong similarity between the three-dimensional structures of *S. ruminantium* LDC and eukaryote ODCs. Grishin et al. (6) proposed a topographic model of the PLP-binding domain of eukaryotic ODC through the analysis of known barrel structures of ODC. They classified the PLP-utilizing enzymes into seven fold types from the predicted secondary structures, and they showed that the eukaryotic ODCs belong to fold type III while bacterial ODCs and LDCs belong to fold type I. We propose that *S. ruminantium* LDC belongs to fold type III.

Most recently, whole genome sequences of *Aquifex aeolicus* (4) and *Thermotoga maritima* (22), which have been placed as the deepest and most slowly evolving lineages among the *Eubacteria* (24), were reported. After a homology search of the amino acid sequences corresponding to putative ODCs from these bacteria, we noticed that the putative ODCs of these bacteria show 35% identity overall with *S. ruminantium* LDC. Interestingly, in *T. maritima* ODC, 22 of the 26 amino acid residues, K69, D88, E94, D134, R154, K169, H197, G235, G236, G237, F238, E274, G276, R277, Y278, K294, C360, D361, D364, G387, Y389, and F397 (mouse ODC numbering), which were described above as being essential for ODC activity, are also conserved, with D233 and Y323 of *S. ruminantium* LDC being replaced with N and F in *T. maritima* ODC. Taken together with the 35% sequence identity between *S. ruminantium* LDC and eukaryotic ODC, these observations indicate the occurrence of a bacterial group which includes the eukaryotic ODC in the putative bacterial ODCs. The data also suggest that the origin of the ODCs of *S. ruminantium* and the two species of eubacteria mentioned above is completely different from that of the ODCs of general bacteria, including *E. coli*, and that eukaryotic ODC and *S. ruminantium* LDC have the same origin.

Identification of the amino acid residues conferring substrate specificity upon *S. ruminantium* LDC. Eukaryotic ODC has absolute specificity as a decarboxylase for L-ornithine. In contrast, *S. ruminantium* LDC decarboxylates both L-lysine and L-ornithine with similar K_m and V_{max} values (32). Here, we identified the amino acid residue(s) in the catalytic domain of LDC that confers decarboxylase activity toward L-ornithine and/or L-lysine. To identify the amino acid residue(s) conferring substrate specificity on *S. ruminantium* LDC, we consid-

ODC and LDC activities of *E. coli* itself were almost negligible. Each value is the mean \pm the standard deviation. In parentheses, n represents the number of experiments. Asterisks indicate mutations that caused loss of specific activities. The ODC activities of mutants 7 and 11 (*) and mutants 12 and 13 (**) were about 1/20 and 1/4, respectively, of that of wild-type LDC. N.D., not detected.

TABLE 2. Kinetic parameters for the decarboxylation of lysine and ornithine by wild-type and mutant LDCs and mouse ODC^a

Line no.	Mutant no.	Enzyme	K_m (mM)		k_{cat} (s ⁻¹)		k_{cat}/K_m ratio		Substrate specificity
			Lysine	Ornithine	Lysine	Ornithine	Lysine	Ornithine	
1		Mouse ODC ^b	5.2	0.090	1.8	7.8	350	87,000	250
2		Wild-type rLDC	1.5	0.96	16	8.6	11,000	8,900	0.83
3	2	A44V/G45T/V46P	3.0	0.98	13	8.4	4,300	8,600	2.0
4	5	A52C	1.9	1.3	10	7.0	5,500	5,500	1.0
5	6	P54D	5.7	2.1	16	13	2,900	6,300	2.2
6	9	A52C/P54D	2.4	1.6	6.8	7.2	2,900	4,500	1.6
7	13	M50V/A52C/P54D/T55S	3.6	4.5	2.6	4.7	700	1,000	1.5
8	15	A44V/G45T/V46P/P54D	8.0	3.1	9.8	14	1,200	4,700	3.8
9	19	G319W	4.0	1.3	5.6	7.2	1,400	5,400	3.9
10	23	S322T/I326L	17	1.5	21	24	1,200	16,000	13
11	24	S322A	6.7	1.2	4.8	20	720	17,000	24
12	26	A44V/G45T/V46P/P54D/S322T/I326L	22	3.3	19	36	850	11,000	13
13	29	A44V/G45T/V46P/P54D/S322A	270	4.0	5.5	4.8	21	1,200	58

^a Each reaction was carried out in 0.5 M sodium acetate buffer containing 50 μ M PLP and 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; pH 6.0) at 30°C. Substrate specificity was defined as the k_{cat}/K_m ratio obtained with ornithine relative to that obtained with lysine as the substrate.

