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Isolation and Characterization *Salmonella typhimurium* Mutants Lacking a Tripeptidase (Peptidase T)

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Salmonella typhimurium contains an enzyme, peptidase T, that hydrolyzes a variety of tripeptides. Specificity studies with a peptidase activity stain after gel electrophoresis of crude cell extracts showed that peptidase T hydrolyzes tripeptides containing N-terminal methionine, leucine, or phenylalanine. Little or no activity could be detected against dipeptides, N-blocked or C-blocked tripeptides, and tetrapeptides. Analysis of reaction products by high-pressure liquid chromatography showed that peptidase T removes the N-terminal amino acid from tripeptides. Mutants lacking peptidase T were isolated by screening microcultures grown in the wells of plastic microtitration plates for hydrolysis of Met-Ala-Ser or Met-Gly-Gly. Mutations (*pepT*) that eliminate this enzyme were found to be phage P22 cotransducible with *purB* at approximately 25 map units on the *S. typhimurium* map. Comparison of the growth properties of mutant and wild-type strains suggests that peptidase T does not function in utilization of tripeptides to provide amino acids during growth.

Salmonella typhimurium contains a variety of distinct peptidases with unique but overlapping substrate specificities. Mutants lacking six of these enzymes have been described (19, 21). Three of these enzymes, peptidases N, A, and B, are broad-specificity aminopeptidases. Peptidase D is a broad-specificity dipeptidase. Peptidases P and Q are the only enzymes present in this organism that hydrolyze peptides containing proline next to the N terminus (19). All of these enzymes enable the cell to use particular peptides as amino acid sources (13, 19, 21). In addition, they function in the degradation of endogenous peptides generated by intracellular proteolysis (20, 29).

To identify other peptidases present in *S. typhimurium*, we have tested various peptides for their ability to supply a required amino acid to auxotrophic strains lacking peptidases N, A, B, D, P, and Q. We have found that the peptides Met-Ala-Ser, Met-Ala-Met, and Met-Thr-Met can serve as methionine sources to strains lacking these six peptidases, but a number of other N-terminal methionine peptides cannot serve as methionine sources (see below). This apparent specificity for N-terminal methionine peptides with alanine or threonine in the second position is the substrate specificity that has been predicted for the enzyme that removes methionine from the N terminus of nascent protein (6, 28).

It was previously reported that cell extracts of *S. typhimurium* mutants lacking peptidases N, A, B, and D contain hydrolytic activity against Met-Ala-Ser (21). An activity capable of hydrolyzing Met-Ala-Ser is also present in *Escherichia coli* K-12 (10, 22). This enzyme, tripeptidase TP, has been partially purified and shown to be an aminotripeptidase that specifically removes the N-terminal amino acid from tripeptide substrates (10). It is therefore unlikely that this enzyme is responsible for the removal of N-terminal methionine from nascent peptide chains because this N-terminal maturation occurs when the growing chain contains approximately 50 amino acids (2, 12).

This paper addresses the following questions. (i) Is the Met-Ala-Ser–hydrolyzing activity observed in *S. typhimurium* extracts responsible for the utilization of N-terminal methionine tripeptides as methionine sources? (ii) Does the specificity of the *S. typhimurium* activity correspond with that expected of the enzyme that removes N-terminal methionine from nascent peptide chains? (iii) What is the relationship between the *S. typhimurium* activity and the aminotripeptidase reported in *E. coli* K-12? The approach taken has been to isolate mutants lacking the *S. typhimurium* Met-Ala-Ser–hydrolyzing activity, to study the peptide utilization phenotype of these mutants, and to characterize the specificity of the activity toward a variety of peptide substrates.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are derived from *S. typhimurium* LT2 and are listed in Table 1.

Media and growth conditions. E medium (24) supple-

TABLE 1. Bacterial strains[a]

Strain	Genotype
TN1146	Δ(*leuBCD*)*485 proB25 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1*
TN1147	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*)
TN1152	Δ(*leuBCD*)*485 proB25 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepT1*
TN1214	Δ(*leuBCD*)*485 proB25 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepT*$^+$ *zce850*::Tn*10*
TN1215	Δ(*leuBCD*)*485 proB25 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepT1 zce850*::Tn*10*
TN1281	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *pepT*$^+$ *zce-850*::Tn*10*
TN1282	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *pepT1 zce-850*::Tn*10*
TN1285	*purB13 pepT1 zce-850*::Tn*10*
TN1286	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *purB13 pepT1 zce-850*::Tn*10*
TN1304	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1*
TN1319	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *pepT2*
TN1320	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *pepT3*
TN1321	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *pepT4*
TN1322	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *pepT5*
TN1324	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 pepT2*
TN1325	Δ(*leuBCD*)*485 metE338 zie-822*::Tn*5 pepN10 pepA1 pepB1 pepP1 pepQ1 pepT3*
TN1508	Δ(*leuBCD*)*485 metA15 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT1*
TN1641	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT6*::Mu d1(Ap^r *lac* cts62)
TN1642	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT7*::Mu d1(Ap^r *lac* cts62)
TN1703	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT8 zce-850*::Tn*10*
TN1704	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT9 zce-850*::Tn*10*
TN1705	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT10 zce-850*::Tn*10*
TN1706	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT11 zce-850*::Tn*10*
TN1707	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT12 zce-850*::Tn*10*
TN1708	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT13 zce-850*::Tn*10*
TN1709	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT14 zce-850*::Tn*10*
TN1710	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT15 zce-850*::Tn*10*
TN1727	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *dcp-1 zxx-845*::Tn*10 optA1 zxx-848*::Tn*5*
TN1931	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 supQ302* Δ(*proAB-pepD*) *dcp-1 zxx-845*::Tn*10 optA1 zxx-848*::Tn*5 pepT1*
TN1850	Δ(*leuBCD*)*485 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT7*::Mu d1(Ap^r *lac* cts62 X)
TN2023	*metE338*[b]
TN2025	*arg*::Mu d1(Ap^r *lac* cts62)[c]
TN2024	*purB13*[b]

