JOURNAL OF BACTERIOLOGY, Jan. 1994, p. 166–172
0021-9193/94/$04.00+0

Vol. 176, No. 1

Cloning and Nucleotide Sequence of the Cyclic AMP Receptor Protein-Regulated *Salmonella typhimurium pepE* Gene and Crystallization of Its Product, an α-Aspartyl Dipeptidase

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The *Salmonella typhimurium pepE* gene, encoding an N-terminal-Asp-specific dipeptidase, has been cloned on pBR328 by complementation of the Asp-Pro growth defect conferred by a *pepE* mutation. Strains carrying the complementing plasmids greatly overproduce peptidase E. The enzyme has been purified from an extract of such a strain, its N-terminal amino acid sequence has been determined, and crystals suitable for X-ray diffraction have been grown. A new assay using L-aspartic acid *p*-nitroanilide as a substrate has been used to determine the pH optimum (≈7.5) and to test the effect of potential inhibitors. Insertions of transposon γδ (Tn*1000*) into one of the plasmids have been used to localize the gene and as sites for priming sequencing reactions. The nucleotide sequence of a 1,088-bp region of one of these plasmids has been determined. This sequence contains an open reading frame that predicts a 24.8-kDa protein with an N-terminal sequence that agrees with that determined for peptidase E. The predicted peptidase E amino acid sequence is not similar to that of any other known protein. The nucleotide sequence of the region upstream from *pepE* contains a promoter with a cyclic AMP receptor protein (CRP) site, and the effects of growth medium and of a *crp* mutation on expression of a *pepE-lacZ* fusion indicate that *pepE* is a member of the CRP regulon. The unique specificity of peptidase E and its lack of sequence similarity to any other peptidase suggest that this enzyme may be the prototype of a new class of peptidases. Its regulation by CRP and its specificity suggest that the enzyme may play a role in allowing the cell to use peptide aspartate to spare carbon otherwise required for the synthesis of the aspartate family of amino acids.

Salmonella typhimurium and *Escherichia coli* contain more than 10 enzymes capable of hydrolyzing small peptides. Several of these enzymes have been shown to function not only in the catabolism of peptides but also in the breakdown of intracellular proteins (30, 31). While some of these enzymes are able to hydrolyze peptide bonds between many different amino acids, several others are highly specific, acting only on bonds involving one or a few amino acids. Peptidases P and Q, for example, cleave only X-Pro bonds (18), and peptidase M (or methionine aminopeptidase) specifically removes N-terminal Met but only when the second amino acid in the chain is small (21).

Peptidase E (α-aspartyl dipeptidase), another highly specific peptidase, acts only on dipeptides containing N-terminal Asp residues (3). This activity has been partially purified from *S. typhimurium* and shown to be an EDTA-insensitive enzyme with no activity toward peptides with Glu, Gln, or Asn at their N termini. Mutants lacking peptidase E have been isolated, and their mutations have been mapped (3). Although the loss of peptidase E by mutation conferred no detectable phenotype on a wild-type strain, introduction of a *pepE* mutation into a multiply peptidase-deficient (*pepN pepA pepB pepD pepP pepQ*) strain led to loss of the ability to use Asp-Pro as a source of proline to supplement an auxotrophic requirement. Other N-terminal Asp dipeptides were still utilized by such a strain because two additional Asp-X-specific peptidases remain in the mutant strain (3). The physiological functions of these enzymes are not known.

Because the specificity of peptidase E differs from that of any other peptidase, it seemed likely that it might have interesting structural features. To begin the structural characterization of this enzyme, we have cloned its gene and determined its nucleotide sequence. The enzyme has been purified from an extract of a strain carrying the cloned gene, and crystals suitable for X-ray diffraction studies have been grown.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, and growth conditions. The bacterial strains used in this work are derivatives of *S. typhimurium* LT2 unless otherwise noted. Strains not previously described are listed in Table 1. LB was used as a rich medium, and E medium with 0.4% glucose was used as the standard minimal medium with antibiotic supplements as described previously (6). In some experiments cyclic AMP (cAMP) (sodium salt; Sigma Chemical Co.) was added to glucose minimal medium to a final concentration of 5 mM. Succinate minimal medium was NCE (8) supplemented with 1.5% disodium succinate and with 0.1 mM $FeCl_2$. Asp-Pro (Bachem) was used to supplement an auxotrophic requirement for proline at a final concentration of 0.2 mM. All incubations were at 37°C.

