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Purification and Properties of *Selenomonas ruminantium* Lysine Decarboxylase

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Selenomonas ruminantium, a strictly anaerobic, gram-negative bacterium isolated from sheep rumen, contains lysine decarboxylase (Y. Kamio et al., J. Bacteriol. **145**:122–128, 1981). This report describes the synthesis, purification, and characterization of the enzyme. Lysine decarboxylase was synthesized in cells grown in chemically defined medium without lysine. The enzyme was purified approximately 1,800-fold to electrophoretic homogeneity. The native enzyme of approximate molecular weight 88,000 consisted of two identical subunits, each with a molecular weight of 44,000. Several properties of the enzyme were determined and compared with those of the lysine decarboxylases from *Escherichia coli* and *Bacterium cadaverisis*.

There are two types of bacterial amino acid decarboxylase, constitutive and inducible. The former is produced constitutively in a low amount and includes decarboxylases for L-ornithine, L-arginine, *S*-adenosyl-L-methionine, and diaminopimelic acid (4). A previously characterized lysine decarboxylase that participates in the synthesis of cadaverine, is inducible by adding lysine to the medium (4, 16).

Kamio and Takahashi (8, 9) and Kamio et al. (7) previously reported that the wild type of *Selenomonas ruminantium* subsp. *lactilytica*, a strictly anaerobic gram-negative bacterium, contains neither the free nor bound form of cell envelope lipoprotein, but does contain cadaverine as a component of its peptidoglycan. We have shown (6) that a primary unit of the peptidoglycan in *S. ruminantium* is composed of *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanine and that cadaverine is covalently linked to an α-carboxyl group of a D-glutamic acid residue of the peptidoglycan. Furthermore, we showed recently (10) that cadaverine is synthesized from L-lysine and transferred to the α-carboxyl group of the D-glutamic acid residue of the peptidoglycan lipid intermediate in *S. ruminantium*. These findings suggest that lysine decarboxylase should be produced constitutively in this bacterium. In this paper, we show that the lysine decarboxylase of *S. ruminantium* is a constitutive enzyme and report some of its properties.

MATERIALS AND METHODS

Reagents. L-[U-^{14}C]lysine hydrochloride, Protosol, and Omnifluor were purchased from New England Nuclear Corp., Boston, Mass. L-[1-^{14}C]ornithine hydrochloride and DL-*meso*-2,6-diamino[*G*-^{3}H]pimelic acid dihydrochloride were obtained from Radiochemical Centre, Amersham, England. Other chemicals used were of the best grade commercially available.

Sea sand (15 to 200 mesh) was purchased from Junsei Chemicals Ltd., Tokyo, Japan. It was washed with 0.1 N hydrochloric acid and distilled water and dried.

Bacterial strain. *S. ruminantium* subsp. *lactilytica*, described previously (10), was used.

Media and culture conditions. *S. ruminantium* cells were grown in chemically defined medium (CD medium) or a yeast extract-glucose medium (YGV medium) (Table 1) at 37°C under anaerobic conditions (8).

Preparation of cell paste. Large-scale batch cultures of *S. ruminantium* were grown in 1,000 liters of YGV medium. The cells were incubated at 37°C, harvested at mid-exponential phase by continuous centrifugation, and washed once with 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 5 mM magnesium chloride. The packed cells were stored at −80°C until preparation of the enzyme.

Incubation mixture and enzyme assay. Two methods were used to measure lysine decarboxylase activity. The first method was a modification of that described by Morris and Pardee (13). The incubation mixture contained 100 mM potassium phosphate buffer (pH 6.0), 10 mM L-[*U*-^{14}C]lysine (231,000 cpm), 0.1 mM pyridoxal-5′-phosphate (PALP), and the enzyme fraction in a total volume of 100 μl. The reaction mixture was incubated at 41°C for 30 min. The reaction was stopped by adding 300 μl of 50% H_3PO_4, and the radioactivity of $^{14}CO_2$ adsorbed in a Protosol-soaked filter paper was counted in a Packard 3255 liquid scintillation counter with toluene scintillation fluid containing Omnifluor.

