JOURNAL OF BACTERIOLOGY, May 1978, p. 361-374
0021-9193/78/00134-0361$02.00/0

Vol. 134, No. 2
Printed in U.S.A.

Salmonella typhimurium Peptidase Active on Carnosine†

MARVIN KIRSH,†† DAVID R. DEMBINSKI,[1] PHILIP E. HARTMAN,[1]* AND CHARLES G. MILLER[2]

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218,[1] and Department of Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106[2]

Received for publication 28 December 1977

Wild-type *Salmonella typhimurium* can use carnosine (β-alanyl-L-histidine) as a source of histidine, but carnosine utilization is blocked in particular mutants defective in the constitutive enzyme peptidase D, the product of the *pepD* gene. Biochemical evidence for assigning carnosinase activity to peptidase D (a broad-specificity dipeptidase) includes: (i) coelution of carnosinase and dipeptidase activity from diethylaminoethyl-cellulose and Bio-Gel P-300 columns; (ii) coelectrophoresis of carnosinase and dipeptidase on polyacrylamide gels; and (iii) inactivation of carnosinase and dipeptidase activities at identical rates at both 4 and 42°C. Genetic evidence indicates that mutations leading to loss of carnosinase activity map at *pepD*. Several independent *pepD* mutants have been isolated by different selection procedures, and the patterns of peptide utilization of strains carrying various *pepD* alleles have been studied. Many *pepD* mutations lead to the production of partially active peptidase D enzymes with substrate specificities that differ strikingly from those of the wild-type enzyme. The growth yields of carnosinase-deficient strains growing in Difco nutrient broth indicate that carnosine is the major utilizable source of histidine in this medium.

Salmonella typhimurium contains eight distinct peptidases with broad specificities (12, 15), and chromosomal locations of genes affecting five of the eight activities are known (13). *S. typhimurium* can grow on carnosine (β-alanyl-L-histidine; Car) as a source of histidine (5), but the enzyme(s) involved in Car utilization has not been elucidated. In fact, data pertaining to the specificities of enteric proteases and peptidases are quite limited (7, 14).

We have developed an assay for carnosinase effective on sonically treated extracts and on toluenized bacteria, and we have shown that only one of the *S. typhimurium* peptidases acts on Car. Our physiological, genetic, and biochemical tests indicate that carnosinaseless mutants lack or contain altered peptidase D activity, specified by the *pepD* gene. Peptidase D has been partially purified, a spectrophotometric assay for activity has been developed, and some properties of peptidase D are outlined. Particular *pepD* mutants were used to estimate the Car content of biological materials.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* LT2 was progenitor for all strains listed in Table 1 except for strain SB5401, which is an *S. typhimurium* LT7 derivative. Deletions encompassing the *proAB-gpt* region were transduced into strains as necessary by selection on E medium containing proline and 100 μg of 8-azaguanine per ml (3). Transduction to *pepD* was then possible by selection for the *pro*$^+$ marker and examination of transductional clones for their content of the unselected but jointly transduced *pepD* marker. Strain SB3773 was obtained from strain SB3760 after lysogenization with *lacA7* transducing phage (8) and selection of a clone that had lost P22 helper phage (2).

Phage and transduction tests. P22 phage mutant *int-4* and an HT *int* (high-frequency transduction; integration defective) phage double mutant obtained from John R. Roth (19) were grown and used as described by Ely et al. (2). Dr. Roth also supplied the P22 *lacA7* specialized transducing phage (8).

Media. Minimal salts E medium (25) was supplemented with 0.2% glucose and, when required, with L-amino acids or peptides containing L-amino acids at 20 μg/ml. Nutrient broth (Difco) served as a rich medium except in one series of experiments involving comparison of *rel* and *rel*$^+$ bacteria; in this comparison, the medium contained 1% tryptone (Difco), 0.5% NaCl, 0.2% Casamino Acids, and 0.2% glucose at pH 7.2 (26). Solid media contained 1.5% agar (BBL).