^b Described in reference 25.

ered 15-residue and 12-residue segments neighboring K51 (K69 in mouse ODC) and C323 (C360 in mouse ODC), respectively, of the catalytic domain of *S. ruminantium* LDC (Fig. 5) as candidates for regulators of the substrate specificity of *S. ruminantium* LDC. In the PLP-binding domain, residue K51 (K69 in mouse ODC) could be crucial for binding to PLP by forming an internal Schiff base between the ϵ -NH₂ of K69 and the aldehyde group of PLP based on the evidence from eukaryotic ODCs (19, 28). The internal Schiff base is replaced with an external Schiff base between L-ornithine and PLP when the enzyme is incubated with L-ornithine as a substrate. In the 15-residue alignment centered around K51 and K69 between *S. ruminantium* LDC and mouse ODC, two regions that contain different amino acid residues, i.e., (i) A44 G45 V46 and V62 T63 P64 (box A in Fig. 5A) and (ii) M50 K51 A52 N53 P54 T55 and V68 K69 C70 N71 D72 S73 (box B in Fig. 5A) (residues differing between them are underlined), were found. On the other hand, four different amino acid residues (underlined), i.e., F318 G319 G320 P321 S322 C323 D324 G325 I326 and I355 W356 G357 P358 T359 C360 D361 G362 L363 were found in the 12-residue segment including C323 (Fig. 5B). To find whether or not the residues conferring the dual-specificity decarboxylase activity on *S. ruminantium* LDC exist in these segments, we generated 29 mutant LDCs corresponding to the amino acid residues in boxes A, B, and C individually or all together by constructing plasmids containing a series of mutant genes by site-directed mutagenesis using the primers shown in Table 1. As a result of the manipulations, 29 different plasmids which correspond to the mutant proteins listed in Fig. 5A, B, and C were obtained. The mutant proteins were expressed in *E. coli* DH5 α harboring the appropriate plasmid. First, we measured the decarboxylase activities of mutant LDCs 1 through 13 toward L-lysine and L-ornithine after 10 min of incubation at 37°C, using the sonicated extracts, and calculated the ODC/LDC activity ratio. With respect to the mutations in boxes A and B, mutants 1, 3, and 4, mutant 7, mutants 8 and 10, and mutants 11 and 12 showed ODC/LDC activity ratios similar to those of wild-type LDC, mutant 5, mutant 6, and mutant 9, respectively (Fig. 5A). Therefore, mutant proteins 2, 5, 6, 9, and 13 were purified to electrophoretic homogeneity from the crude extract of the recombinant *E. coli* cells and used in kinetic analyses. The purified recombinant wild-type and mutant protein preparations were analyzed by CD spectrometry to determine whether global structural changes were induced

by the mutations. The far-UV CD spectra of the mutant LDCs were similar to that of wild-type LDC (data not shown). These results suggest that no major conformational alterations occurred in the mutant enzymes.

By determination of the k_{cat}/K_m ratio (catalytic efficiency), one can obtain a lower limit of the second-order rate constant for conversion of a substrate to a product (25). Combining effects due to substrate binding and transition state stabilization, this parameter is useful for assessment of altered substrate specificity. The kinetic parameters for decarboxylation of L-lysine and L-ornithine by wild-type and mutant LDCs were determined from the initial rate measurements, and the results are listed in Table 2. When the substrate specificity was defined as the k_{cat}/K_m ratio obtained with L-ornithine relative to that obtained with L-lysine as the substrate, the following findings became evident. (i) Mutant 2, in which all three of the amino acid residues of *S. ruminantium* LDC in box A were replaced with the corresponding ones of mouse ODC, showed a substrate specificity ratio of 2.0 (Table 2, line 3). (ii) Mutant 13, in which residues M50, A52, P54, and T55 of LDC were replaced with corresponding residues V68, C70, D72, and S73 of mouse ODC, converting the entire series of residues in box B of LDC to the corresponding ones of ODC, had a substrate specificity ratio of 1.5 (Table 2, line 7). (iii) Box B single mutants 5 and 6 showed substrate specificity ratios of 1.0 and 2.2, respectively (Table 2, lines 4 and 5), suggesting that P54 is involved in conferring substrate specificity on *S. ruminantium* LDC. (iv) Double mutant 9 (A52C/P54D) had a substrate specificity ratio of 1.6 (Table 2, line 6). These data suggest that the three-residue segment of box A and P54 of box B together are involved in conferring decarboxylase activities toward both L-lysine and L-ornithine on *S. ruminantium* LDC. Accordingly, we created mutant A44V/G45T/V46P/P54D, in which all of the amino acid residues of *S. ruminantium* LDC in box A and P54 in box B were replaced with the corresponding residues of mouse ODC. The mutant protein was expressed in *E. coli* with plasmid pA44V/G45T/V46P/P54D, purified to electrophoretic homogeneity, and then analyzed for decarboxylase activity toward both substrates. The resulting mutant, 15, showed a substrate specificity ratio of 3.8, which is 4.6 times that of wild-type *S. ruminantium* LDC (Table 2, line 8). Mutant 18, in which all of the residues of LDC in both boxes A and B were replaced with the corresponding residues from mouse ODC, had no enzyme activity toward either L-lysine or L-ornithine. Thus, it