The second method measured the amount of cadaverine formed from lysine. For quantitative determination of cadaverine, a portion of the reaction mixture

TABLE 1. Composition of the media used

Component	Concn (%) in:	
	CD medium	YGV medium
Ammonium sulfate	0.09 (wt/vol)	0.09 (wt/vol)
Mineral solution 3[a]	5.0 (vol/vol)	5.0 (vol/vol)
Trace mineral solution[b]	0.5 (vol/vol)	—[c]
Ferrous sulfate solution (0.22%)	0.2 (vol/vol)	—
Vitamin solution[a]	0.5 (vol/vol)	—
Sodium carbonate	0.3 (wt/vol)	0.3 (wt/vol)
Resazurin solution (0.1%)	0.1 (vol/vol)	0.1 (vol/vol)
Cysteine hydrochloride	0.075 (wt/vol)	0.075 (wt/vol)
Glucose	0.5 (wt/vol)	0.5 (wt/vol)
Yeast extract	—	0.05 (wt/vol)
Sodium *n*-valerate	0.01 (wt/vol)	0.01 (wt/vol)
CO_2 gas phase	100	100

[a] See reference 20.
[b] See reference 17.
[c] —, No addition.

was applied to a cellulose thin-layer plate for electrophoresis, with cadaverine used as the standard (7). When L-ornithine, L-arginine, and diaminopimelic acid were used as the substrates, the concentration of each was 10 mM. Decarboxylation of diaminopimelic acid was assayed by cellulose thin-layer electrophoresis, and the radioactivity incorporated into the product was counted.

Specific activity. One unit of enzyme activity was defined as 1 μmol of CO_2 formed from L-lysine at 41°C in 1 min. Specific activity is given as units per milligram of protein.

Protein determination. Protein content was determined by the method of Lowry et al. (11), with bovine serum albumin used as the standard.

PAGE and SDS-PAGE. Slab polyacrylamide gel electrophoresis (PAGE) of purified lysine decarboxylase was carried out in an 8% gel prepared in 0.1 M sodium phosphate buffer (pH 7.2); the gel was electrophoresed overnight before the sample was applied. Protein was detected by staining the gel with 0.0025% Coomassie brilliant blue in 25% ethanol–10% acetic acid. Decarboxylase activity was determined on 1-mm gel slices dispersed in the assay buffer overnight at 4°C. Sodium dodecyl sulfate (SDS)-PAGE was carried out as described by Weber and Osborn (21).

Estimation of molecular weight. The molecular weight of the native form of the enzyme was estimated by gel filtration with Sephadex G-100. The following potassium phosphate buffers with different ionic strength (μ) and pH were used as elution buffers. Buffer A: μ, 0.02 (pH 7.8); buffer B: μ, 0.04 (pH 7.5); buffer C: μ, 0.10 (pH 7.0); and buffer D: μ, 0.10 (pH 6.5). The subunit molecular weight was determined by SDS-PAGE.

Isoelectric focusing. The purified enzyme preparation was placed in the middle of a glycerol gradient containing 2% Ampholine (comprising 0.4% pH 3.5–10, 0.8% pH 4–6, and 0.8% pH 6–8) in a 110-ml LKB electrofocusing column. Electrofocusing was carried out at 4°C at 400 V for 18 h and then at 500 V for an additional 2 h. Fractions were collected and analyzed for enzyme activity and pH.

Amino acid analysis. Amino acid analysis was performed on a Hitachi (Tokyo, Japan) 835 automatic amino acid analyzer. The purified enzyme preparation was hydrolyzed in 6 N HCl for 24 and 48 h at 110°C in a vacuum. Cysteine plus cystine content was determined after performic oxidation and hydrolysis of the enzyme preparation for 24 h by the method of Moore (12). Determination of free sulfhydryl groups was carried out by titration with 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 8 M urea by the method of Ellman (5). Determination of tryptophan levels was carried out in 6 M guanidine hydrochloride by the method of Bencze and Schmid (1).