Chemicals. 8-Azaguanine, Car, and several peptides were purchased from Calbiochem; other peptides originated from Cyclo, Bachem, Sigma, Vega-Fox, and Biopolymers. Amino acids in peptides were of the L-configuration. Because it elicited an unusual growth response, Gly-Pro-Ala (Bachem) was examined for purity. The Gly-Pro-Ala produced only one ninhydrin-positive spot after descending paper chromatography with 72% phenol and 28% ammonia (4). Analysis by Manuel Ricardo on a Beckman Instruments model 89B automated amino acid sequencer indicated the

† Contribution no. 946 of the Department of Biology, The Johns Hopkins University.

†† Present address: Division of Biological Sciences, University of Missouri, Columbia, MO 65201.

TABLE 1. *S. typhimurium strains*

Strain designation	Genotype	Origin or reference
	ΔhisBH22	(5)
	hisG46	Stock
	Δleu-39	Stock
	leu-485	
SB1333	*ara-9 metE338*	(15)
SB3041	*pepD101 ΔhisBH22*	2AP + Pen + Car in *hisBH22*[a]
SB3095	*fla-2055 hisG46 pepD102*	Spont. in *hisG46*[a]
SB3436	*hisT1504*	(26)
SB3437	*hisT1504 relA1*	(26)
SB3760	*his-6853 leu-485 pepA1 pepB1 pepD1 pepD105 pepN10*	DES + Pen + Gly-His in TN277[a]
SB3761	*his-6853 leu-485 pepA1 pepB1 pepD1 pepN10 Δ(supQ357-pro-gpt)*	TN277 × JK1357[b]
SB3773	*his-6853 leu-485 pepA1 pepB1 pepD1 pepD105 pepN10* (P22 *lac*$^+$ *pro*$^+$ *pepD*$^+$)	SB3760 + *lacA7* (see text)
SB3775	*his-6853 leu-485 pepA1 pepB1 pepN10 Δ(supQ357-pro-gpt)*	TN277 × JK1357[b]
SB3796	*his-6853 leu-485 pepA1 pepB1 pepN10 Δ(proAB47-gpt-pepD)*	SB3760 × SB5401[b]
SB3797	*his-6853 leu-485 pepA1 pepB1 pepN10*	TN277 × TN274[b]
SB3803	*his-6853 leu-485 metE527 pepA1 pepB1 pepD1 pepD105 pepN10*	2AP + Pen + Met in SB3760[a]
SB3804	*his-6853 leu-485 metE527 pepA1 pepB1 pepD1 pepD105 pepN10 pro-520*	DES + Pen + Pro in SB3803[a]
SB3843	*his-6853 leu-485 metE527 pepA1 pepB1 pepN10 pepD105*	SB3804 × SB3041[b]
SB3844	*his-6853 leu-485 pepA1 pepB1 pepN10 pepD102*	SB3761 × SB3095[b]
SB3845	*his-6853 leu-485 pepA1 pepB1 pepN10 pepD101*	SB3761 × SB3041[b]
SB4044	*his-6853 leu-485 pepA1 pepB1 pepN10 pepD3*	SB3796 × TN360[b]
SB4045	*his-6853 leu-485 pepA1 pepB1 pepN10 pepD4*	SB3796 × TN362[b]
SB5401	*hisI401 Δ(proAB47-gpt-pepD)*	(4, 9)
TN213	*leu-485 pepA1 pepD1 pepN10*	(15)
TN215	*leu-485 pepA1 pepB1 pepD1 pepN10*	(15)
TN274	*leu-485 pepA1 pepB1 pepN10*	(15)
TN277	*his-6853 leu-485 pepA1 pepB1 pepD1 pepN10*	DES + Pen in TN215[a]
TN279	*leu-485 pepA1 pepB1 pepD1 pepN10 phe-56*	DES + Pen in TN215[a]
TN360	*leu-485 pepA1 pepD3 pepN10*	As TN213 (15)
TN362	*leu-485 pepA1 pepD4 pepN10*	As TN213 (15)
TN417	*leu-485 pepA1 pepB1 pepD1 pepD106 pepN10*	TN215, DES + small colony[c]
TN419	*leu-485 pepA1 pepB1 pepD1 pepD107 pepN10*	TN215, DES + small colony[c]
TN420	*leu-485 pepA1 pepB1 pepD1 pepD108 pepN10*	TN215, DES + small colony[c]
TN421	*leu-485 pepA1 pepB1 pepD1 pepD109 pepN10*	TN215, DES + small colony[c]
TN422	*leu-485 pepA1 pepB1 pepD1 pepD110 pepN10*	TN215, DES + small colony[c]
TN429	*leu-485 pepA1 pepB1 pepD1 pepD111 pepN10*	TN215, DES + small colony[c]
TN430	*leu-485 pepA1 pepB1 pepD1 pepD112 pepN10*	TN215, DES + small colony[c]
TN431	*his-6853 leu-485 pepA1 pepB1 pepD1 pepD113 pepN10*	TN277, DES + small colony[c]
TN432	*his-6853 leu-485 pepA1 pepB1 pepD1 pepD114 pepN10*	TN277, DES + small colony[c]
TN493	*leu-485 pepA1 pepB1 pepD1 pepD115 pepN10*	TN215, DES + small colony[c]
TN494	*leu-485 pepA1 pepB1 pepD1 pepD116 pepN10*	TN215, DES + small colony[c]
TN495	*leu-485 pepA1 pepB1 pepD1 pepD117 pepN10*	TN215, DES + small colony[c]
TN496	*leu-485 pepA1 pepB1 pepD1 pepD118 pepN10 phe-56*	TN279, DES + small colony[c]
TN497	*leu-485 pepA1 pepB1 pepD1 pepD119 pepN10 phe-56*	TN279, DES + small colony[c]
TN498	*leu-485 pepA1 pepB1 pepD1 pepD120 pepN10 phe-56*	TN279, DES + small colony[c]
TN516	*leu-485 pepA1 pepB1 pepD1 pepD122 pepN10*	TN215, DES + small colony[c]
JK1357[d]	*Δ(leuD798-ara) Δ(supQ357-pro-gpt)*	(9)