[a] *zie-822*::Tn*5* is cotransducible with *metE*, *zxx-845*::Tn*10* is cotransducible with *dcp*, and *zxx-848*::Tn*5* is cotransducible with *optA*.

[b] From J. Roth.

[c] From C. Turnbough.

mented with 0.4% glucose and, when required, 0.4 mM L-amino acids or 0.4 mM adenine (or both) was used as a minimal medium. Nutrient broth (Difco Laboratories) containing 0.5% NaCl or Luria broth (23) was used as a rich medium. Solid media contained 1.5% agar (Difco). Liquid cultures were aerated by shaking. All incubations were at 37°C except for strains containing Mu d1(Ap^r *lac* cts62), which were grown at 30°C. The ability of strains to utilize peptides as amino acid sources was tested by placing a few crystals of the peptide on the surface of a minimal glucose plate overlaid with a 2.5-ml soft-agar layer containing 0.1 ml of an overnight culture of the strain to be tested. Growth was scored after overnight incubation. Peptides were obtained from commercial sources and were tested by thin-layer electrophoresis or high-pressure liquid chromatography to ensure that only one ninhydrin-positive or trinitrobenzenesulfonic acid-derivatizable compound was present. Tetracycline (Sigma Chemical Co.) and ampicillin (Sigma) were used at 25 μg/ml. Bochner medium (4) containing fusaric acid (Sigma) was used to select tetracycline-sensitive strains.

Mutagenesis and transduction. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) was performed by the method of Roth (24). Localized mutagenesis with hydroxylamine was performed as described by Hong and Ames (11). Bacteriophage Mu

d1(Ap^r *lac* cts62) was used as a mutagen by growing P22 (HT 12/4 *int-4*) on a *S. typhimurium* Mu d1 lysogen (obtained from Charles Turnbough, University of Alabama) and using the resulting lysate to infect recipient cells at a multiplicity of infection of 1 (8). Plating 10^8 PFU with 0.1 ml of overnight culture on nutrient agar-ampicillin plates gave approximately 100 Amp^r colonies per plate.

Transduction was performed with P22 (HT 12/4 *int-4*) as described by Roth (24). For transduction to antibiotic resistance, bacteria and phage were mixed and preincubated for at least 30 min before plating on selective medium.

Mutant screening. Mutagenized clones were screened for lack of peptidase activity by a modification of a procedure developed by Vimr et al. (26). Sterile 144- or 96-well conical bottom microtitration plates (Linbro) were filled with 200 μl of appropriately supplemented E medium per well. (Cells grown in this medium gave a better distinction between *pepT*$^+$ and *pepT* strains than did cells grown in nutrient or Luria broth.) Colonies to be screened were inoculated with sterile toothpicks into wells. Plates were incubated overnight at 37 or 30°C. After incubation, replicas of the dishes were made on agar plates (150-mm diameter, nutrient or Luria agar). When the colonies to be screened were transductants, we found it easier to recover phage-sensitive strains if this transfer was performed immediately after inoculation of the multiwell plates. The liquid cultures in the multiwell plates were centrifuged, washed with buffer (50 mM Tris-hydrochloride, pH 7.5), and suspended in 30 μl of lysis buffer (1 mg of lysozyme per ml in buffer). After three freeze (−70°C) and thaw (37°C) cycles, 100 μl of peptide solution (1 mg Met-Ala-Ser, Met-Thr-Met, or Met-Gly-Gly per ml and 1 mM $MnCl_2$ in buffer) per well was added. The plates were then incubated at 37°C for 30 minutes, and to each well as added 20 μl of buffer containing 0.6 mg of L-amino acid oxidase (Sigma, type 1) per ml, 1.2 mg of horseradish peroxidase (Sigma) per ml, and 0.6 mg of *o*-dianisidine (Sigma) per ml. Plates were held at room temperature until brown color developed (15 to 30 min). Wells lacking color were putative mutants. These strains were cultured from the agar plate replicas and retested by gel electrophoresis of crude soluble cell extracts and staining for peptidase activity with Met-Ala-Ser or Met-Gly-Gly as substrate.