Preparation of pyridoxal phosphate-Sepharose 4B. Pyridoxal phosphate-Sepharose 4B was prepared by the method of Ryan and Fottrell (15). Cyanogen bromide-activated Sepharose 4B was prepared by the method of Porath (14).

Purification of lysine decarboxylase from *S. ruminantium*. All operations were carried out at 4°C or in an ice bath except as noted. The concentration of NaCl or potassium phosphate was determined by conductivity measurements.

Frozen cell paste (100 g) derived from 100 liters of culture was ground for 5 min with 200 g of sea sand at 4°C. To the ground materials was added 300 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 5 mM $MgCl_2$. The suspension was centrifuged at 10,000 × *g* for 30 min, and the supernatant was collected. Crude extract was also prepared after cells were disrupted with either a sonic oscillator or a French pressure cell. The specific activity of lysine decarboxylase in these preparations was lower than that obtained in those prepared with sea sand.

Purification steps. (i) Treatment with ammonium sulfate. Solid ammonium sulfate was added with stirring to the enzyme fraction to 40% saturation. After 30 min, the supernatant was collected by centrifugation and brought to 60% saturation in the same manner. After 1 h, the precipitate was collected by centrifugation and dissolved in 1 liter of 20 mM potassium phosphate buffer (pH 6.5) (buffer A). The enzyme preparation was dialyzed three times against 20 liters of buffer A.

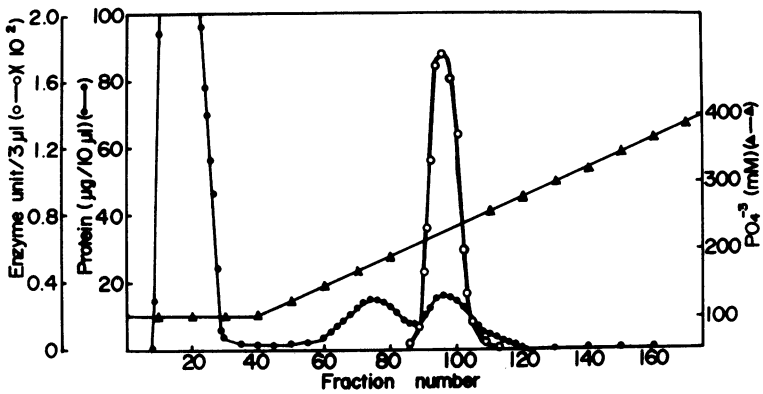

FIG. 1. Purification of lysine decarboxylase from *S. ruminantium* on a hydroxylapatite column chromatography. The conditions are described in the text. Symbols: O, enzyme activity; ●, protein; ▲, PO_4^{-3} concentration.

(ii) DEAE-cellulose and DEAE-Bio-Gel chromatography. The enzyme fraction obtained by ammonium sulfate treatment was applied to a DEAE-cellulose column (4.5 by 140 cm) previously equilibrated with buffer A. The column was washed with 4 liters of buffer A and then with 4 liters of 0.1 M NaCl in buffer A. The enzyme was then eluted with a linear gradient created by mixing 2 liters of 0.1 M NaCl in buffer A and 2 liters of 0.5 M NaCl in buffer A. The enzyme fractions which eluted from 0.23 to 0.28 M NaCl were pooled. After being dialyzed against buffer A, the enzyme preparation was placed onto a DEAE-Bio-Gel column (3.5 by 85 cm) equilibrated with buffer A. The column was washed with 0.1 M NaCl in buffer A and then eluted with a linear gradient of NaCl in buffer A from 0.1 to 0.35 M. The enzyme fractions which eluted from 0.15 to 0.18 M NaCl were pooled. The enzyme preparation thus obtained was precipitated by ammonium sulfate and dialyzed against buffer A.

(iii) Acid precipitation. The enzyme preparation obtained by DEAE-Bio-Gel chromatography was adjusted to pH 4.4 with 0.1 M KH_2PO_4. Precipitated protein was removed by centrifugation. Most of the lysine decarboxylase activity remained in the supernatant.