DNA manipulations. The DNA sequence was determined by using the dideoxy nucleotide chain termination method (24), double-stranded plasmid templates (5), and Sequenase (United States Biochemicals Inc.) according to the manufacturer's directions. Template DNA was isolated by using Qiagen columns (Qiagen Inc.) according to the manufacturer's direc-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype
Strains	
TN1246	*leuBCD485 pepN90 pepA16 pepB11 supQ302* (Δ*proAB pepD*) *pepP1 pepQ1 pepT1*
TN1547	TN1246 *pepE1*
TN2401	TN1547/pJG29
TN2402	TN1547/pJG30
TN2654	TN1547 *recA srl*::Tn*10*
TN2718	TN1246 *pepE8*::MudJ
TN2719	*leuBCD485 pepE8*::MudJ
TN4460	*leuBCD485*/pCM260
TN4522	*leuBCD485 crp-773*::Tn*10*/pCM260
Plasmids	
pJG29	9.1-kb *Sau*IIIA partial fragment containing *pepE* in *Bam*HI site of pBR328
pJG30	7.1-kb *Sau*IIIA partial fragment containing *pepE* in *Bam*HI site of pBR328
pBK3	pJG29 with *pepE8*::MudJ[a]
pCM247	2.6-kb *Kpn*I-*Hin*dIII fragment of pJG29 containing *pepE* in pBluescriptIIKS$^+$ (Stratagene)
pCM248	1.3-kb *Kpn*I-*Sal*I fragment from pJG29 in pBluescriptIIKS$^+$
pCM260	250-bp *Bgl*II-*Eco*RI fragment from pCM248 containing the promoter region of *pepE* in *Bam*HI-*Eco*RI-digested pRS528 (26)

[a] This insertion is associated with a deletion of approximately 3 kb.

tions. Primers for sequencing from the γδ (Tn*1000*) insertions were those described by Liu et al. (15). Both strands were completely sequenced. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories (Life Technologies Inc.). Other manipulations were performed by standard techniques (23).

Isolation of complementing plasmids and insertion derivatives. A library containing *Sau*3A fragments of chromosomal DNA from TN1246 (3) inserted into the *Bam*HI site of pBR328 was transformed into strain TN1547 (*pepE1* [3]) with selection for resistance to chloramphenicol and utilization of Asp-Pro as a source of proline to satisfy the auxotrophic requirement conferred by the *supQ302* mutation. Positive clones were purified and retested for peptide use. Previously described methods were used to isolate insertions of MudJ (13) and γδ (12) into these plasmids. Insertions which inactivated *pepE* were identified by loss of the ability to use Asp-Pro as a proline source or by decreased ability to hydrolyze Asp-Leu (3). The detection of peptidase activity in nondenaturing polyacrylamide gels has been described previously (20).

Sequence analysis. Sequence analysis was carried out by using the programs in the Wisconsin Genetics Computer Group package (9) or the Pustell/IBI programs. GenBank searches of the nonredundant NCBI data base used the BLAST program (1).

Purification of Peptidase E. Strain TN2401 was cultured in 50 liters of medium containing the following (in grams per liter): tryptone, 30.0, yeast extract, 10.0; NH_4Cl, 2.5; $Na_2HPO_4 \cdot 2H_2O$, 7.5; KH_2PO_4, 3.0; $Na_2SO_4 \cdot 10H_2O$, 2.5; $MgSO_4 \cdot 10H_2O$, 0.2; chloramphenicol, 0.02; $MnSO_4 \cdot 4H_2O$, 0.001, $FeSO_4 \cdot 7H_2O$, 0.0006; citric acid, 0.0006; and glucose, 40.0. The pH was maintained at 7.0 by using a pHstat and 5 M NaOH. Fermentation was started with a 3% inoculum from a well-grown seed culture. Cells were harvested at an optical density at 620 nm of 13, filtered, and disintegrated in a French press. The supernatant was obtained by centrifugation, and approximately half of this material was used in the subsequent purification scheme.

The crude extract was first subjected to ammonium sulfate precipitation, and the 30 to 50% fraction was collected, dialyzed, and purified batchwise on DEAE-Sephadex by elution with NaCl in concentrations increasing in steps of 0.1 M. Activity was found at between 0.2 and 0.6 M. The eluate was concentrated by ammonium sulfate precipitation (80%), and the suspended precipitate was dialyzed against 25 mM Tris, pH 8.0. Further fractionation was achieved on Accell anion-exchange medium (Waters Chromatography Division, Millipore Corp.) in an HR 16/50 column (Pharmacia LKB Biotechnology) in 50 mM Tris (pH 8.0) with a linear gradient of 0 to 0.5 M NaCl in 250 ml. Half of the sample was applied at a time. The aspartyl dipeptidase-containing fractions were combined and concentrated to 27 ml against a Spectra/Por C 5K ultrafiltration filter (Spectrum Medical Industries, Inc.) under nitrogen pressure and subjected to gel filtration on a column (5 by 75 cm) with Sephacryl S-300 HR (Pharmacia) in 40 mM Tris, pH 8.0. Aliquots of the gel-filtered material were loaded on a Mono Q column (Pharmacia) in 50 mM Tris (pH 8.0) and eluted with a linear gradient of 0 to 0.12 M NaCl in 90 ml. The anion-exchange chromatography on Accell and Mono Q was performed with a liquid chromatography controller LCC-500 and P-500 pumps (Pharmacia) and was initiated with salt-free isocratic elution. The purification scheme is shown in Table 2. Protein concentrations were determined by the Coomassie dye binding protein assay with bovine serum albumin as a standard (28).