Extract preparation. Crude extracts were prepared from stationary-phase cultures grown in E medium or Luria broth. Cells were harvested by centrifugation, washed twice with Tris-hydrochloride (50 mM, pH 7.5), suspended in the same buffer, and broken in a French pressure cell (5,000 to 6,000 lb/in^2) or by sonication. The broken cell suspension was centrifuged for 60 min at 30,000 × *g* in a Sorvall RC2B centrifuge, and the supernatant solution was stored at −70°C.

Gel electrophoresis and stain. Electrophoresis of cell extracts in 7.5% polyacrylamide slab gels and staining for peptidase activity were performed as described previously (21), except that Tris-hydrochloride buffer was used instead of phosphate in the soft agar overlay.

Peptidase assay. Reaction products of peptidase assays were reacted with trinitrobenzenesulfonic acid (Pierce Chemical Co.) to form trinitrophenyl derivatives (25), which were separated and quantitated by high-pressure liquid chromatography on an Altex Ultrasphere-ODS column (T. H. Carter, Ph.D. thesis, 1982, Case Western Reserve University). For assay of Met-Gly-Gly hydrolysis, a linear gradient from 40 to 70% acetonitrile in trifluoroacetic acid was used (18). The trinitrophenyl peptides and amino acids were detected by their absorbance at 350 nm (Hitachi; model 100-10 spectrophotometer) and quantitated by a data processor (Shimadzu; Chromatopac C-R1A). Amino acid and peptide standards were derivatized and chromatographed under the same conditions to identify reaction products. A standard curve was prepared for trinitrophenyl methionine samples of known concentration. All reaction products were expressed as methionine equivalents.

Thin-layer electrophoresis of amino acids and peptides was performed on cellulose or silica plates at 400 V in a buffer of pyridine-acetic acid-water (10:1:89, vol/vol/vol) as described by Vimr and Miller (27). Compounds were visualized with ninhydrin.

DEAE-cellulose column chromatography. Samples (100 mg) of protein of a crude soluble cell extract were applied to a 10- by 30-mm column of Whatman DE-52 equilibrated with 10 mM Tris-hydrochloride (pH 7.5). Proteins were eluted in a 0 to 0.4 M KCl gradient in the same buffer (150-ml total volume) at a flow rate of 30 ml/h. Fractions of 1.5 ml were collected. Samples (20 μl) of every third column fraction were assayed in multiwell plastic plates as described above to identify those fractions containing activity toward Met-Ala-Met. Samples (5 or 20 μl) of fractions containing activity were electrophoresed in nondenaturing gels, which were stained for peptidase activity with Met-Ala-Met as a substrate.

Protein. Protein concentrations were determined by the method of Lowry et al. (17) with bovine serum albumin (Sigma) as a standard.

RESULTS

Peptidase activity in multiply peptidase-deficient strains. The presence of peptidase activity in a strain can be inferred from the ability of the strain to use peptides as amino acid sources. To identify peptidase activities remaining in a strain lacking peptidases N, A, B, D, P, and Q, we have tested auxotrophic derivatives of such a strain for utilization of peptides as amino sources. As shown in Table 2, the *pep*$^+$ strain TN2023 (*metE338*) could use all the methionine peptides tested as methionine sources. The multiply peptidase-deficient strain TN1281 (*metE pepN pepA pepB pepD pepP pepQ*) could only use Met-Ala-Ser, Met-Ala-Met, and Met-Thr-Met as methionine sources. None of the other tripeptides, the dipeptides, the tripeptide amide, or the tetrapeptide tested could supply methionine to this strain. Strains lacking peptidases N, A, B, D, P, and Q could also not use Leu-Leu-Leu or Leu-Gly-Gly as leucine sources, Gly-Gly-Gly as a glycine source, Phe-Gly-Gly as a phenylalanine source, or Ala-Ala-Ala as a carbon or nitrogen source. It thus appears that a tripeptidase activity with some specificity for N-

TABLE 2. Growth on methionine and methionine peptides

Met source	Growth		
	TN2023[a]	TN1281[b]	TN1282[c]
Met	+	+	+
Met-Ala-Ser	+	+	+
Met-Ala-Met	+	+	+
Met-Thr-Met	+	+	+
Met-Gly-Gly	+	−	−
Met-Gly-Met	+	−	−
Met-Met-Met	+	−	−
Met-Met-Ala	+	−	−
Met-Leu-Gly	+	−	−
Met-Phe-Gly	+	−	−
Met-Tyr-Phe-NH_2	+	−	−
Met-Gly-Met-Met	+	−	−
Met-Ala	+	−	−
Met-Gly	+	−	−
Met-Val	+	−	−

[a] TN2023 *metE338*.
[b] TN1281 *metE338 pepN pepA pepB pepD pepP pepQ*.
[c] TN1282 *metE338 pepN pepA pepB pepD pepP pepQ pepT*.

terminal methionine must be present to account for the observed growth pattern.