(iv) Heat treatment. The enzyme preparation obtained by acid treatment was dialyzed against 0.1 M potassium phosphate buffer (pH 6.8) containing 0.1 mM PALP. The dialyzed enzyme preparation was rapidly brought to 60°C by swirling in a 70°C water bath and maintained at this temperature for 5 min. The suspension was then cooled in an ice bath. After centrifugation, the supernatant was collected and dialyzed against buffer A.

(v) Hydroxylapatite chromatography. The dialyzed enzyme preparation (270 mg) was applied to a hydroxylapatite column (1.1 by 20 cm) equilibrated with buffer A. The column was first washed with 50 ml of buffer A and 45 ml of 0.1 M potassium phosphate buffer (pH 6.5) and then eluted with a linear gradient of potassium phosphate buffer (pH 6.5) from 0.1 to 0.4 M. The flow rate for the elution was 0.2 ml/min. Approximately 60% of the enzyme activity applied was recovered in the fraction which was eluted by 0.22 to 0.24 M phosphate (Fig. 1). The enzyme fraction was dialyzed against buffer A.

(vi) Pyridoxal phosphate column chromatography. The enzyme preparation obtained by hydroxylapatite chromatography was applied onto a pyridoxal phosphate column (0.5 by 2.5) which had been equilibrated with buffer A (see Fig. 2). The column was washed with the starting buffer and then with 0.3 M phosphate buffer (pH 6.5). After being washed with 5 mM potassium phosphate buffer (pH 5.5), the column was eluted with the same buffer containing PALP (2 mg/ml). The fractions with high activity were pooled, dialyzed against buffer A, and stored at 4°C.

RESULTS

Lysine decarboxylase activity in various growth conditions. *S. ruminantium* cells were grown in CD or YGV medium to mid-exponential phase, and the lysine decarboxylase activity was determined. The specific activity of the enzyme was 0.0026 and 0.0033 U/mg of protein in the cells grown in CD or YGV medium, respectively. The enzyme content was unchanged by addition of lysine or glucose up to concentrations of 1% (wt/vol). These findings suggest strongly that in *S. ruminantium* cells lysine decarboxylase is produced constitutively.

Enzyme purification. The steps in the enzyme purification procedure and the yield from each step are shown in Table 2. Lysine decarboxylase from *S. ruminantium* cells remained in a pH-4.4 supernatant, in contrast to the enzyme from *Escherichia coli*, which precipitates readily below pH 5.4 (16). Heat treatment without PALP diminished the enzyme activity markedly, as was observed for *E. coli* (16). The heat treatment was very effective in removing an unidentified material that precipitated as a transparent gel, since in subsequent hydroxylapatite chromatography only about 10% of the enzyme activity was recovered when an unheated sample was applied to the column.

Although hydroxylapatite chromatography

TABLE 2. Purification of the lysine decarboxylase from *S. ruminantium*

Step no. and process	Total vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield %
1. Supernatant from crude extract[a]	3,500	578	175,000	0.0033	100
2. Ammonium sulfate (30 to 60%) precipitate	1,500	434	71,000	0.0061	75.1
3. DEAE-cellulose and DEAE-Bio-Gel chromatography	230	262	3,500	0.075	45.3
4. pH-4.4 supernatant and 70% ammonium sulfate precipitate	30	182	700	0.26	31.5
5. Heat-treated supernatant	25	129	270	0.48	22.3
6. Hydroxylapatite chromatography	21	76	17	4.47	13.1
7. Pyridoxal phosphate chromatography	2	44	7.4	5.95	7.61

[a] Prepared from the cells of 1,000-liter culture.

gave about 10-fold purification, some contaminating proteins with molecular weights of about 70,000, 65,000, and 55,000 were found in the enzyme preparation. Therefore, a final purification was achieved by pyridoxal phosphate chromatography. In this step, all of the contaminating proteins were eluted by 300 mM potassium phosphate buffer (Fig. 2). As a result, the enzyme was purified about 1,800-fold, and the specific activity of the purified enzyme was 5.95 U/mg of protein.