Activity, kinetics, and inhibition. The activity of α-aspartyl dipeptidase was measured with L-aspartic acid *p*-nitroanilide (Bachem Feinchemikalien AG) as a substrate at a standard concentration of 0.77 mM (A_{313} = 10.0) in 50 mM imidazole (pH 7.2) at 25°C. Five to 10 microliters of enzyme solution was added to 0.8 ml of substrate solution, and the A_{405} was recorded (22). The slope of the resulting curve represents the relative rate of hydrolysis under these nonsaturating conditions. The ε_{405} for *p*-nitroaniline (obtained from Janssen) was determined to be 9.82×10^3 M^{-1} cm^{-1} (Cary 219 Varian spectrophotometer), and the absorbance of the substrate at this wavelength was neglected. The substrate solution can be stored for months in the refrigerator without a change in A_{313}. More concentrated solutions suffer from crystallization of the dissolved substrate. The kinetic parameters were determined by means of Eadie-Hofstee plots. The substrate concentration was determined with $\varepsilon_{313} = 13 \times 10^3$ M^{-1} cm^{-1}, and the protein concentration was determined with an ε_{280} of 30×10^3 M^{-1} cm^{-1}, calculated from the amino acid composition of the sequence. The glutamyl- and pyroglutamyl-containing peptides were all manufactured by Vega and used to prepare 25 mM buffered stock solutions. L-Aspartic acid, β-alanine, and β-DL-aminoisobutyric acid were from either Sigma or Merck. As-

TABLE 2. Purification of α-aspartyl dipeptidase

Step	Total protein (g)	Relative rate of hydrolysis (rrh)	Specific rrh (rrh/mg of protein)
Raw material (disintegrated bacteria)	44.7	100.0	1.0
Ammonium sulfate precipitation (30 to 50%)	31.1	89.7	1.3
DEAE-Sephadex (batchwise)	6.42	88.3	5.8
Accell[a]	1.42	60.4	19.1
Sephacryl S-300 HR	0.494	49.3	44.6
Mono Q[b]	0.102	43.8	192

[a] Run twice with half of the material from the ammonium sulfate precipitation step each time.
[b] Run with aliquots containing 4.5% of the material from the Sephacryl step.

partyl dipeptidase (A_{280} = 0.16) was incubated with 1 mM diisopropylphosphorofluoridate (DFP) (Fluka) in 0.1 M Tris (pH 7.5) for 6 h (16) and (A_{280} = 0.24) with 10 mM dipicolinic acid (Merck) in 0.1 M Tris (pH 8) for 18 h (14), both at 25°C. Aspartyl dipeptidase (A_{280} = 0.15) was also incubated with 0.4 mM phenylmethanesulfonyl fluoride (PMSF) (Merck; prepared from a 20 mM stock solution in dioxane) in 35 mM imidazole (pH 7.2) for 1 h and (A_{280} = 0.12) with 1 mM $HgCl_2$ in 40 mM imidazole (pH 6.0) at the same temperature (19).

Molecular characterization. The molecular weight was determined by using 8 to 25% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels run on a Pharmacia Phast System instrument. Reference proteins were ribulose-1,5-biphosphate carboxylase (52.7 kDa), bovine carbonic anhydrase III (29.5 kDa), and lysozyme (14.3 kDa). Isoelectric focusing was performed by using gels of pH 3 to 9 on the same instrument and with Pharmacia isoelectric focusing calibration kit pH 3–10 as reference proteins. The N-terminal amino acid sequence was determined by using an Applied Biosystems 477A protein sequencer and a 120A analyzer.

Crystal analysis. Data were collected with a Siemens X-1000 area detector and a Rigaku RU 200BEH rotating anode as beam source. The cell parameters were determined by using the Xengen software package (Siemens). The X-ray fluorescence spectra were recorded with a JEM-2000 electron microscope and a Link An 10000 instrument, using aspartyl dipeptidase crystals and crystals of human carbonic anhydrase (29) that were washed with metal-free mother liquor.

Nucleotide sequence accession number. The *pepE* sequence has been assigned GenBank accession number U01246.

RESULTS

Isolation of plasmids carrying *pepE*. The ability of *S. typhimurium* strains lacking broad-specificity peptidases to utilize Asp-Pro as a proline source requires a functional *pepE* gene (3). We took advantage of this phenotype to screen a pBR328 (27) library of *S. typhimurium* chromosomal DNA fragments for clones that complemented this growth defect. Two complementing plasmids, pJG29 and pJG30, were obtained.