To identify this activity, crude soluble cell extracts were electrophoresed in nondenaturing polyacrylamide gels, and the gels were stained for Met-Ala-Ser–hydrolyzing activity. A single band of activity toward Met-Ala-Ser was detected at R_f 0.2. This band showed strong activity against several tripeptides (Met-Ala-Ser, Met-Thr-Met, Met-Gly-Gly, Met-Met-Met, Phe-Gly-Gly, Leu-Leu-Leu, Leu-Gly-Gly), weak activity against larger peptides (Phe-Gly-Gly-NH_2, Met-Gly-Met-Met), and little or no activity against dipeptides (Met-Ala, Met-Gly). Therefore, the activity appeared to be a tripeptidase with a specificity profile that does not correspond completely with the peptide utilization pattern of the mutant strain. However, because only one band of activity was observed, it seemed likely to us that this activity was responsible for growth of the multiply peptidase-deficient strain on Met-Ala-Ser.

Isolation of mutants lacking tripeptidase activity. To isolate mutants lacking the R_f 0.2 band, we first tried screening for colonies unable to use Met-Ala-Ser as a methionine source after ampicillin selection in medium containing Met-Ala-Ser as the only source of methionine. Seven independent mutants that failed to grown on Met-Ala-Ser were characterized. All of these mutants retained the R_f 0.2 activity. The inability to use a peptide as an amino acid source when peptidase activity was present suggested that the mutants might be defective in peptide transport. We found that the mutations in these strains had the following characteristics: they prevented growth on the peptides Leu-Leu-Leu and Leu-Gly-Gly when present in *pepA*$^+$ strains (peptidase A activity is sufficient to permit growth on these peptides [21]; this indicates that these mutations affect a step in peptide utilization other than peptide hydrolysis), and they conferred resistance to the toxic peptide norleucyl-glycyl-glycine (which has been used to select mutants deficient in peptide transport [1]). Although these mutations did not map near *trp* and therefore were not *opp* (oligopeptide permease) mutations (3, 16), we believe that these mutations caused defects in tripeptide transport. Because no mutants lacking the R_f 0.2 peptidase activity were isolated, this method was abandoned.

In another attempt to isolate mutants lacking the R_f 0.2 activity, strain TN1146 (which lacks the other Met-Ala-Ser–hydrolyzing enzymes, peptidases N, A, and B) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and single-colony isolates were screened for inability to hydrolyze Met-Ala-Ser in a microassay performed in multiwell plastic plates. From 1,600 colonies screened, 15 prospective mutants were tested for the presence of the R_f 0.2 band after gel electrophoresis of cell extracts. One strain (TN1152) was isolated which lacked the R_f 0.2 band. The mutation in this strain was designated *pepT1*, and the activity affected was named peptidase T (for tripeptidase).

Utilization of peptides by *pepT* mutants. The mutant strain (TN1152) retained the ability to use Met-Ala-Ser as a methionine source. Because this strain lacked only peptidases N, A, B, and T, it was possible that one of the other known peptidases (peptidase D, P, and Q) was responsible for the growth of TN1152 on Met-Ala-Ser. To eliminate this possibility, it was necessary to move the *pepT1* mutation into a strain lacking all of the known peptidases. To accomplish this a Tn*10* insertion cotransducible with *pepT1* was isolated by the following procedure. Transducing phage grown on a population of 1,200 random Tn*10* insertion mutants of Tn1147 (*pepN pepA pepB pepD pepP pepQ*) (9, 15) was used to transduce TN1152 (*pepN pepA pepB pepT*) to tetracycline resistance (Tet^r). Approximately 1,000 Tet^r transductants were screened for the ability to hydrolyze Met-Ala-Ser by using the microassay. Fourteen Met-Ala-Ser–hydrolyzing strains were purified by single colony isolation, and transducing phage was grown on them to test the linkage between Tet^r and Met-Ala-Ser–hydrolyzing ability. Five strains were tested for linkage. Two of these strains carried Tn*10* insertions linked to *pepT*. One strain (TN1214) was saved which carries an insertion 75% linked to *pepT* (*zce-850*::Tn*10*).