Purity and molecular weight of the enzyme. PAGE of the purified enzyme preparation showed a single band of protein (Fig. 3A). The enzyme activity was found to comigrate with the protein band.

The molecular weight of the native enzyme was 88,000 as determined by Sephadex G-100 gel filtration with four buffers having various pHs and ionic strengths as described above (Fig. 4A). The molecular weight of its subunit was determined by PAGE of the purified enzyme preparation in the presence of SDS. A single band was found in the gel, and the protein was estimated at 44,000 molecular weight, indicating that the native enzyme contained two subunits identical in molecular weight (Fig. 3B and 4B).

Substrate specificity. The purified enzyme preparation decarboxylated L-lysine. Neither L-ornithine, L-arginine, nor diaminopimelic acid was able to act as substrate. Production of $^{14}CO_2$ from L-[^{14}C]lysine was not inhibited despite the

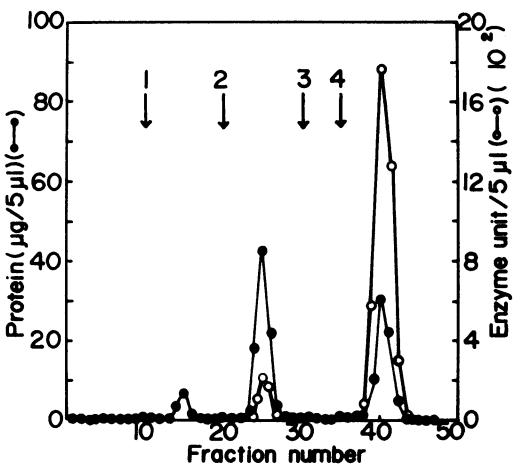

FIG. 2. Adsorption and elution of lysine decarboxylase from *S. ruminantium* on a pyridoxal phosphate-Sepharose 4B column. The conditions are described in the text. At the designated intervals the following reagents were introduced into the column: 1, 0.1 M phosphate buffer (pH 6.5); 2, 0.3 M phosphate buffer (pH 6.5); 3, 5 mM phosphate buffer (pH 5.5); and 4, 5 mM phosphate buffer (pH 5.5) containing pyridoxal phosphate (2 mg/ml). Symbols: ●, protein; ○, enzyme activity.

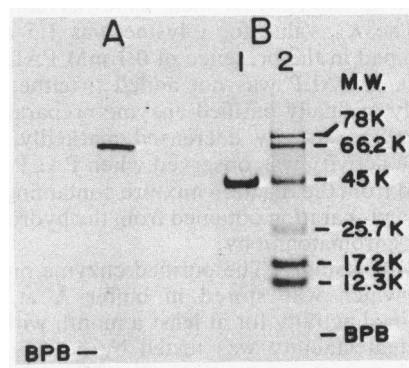

FIG. 3. (A) PAGE and (B) SDS-PAGE patterns of the purified lysine decarboxylase preparation from *S. ruminantium*. Molecular weight (M.W.) standards are described in the legend to Fig. 4. BPB, Bromophenol blue dye.

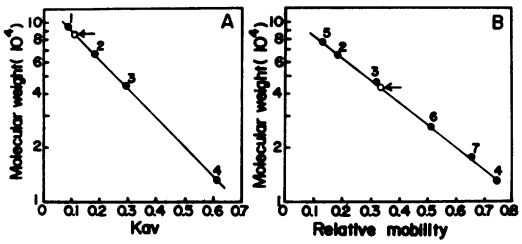

FIG. 4. Determination of the molecular weight of the native (A) and subunit (B) forms of lysine decarboxylase from *S. ruminantium* on Sephadex G-100 and by SDS-PAGE, respectively. Molecular weight standards were filtered individually over the Sephadex G-100 column, and their elutions were monitored by absorbance at 280 nm. The elution volume of each standard was used to calculate the K_{av}, and a standard molecular weight curve was plotted. The relative mobility in SDS-PAGE and the molecular weight of each standard were plotted to calculate the molecular weight of the subunit of the enzyme. Standards used (molecular weight): 1, hexokinase (96,000); 2, bovine serum albumin (66,200); 3, chicken egg ovalbumin (45,000); 4, cytochrome *c* (12,300); 5, chicken egg ovotransferrin (78,000); 6, bovine chymotrypsinogen A (25,700); 7, equine myoglobin (17,200). Open circles represent the native and subunit forms of the enzyme in panels A and B, respectively.