[a] Mutation induction was spontaneous (Spont.), with 2-aminopurine (2AP), or with diethyl sulfate (DES). Penicillin selection (Pen) was used in obtaining several mutants, in some cases performed in the presence of Car or Gly-His.

[b] P22-mediated transductional cross (recipient × donor) and selection for nonlysogenic clone.

[c] Parental culture treated with diethyl sulfate and small colony selected on minimal medium supplemented with excess Gly-Leu (0.2 mM) and a low concentration of L-leucine (0.01 mM).

[d] Other JK strains used in this study and carrying *supQ-pro* deletions are described by Kemper (9).

presence of Gly, Pro, and Ala in a ratio not significantly different from 1:1:1. *o*-Phthaldehyde (*o*-phthalic dicarboxaldehyde) was purchased from Aldrich, 2,4,6-trinitrobenzenesulfonic acid (TNBS) from Pierce, aldolase from Pharmacia, guanosine 5'-diphosphate 3'-diphosphate from P-L Biochemicals, bovine serum albumin from Calbiochem, and L-amino acid oxidase, horseradish peroxidase, and *o*-dianisidine from Sigma.

Growth response survey. Appropriately supplemented E medium plates were spread with a lawn of approximately 2×10^8 bacteria, and then either crystals or drops of solutions of compounds were added. Growth was scored after incubation at 37°C for 24 and 48 h.

Gel electrophoresis and stain. Electrophoresis of cell extracts on 7.0% polyacrylamide slab gels and staining for peptidase activity were performed by previously described procedures (15).

Carnosinase. To obtain sonic extracts, bacteria in 50 ml of medium were grown to about 10^8 cells/ml and sedimented in a Sorvall refrigerated centrifuge. The pellet was resuspended in 4.0 ml of 0.01 M potassium phosphate buffer, pH 7.4, before resedimentation and resuspension in 1.0 ml of the same buffer. The suspension was treated at 1.6 A for 1.0 min in an MSE ultrasonic power unit and clarified by centrifugation in a Beckman model L ultracentrifuge at 60,000 × *g* for 1 h.