Characterization of pJG29 and pJG30. To show that the growth phenotype of *pepE* strains carrying pJG29 and pJG30 was due to production of peptidase E, crude extracts of strains TN2401 and TN2402 were prepared and assayed for peptidase E activity as described previously (3). The ratio of the peptidase E specific activity in extracts of the two plasmid-containing strains to that of the parent strain TN1246 containing only a single chromosomal copy of *pepE* was approximately 180. To show that this activity resulted from peptidase E, crude extracts were electrophoresed on nondenaturing polyacrylamide gels and stained for Asp-Pro-hydrolyzing activity. The position of the band of activity was identical to that of the band of activity in an extract of a $pepE^+$ strain.

Restriction mapping of pJG29 and pJG30 showed that the two plasmids carried inserts of approximately 9.1 kb (pJG29) and 7.1 kb (pJG30) and that all of the sites in pJG30 were contained in pJG29 (Fig. 1).

Isolation of insertions in pJG29. To localize *pepE* in the plasmids, insertions of the mini-Mud element MudJ (MudI1734 [4]) into pJG29 were isolated, and a strain carrying a $PepE^-$ insertion (*pepE8*::MudJ) was saved. Restriction

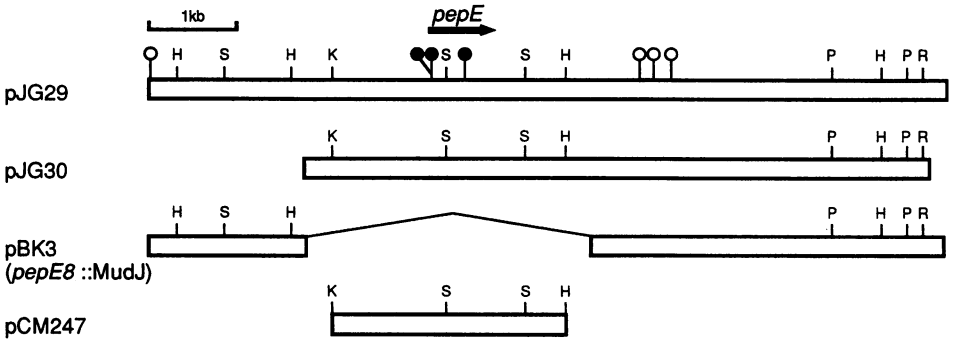

FIG. 1. Restriction maps. Restriction sites within the choromosomal DNA inserts of the indicated plasmids are shown. H, *Hin*dIII; S, *Sal*I; K, *Kpn*I; P, *Pvu*II; R, *Eco*RV. There are no *Eco*RI, *Pst*I, *Sma*I, or *Xba*I sites. Sites of γδ insertions which eliminate *pepE* function are indicated by closed circles, and those which do not are indicated by open circles. The gap in pBK3 indicates the region deleted as a result of MudJ insertion. The location and direction of transcription of the *pepE* gene are indicated by the arrow.

mapping of this plasmid (pBK3) showed that insertion of the MudJ element was accompanied by a deletion of approximately 3 kb (Fig. 1). The occasional formation of deletions associated with the insertion of Mu and its derivatives has long been known. This insertion mutation was transferred to the chromosome, and the resulting strain (TN2719) was used as a recipient in a phage P22 transduction cross with a Tn*10* insertion in *metAH* (*metAH900*::Tn*10*), a locus known to be cotransducible with *pepE* (3). The observed linkage of Tet^r conferred by Tn*10* with the Kan^r conferred by MudJ (1.1% [2 of 174]) falls within the range previously observed for linkage of *metAH* to *pepE*, establishing that pJG29 and pJG30 carry the locus to which *pepE* mutations had been shown to map (3).

Transposon γδ (12) insertions into pJG29 were isolated, and the resulting plasmids were tested for their ability to complement the Asp-Pro growth defect. Three insertions led to loss of complementation, and four more did not (Fig. 1). The insertions that failed to complement were located near the center of the region deleted in *pepE8*::MudJ, defining more precisely the location of *pepE* on the plasmids. A subclone, pCM247, carrying a 2.6-kb *Kpn*I-*Hin*dIII fragment that includes the region defined by the insertions and the deletion complemented the Asp-Pro utilization of TN1547, indicating that this fragment carries a functional *pepE* gene.