simultaneous presence of a 20-fold excess of unlabeled D-lysine in the assay system.

Optimal conditions for enzyme activity. The optimal pH of the enzyme activity was 6.0 when activity was measured over a pH range from 5.0 to 8.0 in 100 mM potassium phosphate buffer. At pH 7.0 and 8.0, the activity decreased to 70% and 25%, respectively. The optimal temperature was estimated to be 41°C. The activity at 41°C was about 2.6 and 1.4 times higher than that at 30 and 37°C, respectively. Liberation of $^{14}CO_2$ from L-[^{14}C]lysine increased linearly with time up to 60 min and gave a linear Lineweaver-Burk plot. The K_m value for L-lysine was 1.5 mM, determined in the presence of 0.1 mM PALP at pH 6.0. If PALP was not added to either the partially or finally purified enzyme preparation, the enzyme activity decreased markedly. No enzyme activity was observed when PALP was omitted from the reaction mixture containing the enzyme preparation obtained from the hydroxylapatite chromatography.

Enzyme stability. The purified enzyme preparation which was stored in buffer A at 4°C maintained activity for at least a month without loss. Heat stability was tested by heating the purified enzyme preparation at 60°C in 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 mM PALP. Portions of the reaction mixture were withdrawn at 1-min intervals, and the enzyme activity was determined. Approximately 70% of the activity remained after 5 min. However, when the enzyme was heated in the absence of PALP, 90% of the activity was lost within 5 min.

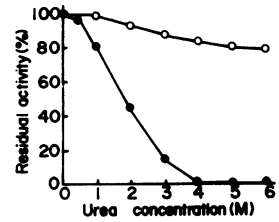

FIG. 5. Effect of urea on the activity of lysine decarboxylase. Enzyme activity with various concentrations of urea in the reaction mixture (●) and after preincubation for 30 min at 30°C with the indicated urea concentration and subsequent 50-fold dilution into the reaction mixture (○).

Effect of urea. By adding urea to the reaction mixture at a concentration of 2 or 4 M, the enzyme activity was inhibited 50 and 100%, respectively. To determine whether the inhibition was reversible, the purified enzyme preparation was incubated for 30 min at 30°C in the presence of various concentrations of urea in 0.1 M potassium phosphate buffer (pH 6.0). Each of the samples was then diluted 50-fold. Approximately 80% of the activity was recovered after diluting the sample containing 6 M urea (Fig. 5).

Amino acid composition. The amino acid composition of the purified enzyme preparation is shown in Table 3. As determined by performic acid oxidation, 2.7 mol of cysteic acid was found

TABLE 3. Amino acid composition of the lysine decarboxylase from *S. ruminatium*

Amino acid residue	Amt (mol/44,000 daltons)	Molar ratio[a]
Lysine	31.7	11.7
Histidine	10.6	3.92
Ammonia	27.5	10.2
Arginine	13.2	4.89
Aspartic acid	39.4	14.6
Threonine	19.6	7.26
Serine	14.9	5.52
Glutamic acid	33.8	12.5
Proline	17.4	6.44
Glycine	31.9	11.8
Alanine	31.0	11.5
Valine	29.2	10.8
Methionine	3.0	1.11
Isoleucine	17.4	6.44
Leucine	34.2	12.7
Tyrosine	20.9	7.74
Phenylalanine	29.4	10.9
Cysteine	2.7	1
Tryptophan	5.3	1.96

[a] Value for cysteine is given as 1.