TABLE 3. Transductional mapping of *pepT*

Donor	Recipient	Selected marker	Unselected marker	Linkage (%)
TN1214 (*pepT*$^+$ *zce-850*::Tn*10*)	TN1152 (*pepT1*)	Tet	PepT	65/92 (70)
TN1215 (*pepT1 zce-850*::Tn*10*)	TN1146 (*pepT*$^+$)	Tet	PepT	45/60 (75)
TN1215 (*pepT1 zce-850*::Tn*10*)	TN2024 (*purB13*)	Pur	Tet	178/360 (54)
TN1146 (*purB*$^+$ *pepT*$^+$)	TN1286 (*purB13 pepT1 zce-850*::Tn*10*)	Pur	PepT	56/92 (61)
		Pur	Tet	51/92 (55)
TN1214 (*pepT*$^+$ *zce-850*::Tn*10*)	TN1641 (*pepT6*::Mu d1)	Tet	Amp	110/150 (73)
TN1214 (*pepT*$^+$ *zce-850*::Tn*10*)	TN1642 (*pepT7*::Mu d1)	Tet	Amp	122/150 (81)

The results of crosses demonstrating linkage of the insertion to *pepT* are shown in Table 3. Transducing phage grown on TN1215 (*pepT1 zce-850*::Tn*10*) was used to transduce TN1147 (*pepN pepA pepB pepD pepP pepQ*) to Tet^r. A transductant able to hydrolyze Met-Ala-Ser was saved as TN1281, and one unable to hydrolyze the peptide was saved as TN1282. TN1282, which lacks seven peptidases, was found to grow on Met-Ala-Ser, Met-Ala-Met, and Met-Thr-Met. Consequently, it has the same growth profile as the isogenic *pepT*$^+$ strain TN1281 (Table 2). This result was surprising because we could not detect hydrolytic activity against Met-Ala-Ser in extracts of TN1281. We conclude that there must be another activity present which cannot be detected under our assay conditions.

In electrophoresis gels peptidase T appears to hydrolyze Met-Gly-Gly, Met-Met-Met, and Met-Met-Ala as well as it hydrolyzes Met-Ala-Ser, Met-Ala-Met, and Met-Thr-Met (see Table 5). Therefore, if peptidase T activity were sufficient for utilization of Met-Ala-Ser as a methionine source, it should also be sufficient for utilization of Met-Gly-Gly, Met-Met-Met, and Met-Met-Ala. The inability of *pepT*$^+$ strains to use these peptides as methionine sources makes it unlikely that peptidase T contributes significantly to the utilization of Met-Ala-Ser by *pepT*$^+$ strains. It thus appears that peptidase T plays no role in utilization of peptides as amino acid sources.

Isolation of additional *pepT* alleles. Because no peptide growth phenotype could be associated with the *pepT1* mutation, it was necessary to isolate other *pepT* alleles by enzyme assay screens. Consequently, localized mutagenesis procedures which rely on the Tn*10* insertion linked to *pepT1* were used to decrease the number of colonies that had to be screened. Because Tn*10* is known to cause deletions and inversions upon excision (15), Tet^s derivatives are likely to possess mutations in loci near the insertion site. A selective medium developed by Bochner et al. (4) allows isolation of Tet^s colonies by virtue of their increased resistance to fusaric acid. Tet^s (i.e., fusaric acid resistant) derivatives of TN1281 (*pepT*$^+$ *zce-850*::Tn*10*) were screened for inability to hydrolyze Met-Ala-Ser in the microassay. Peptidase mutants were found at a frequency of 1/100. Strains TN1319-1322 (*pepT2-5*) were isolated by this procedure. All four of these strains, which lack seven peptidases (peptidases N, A, B, D, P, Q, and T), retained the ability to grow on Met-Ala-Ser.

To obtain point mutations at the *pepT* locus, localized mutagenesis with hyroxylamine was performed on transducing phage grown on TN1214 (*pepT*$^+$ *zce-850*::Tn*10*). The mutagenized phage lysate was used to transduce TN1304 (*pepN pepA pepB pepP pepQ*) to Tet^r. These transductants should have inherited mutagenized DNA flanking the Tn*10* insertion. The Tet^r transductants were screened for inability to hydrolyze Met-Gly-Gly. Mutants unable to hydrolyze the tripeptide were found at a frequency of 1/500. Eight mutants (TN1703 through TN1710, *pepT8* through *pepT15*) were isolated by the hydroxylamine-localized mutagenesis. Two of these alleles, *pepT8* and *pepT11*, appear to be leaky as judged by staining gels for Met-Gly-Gly hydrolysis (Table 4).

To obtain insertion mutants lacking peptidase T, the operon fusion vector Mu d1(Ap^r *lac* cts62) was used as an insertion mutagen. Mu d1 was used in preference to transposons Tn*10* or Tn*5* because Mu insertion is known to be random with respect to DNA sequence at the target site (5), but Tn*10* and Tn*5* are known to exhibit site specificity (14). Consequently, it was likely that a Mu d1 insertion would be found among the number of insertion mutants which could be conveniently tested by the enzyme assay screen. In addition, Mu d1 is capable of generating an operon fusion of *lacZYA* to the promotor of the gene inactivated by the insertion (7), and such a fusion could be useful in studying possible regulation of peptidase T. To obtain the insertion mutants, Mu *d*1 was introduced into strain TN1304 (*pepN pepA pepB pepP pepQ*) via generalized transduction with P22 phage grown on a *S. typhimurium* Mu d1 lysogen. Approximately 6,500 ampicillin-resistant insertion mutants were

TABLE 4. Peptidase T activity in *pepT* mutants[a]