Nucleotide sequence of the *pepE* region. The nucleotide sequence of a 1,088-bp region of pJG29 surrounding the noncomplementing γδ insertions was determined (Fig. 2). This sequence contained an open reading frame (Fig. 2, bp 165 to 851) that predicted a 24,768-Da protein. Data reported below indicate a molecular mass for purified peptidase E of 27 kDa. The N-terminal amino acid sequence of peptidase E purified as described below was determined and agrees completely with that predicted by the open reading frame (Fig. 2). To further confirm the open reading frame, the nucleotide sequence of the *pepE* gene from a strain carrying the *pepE1* mutation (3) was partially determined. This sequence revealed a C-to-T change at position 573, which predicts a Pro-to-Ser change at position 137 in the open reading frame. A search of GenBank identified no protein with significant amino acid similarity to that predicted for peptidase E. Consistent with the fact that peptidase E is a soluble cytoplasmic protein, no signal sequence was present and the hydrophobicity plot revealed nothing of interest. The presence of only a single cysteine residue demonstrates that the enzyme has no disulfide bridges.

The region 5′ to the open reading frame contains a potential ribosome binding site, a possible σ^{70} −10 sequence, and a sequence with significant similarity to a cAMP receptor protein (CRP) site (Fig. 2, bp 67 to 83). We note that the promoter region contains an inverted repeat located near the proposed −10 sequence. We note also the presence of an open reading frame 3′ to *pepE* reading in the opposite direction. A BLAST search of GenBank using the amino acid sequence predicted by this open reading frame revealed nothing.

***pepE* is regulated by CRP-cAMP.** The presence of a possible CRP site suggests that *pepE* may be part of the CRP-cAMP regulon. To test this we cloned a 230-bp *Bgl*II-*Sal*I fragment (Fig. 3) carrying the region containing the putative *pepE* promoter into the promoter detection vector pRS528 (24). In this construct (pCM260) the *E. coli lac* operon should be controlled by the *pepE* promoter. This plasmid was introduced into isogenic *crp*$^+$ and *crp* mutant strains, and β-galactosidase levels were determined for cells grown on glucose and on succinate (Table 3). The results of these experiments indicate that succinate-grown cells contain about threefold-higher β-galactosidase levels than cells grown in glucose. Addition of cAMP to glucose-grown cells leads to a nearly fivefold increase in β-galactosidase levels. This increase is dependent on the presence of a functional *crp* gene. We conclude that *pepE* is indeed regulated by CRP-cAMP.

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                                        BglII
   1 TAATTTTTCAGGCGATACCAGATGGCAGATCTGGCCTGTCGTCACTTTTA
                                  CRP Site
  51 GCGACCGCGCTTTGCCTGTGACACGCGTAACATCATGAGCCATATCAGTA
                                 -10?
 101 AGACATTAAATGGATAATGGTAGCATTATTACGTTTTGACATTTCCTGAT
                     ----->    <-----
      SD
 151 GAGGTGGAGAAGTAATGGAACTGCTTTTATTAAGTAACTCGACGCTGCCG
   1                  M  E  L  L  L  L  S  N  S  T  L  P
 201 GGTAAGGCCTGGCTGGAACACGCTCTGCCGCTGATAGCGAATCAATTGAA
  13  G  K  A  W  L  E  H  A  L  P  L  I  A  N  Q  L  N
      SalI
 251 CGGTCGACGCTCAGCCGTGTTTATTCCGTTTGCCGGCGTCACGCAAACAT
  30   G  R  R  S  A  V  F  I  P  F  A  G  V  T  Q  T
 301 GGGACGAGTACACGGATAAAACCGCAGAGGTGCTTGCGCCATTAGGGGTA
  46 W  D  E  Y  T  D  K  T  A  E  V  L  A  P  L  G  V
 351 AACGTCACAGGGATTCATCGTGTCGCCGATCCGCTTGCGGCGATTGAGAA
  63  N  V  T  G  I  H  R  V  A  D  P  L  A  A  I  E  K
 401 AGCGGAAATCATTATTGTCGGCGGCGGAAATACCTTCCAGCTACTGAAAG
  80   A  E  I  I  I  V  G  G  G  N  T  F  Q  L  L  K
 451 AATCGCGTGAACGCGGGCTGCTGGCGCCGATGGCGGACAGGGTAAAACGC
  96 E  S  R  E  R  G  L  L  A  P  M  A  D  R  V  K  R
 501 GGCGCGCTGTATATCGGCTGGAGCGCGGGCGCCAACCTTGCCTGCCCGAC
 113  G  A  L  Y  I  G  W  S  A  G  A  N  L  A  C  P  T
                                T(pepE1)
 551 TATCCGCACGACCAACGATATGCCGATCGTCGATCCAAACGGTTTCGACG
 130   I  R  T  T  N  D  M  P  I  V  D  P  N  G  F  D
                                S(PepE1)
 601 CGCTGGATCTGTTCCCGCTGCAAATTAATCCTCACTTCACTAACGCCTTG
 146 A  L  D  L  F  P  L  Q  I  N  P  H  F  T  N  A  L
 651 CCGGAAGGTCATAAGGGCGAGACCCGCGAGCAGCGTATTCGCGAACTGCT
 163  P  E  G  H  K  G  E  T  R  E  Q  R  I  R  E  L  L
 701 GGTGGTCGCGCCGGAACTGACAGTGATTGGTTTGCCGGAAGGTAACTGGA
 180   V  V  A  P  E  L  T  V  I  G  L  P  E  G  N  W
 751 TTCAGGTGAGCAACGGCCAGGCGGTTCTCGGCGGCCCGAATACCACCTGG
 196 I  Q  V  S  N  G  Q  A  V  L  G  G  P  N  T  T  W
 801 GTGTTTAAAGCGGGCGAAGAGGCCGTGGCGCTGGAAGCGGGTCATCGCTT
 213  V  F  K  A  G  E  E  A  V  A  L  E  A  G  H  R  F
 851 TTAATCCCAGGCCATATTGAATGCCGAATGACGGGCCTACGCGCATGTTC
 901 TCCGTAGGCCCCGCCATCCGGCATTTCACTTAATACTCGCCCCGTTCCTC
 951 GTCCTGATCCGGCTGTTCCAGTACGCTATAGGCGACCGAACAGAACAGTG
1001 AGTTAAGACGCTTCATATCGCCCAGCAGCCCTAAATGGAGCGAGCTGGTT
1051 TCAATGCTTTGCACGTTCTGCTGGTGCAGGCGGTCGAC 1088
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FIG. 2. Nucleotide sequence of *pepE* and deduced amino acid sequence of peptidase E. A possible CRP site, −10 sequence, and Shine-Dalgarno sequence are indicated. Arrows indicate an inverted repeat of unknown significance. The N-terminal amino acid sequence determined for purified peptidase E is underlined. The *pepE1* mutation and the predicted amino acid change which would result are also shown. The restriction sites used for cloning the *pepE* promoter region (*Bgl*II and *Sal*I) are indicated.