For toluenized cell suspension, bacteria grown and sedimented as in the preparation of sonic extracts were resuspended and washed in 5 ml of distilled water before resuspension in 5 ml of distilled water. After the addition of 0.33 ml of toluene, the mixture was first agitated for 2 min with a Vortex-Genie mixer and then shaken at 37°C for 15 min.

The assay procedure for carnosinase activity was modified from one previously described (16). Attempts to increase the sensitivity of the assay (cf. 1, 24) either led to high blanks or were unsatisfactory for other reasons. In our procedure, an appropriate amount of extract or of toluenized bacteria (about 0.5 mg of protein when Co^{2+} was used as cation and 2.0 mg with Mn^{2+}) was added to 10 mM imidazole buffer, pH 7.2 (I buffer), containing 0.884 mM substrate and cation in a final volume of 10 ml. In some of the earlier experiments, 0.3 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.5 (T buffer), and 0.442 mM substrate were used. During incubation at 37°C, 0.9-ml samples of the reaction mixture were removed at various times, and the reaction was stopped by addition of 0.1 ml of 50% trichloroacetic acid. After the addition of 0.1 mg of bovine serum albumin, the precipitate was sedimented by centrifugation. Neutralization of the supernatant was achieved by addition of 0.1 ml of 12.0 N NaOH. Samples were frozen at −4°C and were assayed within 2 days; assay results were not affected by these storage conditions.

A 1% solution of *o*-phthaldehyde in ethanol was diluted 1:11 in 0.05 M sodium tetraborate buffer, pH 9.5. Samples of 0.2 ml were added to 2.0 ml of the buffer, and the fluorescence was determined after 7 min in an Aminco spectrofluorometer (λ_{excite} = 355 nm, λ_{emit} = 455 nm). Histidine standards were prepared by combining extracts from a Car^- strain, Car, and Co^{2+} and varying amounts of L-histidine. The standards were frozen before assay. Data in this paper are based on reaction rates involving four or more points in which activity was linear with time of enzyme incubation and where activity was proportional to protein concentration. Specific activity is defined as micromoles of histidine released per hour per milligram of protein.

Peptidase D purification. One liter of a nutrient broth culture of strain TN274 was grown to about 10^8 cells/ml (late log phase) and sedimented in a Sorvall refrigerated centrifuge. The pellet was resuspended in 50 ml of 0.01 M Tris-hydrochloride + 0.05 M KCl, pH 7.5, at 25°C (buffer A), resedimented, and resuspended in 10 ml of the same buffer. An MSE ultrasonic power unit was used to treat 1.0-ml aliquots of the cell suspension on ice at 1.6 A for 1.5 min. After centrifugation in a Beckman model L ultracentrifuge at 60,000 × *g* for 1 h, the upper 6 ml was decanted and used as the crude sonic fluid.

Five milliliters of crude sonic fluid containing 80 to 100 mg of protein was applied directly to a gel filtration column (Bio-Gel P-300; dimensions 3 by 40 cm) equilibrated with buffer A. The column was eluted with the same buffer, and 4.4-ml fractions were collected. Fractions containing peptidase activity were identified by using the amino acid oxidase spot test procedure described below.

The most active fractions from the gel filtration column were pooled and applied to a Whatman DE-52 column (2 by 20 cm) previously equilibrated with buffer A. The column was pumped with 1 to 2 bed volumes of buffer A before elution with a linear KCl gradient (0.05 to 0.4 M). Fractions of 4.4 ml were collected and assayed for peptidase activity via the spot test procedure. The peptidase was purified about 20-fold in this two-step procedure, with a yield of approximately 70%.

Leu-Gly and Gly-Leu hydrolysis. In the early stages of peptidase D purification, Leu-Gly- and Gly-Leu-hydrolyzing activities were assayed quantitatively via a modification of the TNBS-Cu-borate method of Payne (18). In our procedure, 0.8 ml of reaction mix (Mn^{2+} and substrate in 0.05 M Tris-hydrochloride, pH 7.5) was prewarmed to 37°C, and the reaction was started by the addition of 0.2 ml of cold enzyme (final concentrations: 1 mM Mn^{2+}; 7.5 mM substrate). At each 15-min interval, a 0.1-ml sample was withdrawn and added to 2.5 ml of a freshly prepared solution containing 1.9 ml of 0.05 M sodium borate, 0.1 ml of 0.6% $CuSO_4$, and 0.5 ml of 0.4% TNBS. After incubation at 37°C for 20 min, the tubes were acidified by the addition of 0.1 ml of 11.6 M HCl, and the optical density at 420 nm was read immediately on a Beckman DU spectrophotometer. Standard curves were constructed by using a similar procedure.