Strain (allele)	Sp act (nmol of Met/min per mg)	Relative activity (%)
TN1304 (*pepT*$^+$)	138	100
TN1508 (*pepT1*)	<0.33	<0.2
TN1324 (*pepT2*)	0.42	0.3
TN1325 (*pepT3*)	<0.33	<0.2
TN1850 (*pepT7*::Mu d1)	0.77	0.6
TN1703 (*pepT8*)	20.4	14.8
TN1704 (*pepT9*)	0.68	0.5
TN1705 (*pepT10*)	1.37	1.0
TN1706 (*pepT11*)	16.3	11.8

[a] Reaction mixtures contained 10 mM Met-Gly-Gly, 0.1 mM $MnCl_2$, and 100 μg (TN1304) or 500 μg (other strains) of protein of a soluble cell extract in 0.5 ml of 50 mM Tris-hydrochloride (pH 7.5). Reaction mixtures were incubated at 37°C for 30 min (TN1304) or 120 min (other strains).

screened for inability to hydrolyze Met-Gly-Gly. Two *pepT*::Mu *d*1 mutants were found (TN1641 and TN1642). Both of these mutants lacked the R_f 0.2 band after gel electrophoresis of soluble cell extracts.

The specific activity of peptidase T in cell extracts of representative *pepT* mutants is shown in Table 4. Most of the *pepT* alleles reduced peptidase T activity to less than 1% of that of the wild type. The two alleles that appeared leaky when tested by gel electrophoresis of cell extracts, *pepT8* and *pepT11*, contained approximately 15 and 12%, respectively, of the wild-type activity.

Map position of the *pepT* locus. The *pepT* locus was mapped by using the Tn*10* insertion (*zce-850*::Tn*10*) linked to the *pepT1* allele. This insertion was introduced into two Hfr strains which were used as donors in Hfr crosses with auxotrophic recipients. Prototrophic recombinants were selected and screened for inheritance of tetracycline resistance encoded by Tn*10*. The highest linkage occurred with the markers *pyrD* (20 map units, 82%), *pyrC* (22 map units, 76%), and *purB* (25 map units, 96%). These three markers were then tested for linkage to the Tn*10* insertion by P22 transduction. Only *purB* was transductionally linked to the Tn*10* insertion. The results shown in Table 3 indicate that *purB* and the Tn*10* insertion are 55% linked. To test the linkage between *purB* and *pepT* we constructed a Tet^r PurB PepT derivative of TN1147, a multiply peptidase-deficient strain (in which the presence or absence of peptidase T could be detected by the microassay). This strain, TN1286 (*pepT1 purB13 zce-850*::Tn*10 pepN pepA pepB pepD pepP pepQ*), was transduced to Pur$^+$ with phage grown on TN1214 (*pepT*$^+$ *purB*$^+$). Pur$^+$ transductants were tested for Tet^r and ability to hydrolyze Met-Ala-Ser. The *purB* locus and *pepT1* were 60% linked (Table 3). Based on the linkages derived from two point crosses (75% between the Tn*10* and *pepT*, 60% between *pepT* and *purB*, and 55% between the Tn*10* and *purB*), the probable order of the markers is *zce-850*::Tn*10*—*pepT*—*purB*. The two Mu d1 insertions are the only other *pepT* alleles that were isolated in a nonlocalized manner (i.e., the mutagenesis and screening procedure did not require linkage to the Tn*10* insertion linked to *pepT1*). These two mutations were therefore tested for linkage to the Tn*10* insertion. As shown in Table 3, the two Mu d1 insertions show approximately the same linkage to the Tn*10* insertion as *pepT1* does. Therefore it is likely that *pepT* is the only locus at which mutations leading to loss of peptidase T activity occur.

Properties of peptidase T. As stated above, peptidase T can be detected in nondenaturing polyacrylamide gels at an R_f of 0.2. It was previously reported that multiply peptidase-deficient strains of *S. typhimurium* contain another band of activity against Met-Ala-Ser at an R_f of 0.4 in these gels (21). During the course of this work it became apparent that detection of this R_f 0.4 band was variable. Although this band was occasionally seen in extracts made from cells grown in nutrient broth, it was never seen in crude extracts of cells grown in E medium or Luria broth. Because no difference in substrate specificity between the R_f 0.2 and 0.4 bands had been detected, it seemed possible that the two bands were electrophoretic forms of peptidase T, perhaps due to alternative conformations of the protein or a posttranslational modification. If so, then the *pepT* mutant should not contain the R_f 0.4 activity band. To test this it was necessary to define conditions that allow detection of the R_f 0.4 band. We have observed that both the R_f 0.2 and 0.4 bands could be detected in column eluents after DEAE-cellulose column chromatography of cell extracts. When an extract containing only the R_f 0.2 band (prepared from strain TN1146 grown in E medium) was chromatographed on DEAE-cellulose, the R_f 0.4 band of activity could be detected in column fractions from the trailing end of the peak of the R_f 0.2 band (Fig. 1). When the same experiment was performed with an extract of TN1152 (*pepT1*) neither the R_f 0.2 nor the R_f 0.4 band could be detected in the column fractions. This is consistent with these two bands being electrophoretic forms of one enzyme.