TABLE 3. β-galactosidase levels

Strain (genotype)	β-Galactosidase level (U) in cells grown in:		
	Glucose	Succinate	Glucose-cAMP
TN4460 (*crp*$^+$)	1,110	3,280	4,880
TN4522 (*crp*)	900		933

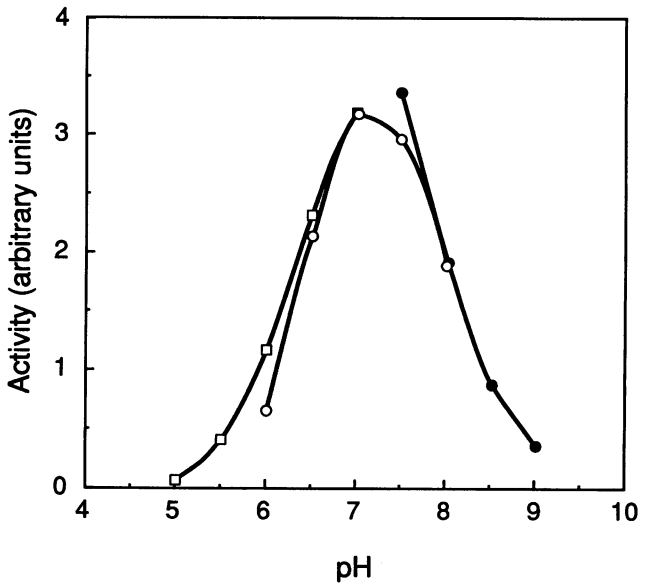

FIG. 3. pH-rate profile for the peptidase E-catalyzed hydrolysis of L-aspartic acid *p*-nitroanilide determined with 90 mM 2-(*N*-morpholino)ethanesulfonate (MES) (□), imidazole-HCl (○), and Tris-HCl (●) buffers.

Purification of peptidase E. Peptidase E was purified as described in Table 2. Approximately half of the crude extract was used in the scheme in Table 2. The partly purified enzyme preparation after the Sephacryl step was stored in the freezer. Aliquots of approximately 4% of the amount from this step (Table 2) were then purified on Mono Q when needed. The pI of the purified enzyme was found to be 4.7 (by isoelectric focusing), and the molecular weight was found to be 27,000 (by SDS-PAGE).

Assay, kinetic parameters, and inhibitors. Figure 3 shows the profile of activity versus pH for aspartyl dipeptidase, with a maximum activity at between pH 7 and 7.5. The choice of 7.2 as the assay pH was made on the basis of these data. The enzyme is inactive in 0.1 M phosphate at pH 6.5, but activity increases with pH up to at least 8 (30% active relative to activity in Tris at the same pH), probably because of an inhibitory effect of the monovalent phosphate anion. Two determinations of the kinetic parameters both gave a k_{cat} of 39 s^{-1} and a K_m of 4.3 mM, with an average correlation coefficient of -0.96. Unfortunately, because of the limited solubility of the substrate, all concentrations used were below the K_m.