TABLE 4. Comparison of different lysine decarboxylases

Lysine decarboxylase source	Property								
	Production	% of crude extract	K_m (mM)	Cofactor	pH optimum	pI	Mol wt (native) (10^4)	Subunit mol wt (10^4)	Subunit structure
S. ruminantium	Constitutive	0.06	1.5	PALP	6.0	4.7	8.8	4.4	Dimer
E. coli	Inducible	6	1.5	PALP	5.7	4.6	78	8.0	Dimer, decamer, and aggregates
B. cadverisis	Inducible	2	0.4	PALP	5.8		100		

per 44,000 g of protein. Furthermore, the cysteic acid content was found to be equal to the number of cysteinyl residues as determined in samples containing 8 M urea.

Isoelectric point. The isoelectric point of the purified enzyme preparation was estimated to be pH 4.7.

DISCUSSION

The occurrence, purification, and properties of inducible lysine decarboxylase have been shown in microorganisms (16, 18). However, there has been no previous report of a constitutive lysine decarboxylase. The results of the present study suggest strongly that the synthesis of lysine decarboxylase is constitutive in *S. ruminantium* cells. Further studies are desirable to clarify this possibility. In *E. coli*, synthesis of the enzyme is not only inducible but also dependent on the concentration of glucose in the medium (16). In contrast, the amount of the enzyme synthesized in *S. ruminantium* cells was independent of the glucose concentration. Cadaverine, a product from lysine, is unmetabolized and secreted out of the cell in *E. coli*. In *S. ruminantium*, cadaverine was not detected in the medium, indicating that the cells do not secrete it into the medium. We previously showed (7) that cadaverine exists as a component of the cell wall peptidoglycan of *S. ruminantium* and identified a particulate enzyme which catalyzes the addition of cadaverine to the peptidoglycan (10). In the present study, we isolated a lysine decarboxylase that may participate in the synthesis of the cadaverine that is found in the cell wall of *S. ruminantium*. We propose that the cadaverine in the peptidoglycan might associate with the outer membrane components, such as acidic protein and phospholipids, by an ionic interaction and might play a role similar to that of the bound form of lipoprotein in *E. coli* and other gram-negative bacteria.

Table 4 shows some properties of the lysine decarboxylase of *S. ruminantium* and of the inducible lysine decarboxylases from *E. coli* (16) and *Bacterium cadaverisis* (18). There are several similarities between the enzymes from *S. ruminantium* and *E. coli*, including K_m value, cofactor requirement, the absence of any cystine residues in the native form, optimal pH, and pI. However, the molecular structure of the enzyme in *S. ruminantium* is markedly different from that in *E. coli*. The molecular weight of the native enzyme of the former was 88,000, and it consisted of two identical subunits, whereas that of the latter is characterized by a decameric structure with a molecular weight of 780,000, consisting of ten identical subunits. The enzyme from *B. cadaverisis* appears to have a structure similar to that from *E. coli*.

It has been shown that the inducible lysine decarboxylase in *E. coli* cells tends to associate or dissociate reversibly as a function of pH and ionic strength. At pH 8.0 and ionic strength 0.02, the enzyme has a dimeric structure (16). Decreasing the pH and increasing the ionic strength cause the enzyme molecule to associate further to form a decameric structure, which is an active form of the enzyme (16). In contrast, the lysine decarboxylase of *S. ruminantium* in the dimeric state was active regardless of the pH and ionic strength. These findings indicate important structural differences between the lysine decarboxylases from *S. ruminantium* and *E. coli*.

Enzymes that are denatured by urea generally require slow removal of the urea, prolonged incubation with the substrates, and the presence of certain ions for reactivation. These conditions are required for unfolded peptide chains to reassemble and regain the native conformation. As shown in Fig. 5, the enzyme from *S. ruminantium* was completely inactivated by 6 M urea. It was reactivated quickly after removal of the urea, although the recovery was partial. This indicates that the native form of the enzyme is relatively stable.

ACKNOWLEDGMENTS

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