To characterize the substrate specificity of peptidase T, soluble cell extracts were prepared from *pepT*$^+$ and *pepT1* strains which have mutations affecting all of the other peptidases which have been studied. These strains carry muta-

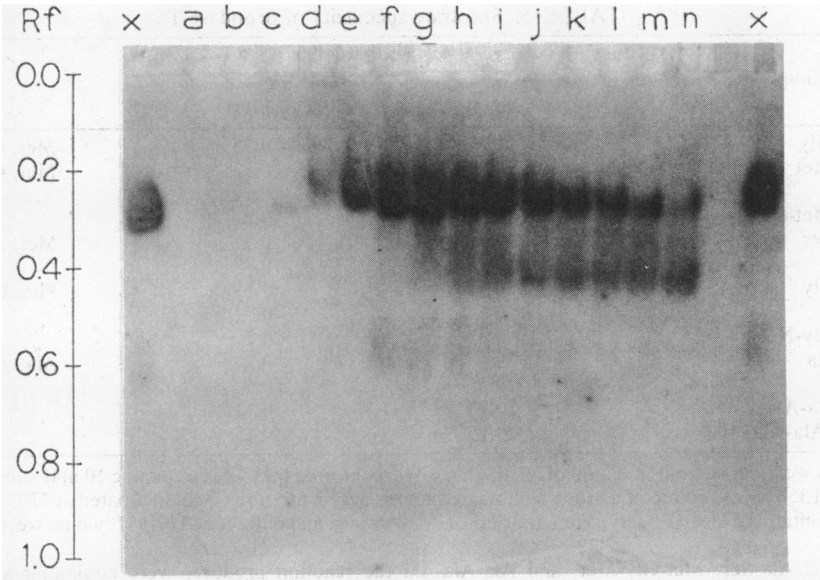

FIG. 1. DEAE-cellulose chromatography of peptidase T. A crude soluble extract of TN1146(*pepT*$^+$) was prepared and chromatographed as described in the text. Lanes marked "×" contained 2 μg of protein of crude extract. Other lanes contained 20 μl of column eluent; a, fraction 45; b, fraction 50; c, fraction 55; d, fraction 60; e, fraction 65; f, fraction 70; g, fraction 75; h, fraction 80; i, fraction 85; j, fraction 90; k, fraction 95; l, fraction 100; m, fraction 105; n, fraction 110. The activity at R_f 0.6 is not present in a *pepD* strain.

tions eliminating three aminopeptidases (*pepN pepA pepB*), a dipeptidase (*pepD*), two proline-specific peptidases (*pepP pepQ*), a dipeptidyl carboxypeptidase (*dcp* [27]), and an oligopeptidase (*optA* [26]). These mutations eliminate most of the cell's hydrolytic activity toward dipeptides, tripeptides, and tetrapeptides. By comparing the activity of cell extracts of isogenic *pepT*$^+$ and *pepT1* strains lacking these other enzymes, the substrate specificity can be determined. As shown in Table 5, when tested with several homologous series of dipeptides, tripeptides, and tetrapeptides, the dipeptides and tetrapeptides were not hydrolyzed at any detectable rate by the *pepT*$^+$ extract. The rate of hydrolysis of Phe-Gly-Gly-NH_2 was approximately 2.5% the rate of hydrolysis of the homologous tripeptide Phe-Gly-Gly. The tripeptides (Met-Gly-Gly, Met-Gly-Met, Met-Ala-Ser, Phe-Gly-Gly, and Ala-Ala-Ala) were only hydrolyzed by the *pepT*$^+$ extract. The *pepT1* extract had less than 1% of the activity of the pepT$^+$ extract against these tripeptides. The hydrolysis products of these tripeptides were the N-terminal amino acids and the C-terminal dipeptides. We conclude that peptidase T is an aminotripeptidase.

The effect of metal ions on peptidase T activity is shown in Table 6. Mn^{2+} stimulated peptidase T activity, but Co^{2+} and Mg^{2+} did not. Zn^{2+} showed the greatest stimulation of peptidase T, but this occurred in a relatively narrow concentration range. Zn^{2+} had little affect at 0.01 mM, showed fourfold stimulation at 0.1 mM, and was highly inhibitory at 1.0 mM. The activity of peptidase T could be completely inhibited by EDTA at concentrations as low as 0.01 mM. Therefore, peptidase T is a metalloenzyme like most other peptidases in *S. typhimurium*.