Three β-amino acids were tested as inhibitors for aspartyl dipeptidase (Table 4). Modest effects were displayed by β-alanine and L-aspartic acid, whereas β-DL-aminoisobutyric acid had no effect at all. In Table 4, the effects of eight different Glu-containing di- and tripeptides are also listed. Again, only weak inhibition could be observed. About 42% activity remained after 0.75 h of incubation with mercuric chloride, and 41% remained after 2.5 h. The protease classification inhibitors DFP, PMSF, and dipicolinic acid had no effect at all upon the activity of aspartyl dipeptidase.

Crystallization. Aspartyl dipeptidase (6 to 12 mg/ml) can be crystallized at different pH values and temperatures in the presence of $CdSO_4$ with $(NH_4)_2SO_4$ or polyethylene glycol (PEG), but all of our initial experiments produced only thin plates, often growing in clusters. Generally, the best results were obtained in 50 mM 2-(cyxlohexylamino)ethanesulfonate (CHES)–50 mM cacodylate-NaOH (pH 9.5)–1 mM $CdSO_4$–2.5% PEG 20000 vapor equilibrated against 50 mM CHES (pH 9.5)–0.25 M NaCl, where the tendency of crystal twinning is less pronounced. The sides of the plates were frequently grown up to 0.3 mm, but the maximum thickness observed was only about 0.025 mm. These crystals diffracted to 2.6 Å (1 Å = 0.1 nm) and belong to space group P1 with a = 43.5 Å, b = 51.5 Å, c = 64.3 Å, a = 104.8°, b = 89.6°, and g = 115.2°. V_m was determined to 2.3 $Å^3$/Da, assuming two molecules per asymmetric unit (17). An X-ray fluorescence spectrum of a crystal that had been washed with fresh PEG solution was recorded (data not shown) and compared with a similar spectrum of carbonic anhydrase, an enzyme known to contain zinc. Our interpretation of these qualitative spectra is that α-aspartyl dipeptidase does not contain zinc, since the zinc ion in carbonic anhydrase is easily detected by this technique, whereas the spectrum of α-aspartyl dipeptidase lacks any signal.

A very large crystal was found after 1.5 years of incubation in 12.5 mM Tris-cacodylate (pH 7.5)–2 mM $CdSO_4$–0.5% PEG 20000 (7.5 mg of enzyme per ml) equilibrated against 50 mM Tris-cacodylate (pH 7.5)–60 mM NaCl. A piece of this, approximately 0.1 × 0.1 × 0.1 mm^3, diffracted below 2.0 Å with virtually the same cell parameters as given above.

TABLE 4. Inhibition with amino acids or peptides

Inhibitor	I_{50} (mM)[a]	% Remaining activity[b]
L-Aspartic acid	7–8	
β-Alanine	7–8	
DL-Aminoisobutyric acid	NI[c]	
L-Glutamyl-L-alanine		76
L-Glutamyl-L-alanyl-L-alanine		72
L-Glutamyl-L-glutamic acid		72
L-β-Glutamyl-L-leucine		105
L-Glutamyl-L-tyrosine		64
L-Glutamyl-L-valine		39
L-Pyroglutamyl-L-alanine		105
L-Leucyl-L-glutamic acid		112

[a] Concentration giving 50% inhibition.
[b] Relative rate of hydrolysis in the presence of 3 mM inhibitor, expressed as percentage of the hydrolysis rate in the absence of peptide.
[c] NI, no inhibition.

DISCUSSION

The deduced amino acid sequence of peptidase E shows that its unique substrate specificity is indeed a reflection of a primary structure unrelated to that of any other peptidase. A substantial number of peptidase amino acid sequences have now been determined, and for many of them primary sequence relationships to other enzymes with related functions or specificities have been identified. It seems a reasonable guess that other enzymes structurally related to peptidase E remain to be discovered. It will be interesting to learn whether the other two Asp-X-specific peptidases in *S. typhimurium* are structurally similar to peptidase E.

Several other properties of peptidase E differ from those of many other peptidases. On the basis of its insensitivity to EDTA, we have proposed (3) that peptidase E, in contrast to most of the other *Salmonella* peptidases (21), is not a metalloenzyme. This suggestion is strongly supported by the results presented in this paper. In addition to its insensitivity to EDTA, peptidase E is not inhibited by dipicolinic acid. The latter was found to be the more efficient of the two chelators in demineralizing carbonic anhydrase (14), presumably for steric reasons since it is distinctly smaller than EDTA. In addition,

the X-ray fluorescence spectrum of a peptidase E crystal indicates that the enzyme does not contain zinc or any other metals. The two active-site serine reagents DFP and PMSF were both unable to inactivate peptidase E. The specificity of α-aspartyl dipeptidase for a negatively charged residue on the amino-terminal side of the scissile bond is rather unusual among peptide-bond-hydrolyzing enzymes. We cannot exclude the possibility that the peptidase actually has an active-site serine but that the relatively hydrophobic active-site serine reagents are repelled from the active site by the substrate-binding residues. The staphylococcal serine protease, cleaving peptide bonds C terminal to glutamyl and aspartyl residues, is inactivated by DFP (10), but the number of investigated proteases with such specificity is quite small. The active site of peptidase E must accommodate three charged groups close to the scissile bond of its substrates.