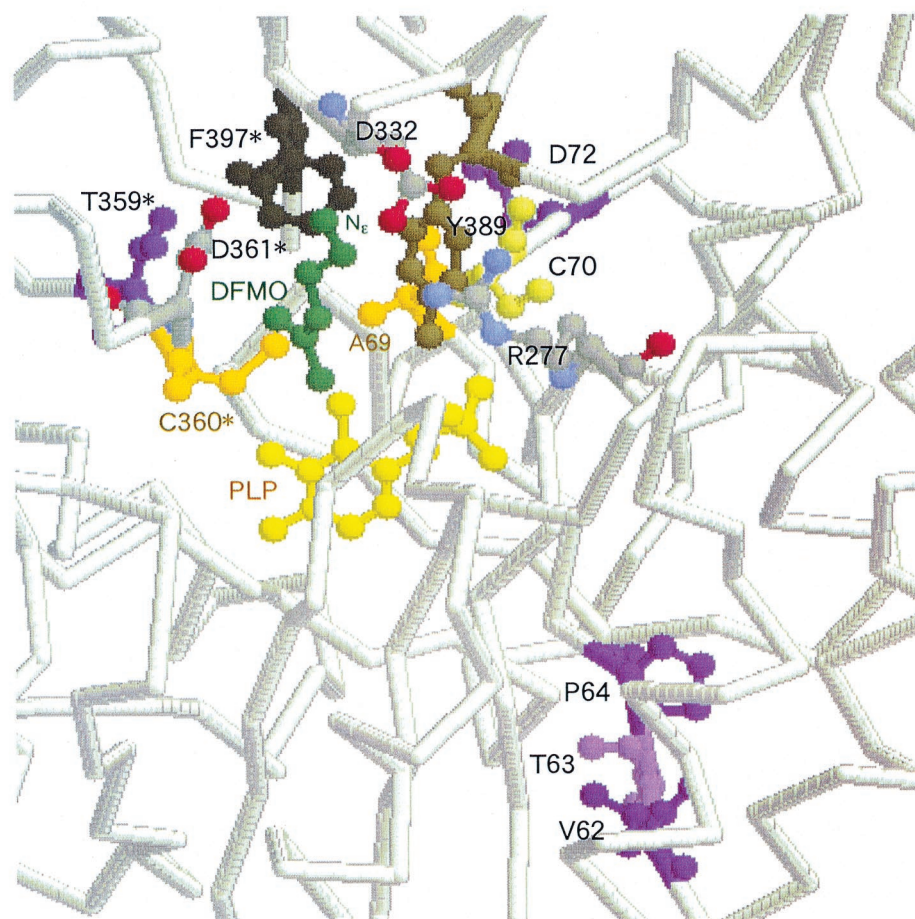


FIG. 6. Active site of K69A *T. brucei* ODC in complex with DFMO. The figure was drawn based on the data of reference 7 using the RasMol 2.6 Molecular Graphics Visualisation tool (Glaxo Wellcome Research & Development Co., Stevenage, Herts., United Kingdom). In reference 7, the N terminus of recombinant *T. brucei* ODC corresponds to the 21st residue of intact ODC (Fig. 3). Residues (V62, T63, P64, D72, and T359) which correspond to the residues mutated in this study and were identified as functionally important for substrate specificity in *S. ruminantium* LDC are purple and violet. The atoms of PLP are yellow, the atoms of DFMO are green, A69 and C360 are orange, and Y389 and F397, which cradle the aliphatic portion of DFMO, are brown. R277, D332, and D361 are also shown, and nitrogen and oxygen atoms of these residues are blue and red, respectively. The asterisked residues belong to the other subunit of the dimer.

was concluded that in the N-terminal domain of the active site of *S. ruminantium* LDC, residues A44, G45, V46, and P54 together confer higher decarboxylating activity toward L-lysine than toward L-ornithine upon *S. ruminantium* LDC.