DISCUSSION

The evidence presented in this paper shows that *S. typhimurium* contains, in addition to peptidases that have already been characterized, a tripeptidase. This enzyme has been designated peptidase T to emphasize that the enzyme is a tripeptidase and to be consistent with the nomenclature for peptidases and *pep* genes which has been used for the other peptidases of *S. typhimurium*. A tripeptidase of *E. coli* K-12, tripeptidase TP, has been partially purified and characterized by Hermsdorf (10). Like peptidase T, tripeptidase TP is a metalloenzyme. Tripeptidase TP electrophoreses in native gels at an R_f of 0.47, similar to the R_f 0.4 electrophoretic form of peptidase T. In addition, tripeptidase TP shows relative specific activity against tripeptides of the order Met-Gly-Met > Met-Gly-Gly > Met-

TABLE 5. Substrate specificity of peptidase T[a]

Substrate	% Hydrolyzed by: TN1931 (*pepT*$^+$)	% Hydrolyzed by: TN1727 (*pepT1*)	Products[b]
Met-Gly-Gly	46	0.25	Met, Gly-Gly, Gly
Met-Gly-Met	99	<0.13	Met, Gly-Met
Met-Gly	<0.12	<0.12	
Met-Gly-Met-Met	<0.8	<0.8	
Met-Ala-Ser	35	0.33	Met, Ala-Ser
Met-Ala	<0.17	<0.17	
Phe-Gly-Gly	10	<0.15	Phe, Gly-Gly, Gly
Phe-Gly	<0.5	<0.5	
Phe-Gly-Gly-NH_2	0.33	<0.2	
Ala-Ala-Ala	29	0.14	Ala, Ala-Ala
Ala-Ala	<0.5	<0.5	
Ala-Ala-Ala-Ala	<0.13	<0.13	
N-Acetyl-Ala-Ala-Ala	<0.15	<0.15	

[a] Assays were carried out in 0.5 ml of 50 mM Tris-hydrochloride (pH 7.5) containing 10 mM substrate, 0.1 mM $MnCl_2$, and 350 μg of protein of a crude soluble extract. Reaction mixtures were incubated at 37°C for 3 h, except for those containing Met-Gly-Gly, Met-Ala-Ser, or Ala-Ala-Ala and extract of TN1931, which were incubated for 2 h.

[b] For Met-Ala-Ser, Met-Gly-Met, and Ala-Ala-Ala the reaction products were equimolar amounts of N-terminal amino acid and dipeptide. For Met-Gly-Gly and Phe-Gly-Gly the reaction products contained a mixture of Gly-Gly and Gly due to a glycl-glycine peptidase (K. L. Strauch, unpublished data) present in these extracts. Gly-Gly is not a substrate for peptidase T.

Ala-Ser > Phe-Gly-Gly (10). Our results with peptidase T show the same relative preference for these substrates. These similarities suggest that peptidase T and tripeptidase TP are homologous enzymes in these two organisms. More information on the comparative properties of purified enzymes from the two organisms will be necessary to establish the validity of this suggestion. The substrate specificity of peptidase T does not suggest any role for this enzyme in the removal of methionine from the N terminus of nascent protein.

We have not been able to show that peptidase T confers the ability to use any peptide as an amino acid source. Of the methionine-containing tripeptides which have been tested, only Met-Ala-Ser, Met-Ala-Met, and Met-Thr-Met can be used as methionine sources by multiply peptidase-deficient, but *pepT*$^+$, strains. Because *pepT*$^+$ strains are unable to use Met-Gly-Gly or Met-Gly-Met as amino acid sources and these two tripeptides are better substrates for peptidase T than Met-Ala-Ser, it is unlikely that peptidase T is involved in the utilization of Met-Ala-Ser, Met-Ala-Met, and Met-Thr-Met. The ability of *pepT* mutants to utilize these peptides suggests that another enzyme capable of hydrolyzing these tripeptides is present but cannot be detected under the conditions used for native gels or for assaying peptidase T.

Peptidase T is the first peptidase of *S. typhimurium* which has not been found to confer a peptide growth phenotype. In another communication (K. Strauch, T. Carter, and C. G. Miller, manuscript in preparation) we report that mutants that overproduce peptidase T are able to utilize a variety of tripeptides as amino acid sources. Apparently, the wild-type level of peptidase T activity is insufficient to allow growth on tripeptide substrates.

TABLE 6. Effect of metal ions on peptidase T activity[a]

Addition (mM)	nmol of Met/min per mg	Fold stimulation
No addition	42	1.00
Mn^{2+} (1.0)	105	2.5
(0.1)	56	1.3
(0.01)	38	0.90
Mg^{2+} (1.0)	30	0.72
(0.1)	41	0.97
(0.01)	43	1.03
Co^{2+} (1.0)	42	1.00
(0.1)	39	0.94
(0.01)	41	0.98
Zn^{2+} (1.0)	54	0.13
(0.1)	183	4.35
(0.01)	47	1.12
EDTA (1.0)	<1.6	<0.039
(0.1)	<1.6	<0.039
(0.01)	<1.6	<0.039

[a] All ions were present as chloride salts. Reaction mixtures contained 10 mM Met-Gly-Gly and 100 μg of protein of a crude soluble extract of TN1931 in 0.5 ml of 50 mM Tris-hydrochloride (pH 7.5). All incubations were performed at 37°C.

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