After the second step in the purification procedure (P-300 chromatography), the enzyme preparations were sufficiently pure to permit use of the spectrophotometric peptidase assay of Schmitt and Siebert (20) to monitor Leu-Gly- and Gly-Leu-hydrolyzing activities. In our procedure, Gly-Leu activity was assayed by addition of 0.25 ml of enzyme preparation to 1.25 ml of reaction mixture containing substrate and cation in 0.05 M Tris-HCl, pH 7.5 (final concentrations: 6.667

mM Gly-Leu; 0.1 mM Co^{2+}). The change in absorbancy at 228 nm was then monitored at room temperature in a Beckman DU spectrophotometer using a Gilford chart recorder.

Leu-Gly-hydrolyzing activity was assayed via a similar procedure except that: (i) 0.4 ml of enzyme preparation was added to 1.6 ml of reaction mixture; (ii) final substrate concentration was 10 mM; and (iii) a wavelength of 225 nm was used.

Spot tests. Peptidase D activity was detected via a modification of the method of Miller and MacKinnon (15). One drop of each fraction was incubated at 37°C for 30 min in a plastic depression plate with 2 drops of a solution made by mixing 1.4 ml of 20-mg/ml substrate (Leu-Gly or Gly-Leu), 0.6 ml of 0.01 M $MnCl_2$, and 18 ml of 0.05 M Tris-hydrochloride, pH 7.5. One drop of a mixture of 5 mg of L-amino acid acid oxidase, 5 mg of *o*-dianisidine dihydrochloride, and 10 mg of horseradish peroxidase (all in 16 ml of 0.05 M Tris-hydrochloride, pH 7.5), was then added. Activity was indicated by the formation of brown oxidized dianisidine after 5 to 10 min at room temperature (about 23°C).

Protein. Protein was measured with the Folin phenol reagent (11).

RESULTS

Origin and growth responses of mutants. Considerable overlaps in substrate specificity among *Salmonella* peptidases generally require that peptidaseless mutants be isolated in sequential steps, using carefully selected substrates (15; Fig.1, part I). On the other hand, we were able to isolate, by simple penicillin selection, a mutant defective in Car utilization and detected another such strain in a histidine-requiring stock that had been put through single-colony isolation (Table 1; Fig. 1, part II). Because of the overlapping specificities of multiple peptidases for many peptides other than Car, much of our work has been performed with bacteria with a *pepA pepB pepN* genetic background. Results also are shown for other strains because the data provide indications of the substrate specificities of some of the other peptidases. Growth responses of *pep*$^+$ and various singly and multiply mutant *pep* strains are indicated for peptides containing histidine (Table 2), proline (Table 3), methionine (Table 4), and leucine (Table 5). Not shown in Table 5 is the observation that strains *leu-39* (*pep*$^+$) and TN274 (*pepD*$^+$)cannot utilize β-Ala-Leu as a leucine source; *leu-39* can utilize Leu-β-Ala, but this peptide was not studied further.

Carnosinase is peptidase D. The following data indicate that carnosinase activity (and, thus, the ability to utilize Car as a histidine source) resides with peptidase D, the product of the *pepD* gene, and that this enzyme is the only peptidase in *S. typhimurium* capable of hydrolyzing Car.

(i) Gene *pepD* is encompassed by the *proAB47* deletion (13). Strain SB5401, which is *pepA*$^+$ *pepB*$^+$ *pepN*$^+$ and carries the *proAB47* deletion, failed to respond to Car as a histidine source (Table 2, column 13), whereas Car could serve as a histidine source when a comparable strain was lysogenized with P22 *lacA7* specialized transducing phage (strain SB3773; Table 2, column 7), known to carry the *Escherichia coli* *pepD*$^+$ gene (8, 13).