Next, the C-terminal domain in the active center of *S. ruminantium* LDC was examined. Sonic extracts from the cells in which the mutant LDCs were expressed were used as enzyme preparations, the decarboxylase activities of mutant LDCs 19 through 24 toward L-lysine and L-ornithine after 10 min of incubation at 37°C were measured, and the ODC/LDC activity ratio was calculated. Mutant LDCs 19 to 23 showed the same ODC/LDC activity ratio, which was twice that of wild-type LDC (Fig. 5B). Mutant 24 (S322A) had about 5.7 times the L-ornithine decarboxylation activity of wild-type LDC. Mutant proteins 19, 23, and 24 were purified to electrophoretic homogeneity, and their decarboxylase activities toward both substrates were measured; the substrate specificity ratios were 3.9, 13, and 24, respectively. Thus, we concluded that in the C-terminal domain of the active site of *S. ruminantium* LDC, residue S322 also contributes to the higher decarboxylating activity toward L-lysine.

Finally, we generated mutants 26 and 29 (double mutants of mutants 15 and 23 and mutants 15 and 24, respectively). The

two mutant proteins were purified, K_m and k_{cat} were measured, and the substrate specificity ratios were calculated. Surprisingly, mutant 29 showed a K_m value of 270 for L-lysine, 180 times that of wild-type rLDC, resulting in a substrate specificity ratio of 58, which is approximately 70 times that of wild-type rLDC. The substrate specificity of mutant 26 was the same as that of mutant 23. Thus, it was concluded that five amino acid residues (A44, G45, V46, P54, and S322) confer the dual decarboxylation activities toward both L-lysine and L-ornithine in the case of *S. ruminantium* LDC and to similar extents. Consequently, we have succeeded in converting *S. ruminantium* LDC to an enzyme with a preference for L-ornithine with a substrate specificity ratio of approximately 58 by constructing mutant protein A44V/G45T/V46P/P54D/S322A.

The role of each substitution may be proposed from an analysis of the X-ray crystal structure of *T. brucei* ODC (TbODC) complexed with a substrate analog, DFMO (Fig. 6 and reference 7). The N_ϵ of DFMO is bound between two acidic residues, D361 and D332, which are contributed to the active site by opposite subunits. S322 in box C (T359 in TbODC) might influence substrate binding through second-shell interaction with the D361 loop. Since the other residues in boxes A and B, A44, G45, V46, and P54, which correspond

to V62, T63, P64, and D72 in TbODC, respectively, are positioned farther away from the substrate, it is more difficult to speculate on their effects. The X-ray structure of TbODC also shows that the aliphatic portion of DFMO is cradled by the aromatic rings of F397 and Y389 and also forms a hydrogen bond with the PLP phosphate and that R277 is also involved in substrate binding as a second-shell residue. A salt bridge is formed between R277 and D332 (2.9 Å), the residue that forms a hydrogen bond with N_ε of DFMO. A52 in box B (C70 in TbODC) may therefore influence substrate binding through second-shell interactions with Arg277 and/or Y389. However, mutation of P54, the residue positioned farther than A52 from the substrate, had a more significant effect on substrate specificity (Table 2, lines 4 and 5). Because the residues in box A are 12 Å below the PLP ring, it is impossible to speculate on the effect of the A44V/G45T/V46P substitutions. It is likely that the residues which seem not to be in a position to influence substrate binding have an effect on substrate specificity alteration. Yano et al. (34) modified the substrate specificity of aspartate aminotransferase, and they identified six amino acid residues which contributed to the substrate specificity change. Interestingly, only one of the six residues was located at a distance allowing direct interaction with the substrate and the other residues were positioned farther away from the substrate, even though one of these was located >10 Å from the substrate-binding site.

Based on the X-ray structure of TbODC, Grishin et al. (7) have suggested that the distance between the Schiff base nitrogen and the D361-D332 pair may act as a ruler to select for a side chain with a length equivalent to that of L-ornithine. Our studies are focused on the X-ray diffraction analysis of wild-type DFML-bound and uncomplexed *S. ruminantium* rLDC and mutant 29 in order to improve our understanding of the relationship between the protein structure and L-lysine and L-ornithine recognition of *S. ruminantium* LDC.

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