Peptidase E thus may not belong to any of the four protease classes, since (i) it is only partially inactivated by mercuric chloride, (ii) it is not inhibited by metal chelators, (iii) it is not inhibited by active-site serine reagents, and (iv) neither the amino acid sequence nor the molecular mass is in agreement with what is found for the acid proteases, which usually contain a Phe-Asp-Thr-Gly-Ser sequence within the 40 N-terminal residues and have a molecular mass of about 35 kDa (7). The pH-activity profile for peptidase E shows a much more alkaline optimum than is observed for the known acid proteases. Most of these enzymes are active at low pH, although renin has a pH optimum of between 5.5 and 7.5 (25). Partial inhibition by mercuric chloride suggests that the cysteine residue can be modified but is not essential. A single cysteine accessible to mercuric ions makes this derivative a possible candidate for isomorphous replacement in order to solve the crystallographic phase problem.

None of the β-amino acids or glutamic acid peptides we tested were sufficiently strong inhibitors to be used in crystallography or affinity chromatography. We also note that the binding to subsite 1 (the subsite on the N-terminal side of the bond to be cleaved) appears to be restricted to a carboxymethyl group, since (i) glutamic acid peptides are neither hydrolyzed nor bound by the enzyme, (ii) β-alanine has the same inhibitory effect as L-aspartate (which also indicates that the carboxymethyl group and the α-amino group are the sole contributors to the subsite 1 binding energy), and (iii) the inhibitory effect of β-alanine is abolished upon introduction of a methyl group on the β-carbon, as in aminoisobutyric acid. We therefore expect a good inhibitor of peptidase E to consist of an amino-terminal aspartic acid residue connected to a second amino acid by something other than a peptide bond, e.g., a compound in which the amide nitrogen is replaced with a methylene group and/or the carbonyl oxygen is reduced (7).

pepE is the first peptidase gene shown to be regulated by CRP-cAMP. The physiological significance of this finding is not clear. Although aspartate cannot be used as the sole carbon source by *S. typhimurium* LT2, several other amino acids (alanine, cysteine, proline, and serine) can serve as sole carbon sources (11). Peptidase E is known to hydrolyze N-terminal Asp dipeptides containing Ala, Pro, or Ser, and there is no reason to think that Asp-Cys would not also be a substrate. It is possible, therefore, that some environment that *S. typhimurium* encounters contains a sufficient supply of Asp dipeptides which also contain an amino acid utilizable as a carbon source. Another possible function for peptidase E involves the carbon sparing that might be achieved by production of aspartate from peptides. The availability of aspartate spares the cell the necessity of contributing most of the carbon found in aspartate, asparagine, lysine, methionine, threonine, isoleucine, and diaminopimelate (2). This might be significantly advantageous under conditions of carbon limitation. Again this presupposes that there are environments in which N-terminal Asp dipeptides are in relatively abundant supply.

We speculate that CRP-cAMP may not be the only regulatory element for *pepE*. The finding that the presence in the cell of a plasmid carrying *pepE* leads to a nearly 200-fold increase in peptidase E levels (much larger than expected on the basis of the copy number of pBR328) suggests to us that there might be a negative regulator of *pepE* expression. The inverted repeat that overlaps the proposed −10 sequence in the promoter is an attractive possibility for a site of action of such an element.

Further work on *pepE* regulation may provide insight into its physiological function, just as further characterization of its structure should provide an understanding of its unique specificity and, perhaps, reveal some new insights into the mechanisms of enzymatic peptide bond hydrolysis.

ACKNOWLEDGMENTS

The contributions of Jane Glazebrook, Beverly Klooster, and Judy Miller to this project in the Miller laboratory are gratefully acknowledged. K.H. thanks Arnthor Aevarsson and Per Berdén for supervision and assistance in the bacterial fermentation and Anders Svensson for help in recording the X-ray diffraction data. We thank Jan-Olov Bovin, Department of Inorganic Chemistry 2, for recording the X-ray fluorescence spectra and Chatarina Svensson and Jakob Donner, BM unit, for determining the amino-terminal sequence.

This work was supported by a grant (AI10033) from the National Institute for Allergy and Infectious Diseases (to C.G.M.) and by the Natural Science Research Council (NRF), the Swedish Council for Planning and Coordination of Research (FRN), the SE-bank, and the Knut and Alice Wallenberg foundation (to K.H. and A.L.).

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