(ii) Strains SB3095 and SB3041 were selected as Car nonutilizers. When the genetic lesions conferring the Car$^-$ phenotype were transduced into a *pepA pepB pepN* genetic background (strains SB3844 and SB3845, respectively), the transductants were unable to grow on a large array of dipeptides, including Gly-Leu and Leu-Gly (Table 2, columns 13 and 14; Table 5, columns 12 and 13).

The *pepD* locus was originally defined by mutants isolated as Leu-Gly nonutilizers in a *pepN pepA* background (Fig. 1). When *pepD* mutants isolated in this way were tested for their ability to use Car as a histidine source, the response ranged from no growth at all (*pepD3*, Table 2, column 15), to weak growth (*pepD4*, Table 2, column 9), to strong growth (*pepD1*, Table 2, column 10). Differences in the responses of these

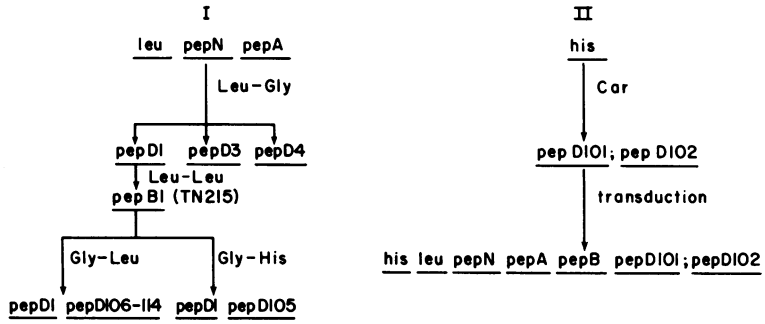

FIG. 1. *Isolation of pepD mutant strains. Mutants were selected for failure to use the indicated peptide as a source of leucine (Leu-Gly, Leu-Leu, Gly-Leu) or of histidine (Gly-His, Car).*

TABLE 3. *Growth responses to prolyl peptides*[a]

Peptide	*pep*$^+$,[b] JK1357[c]	Δ*D*, SB5401	*A1 B1 N10*, SB3775	*A1 B1 N10 D1*, SB3761	*A1 B1 N10 D1 D105*, SB3804	*A1 B1 N10* Δ*D*, SB3796
Ala-Pro	++	++	+	++	++	++
Asp-Pro	++	++	++	++	++	++
Gly-Pro	++	+	++	++	++	++
Gly-Gly-Pro	++	++	0	0	++	0
Gly-Pro-Ala	++	wk	++	++	0	0
Gly-Pro-Pro	++	++	++	++	++	++
Leu-Pro	++	++	++	++	++	++
Met-Pro	++	++	++	++	++	++
Phe-Pro	++	++	++	++	++	++
Pro-Ala	++	++	++	++	wk	0
Pro-Gly	++	++	++	0	++	0
Pro-Gly-Gly	++	++	0	0	0	0
Pro-Ile	++	++	++	0	++	0
Pro-Leu	++	++	++	++	0	0
Pro-Met	++	++	++	++	0	0
Pro-Phe	++	++	++	++	0	0
Pro-Pro	++	++	++	++	++	++
Pro-Trp	++	0	0	0	0	0
Pro-Val	++	++	++	++	++	++
Ser-Pro	++	++	++	++	++	++
Val-Pro	++	++	++	++	++	++
Pro	++	++	++	++	++	++

[a-c] See Table 1.

TABLE 4. *Growth responses to methionyl peptides*[a]

Peptide	*pep*$^+$,[b] SB1333[c]	*A1 B1 N10 D1 D105*, SB3803
Gly-Met	++	++
His-Met	+	0
Leu-Met	++	0
Met-Ala	++	++
Met-Gly	++	wk
Met-Gly-Gly	++	0
Met-His	++	0
Met-Leu	++	0
Met-Met	++	0
Met-Pro	++	++
Pro-Met	++	0
Met	++	++

[a-c] See Table 1.

strains to leucine peptides other than Leu-Gly and to histidine peptides other than Car also were found (see Table 5, columns 8, 9, and 14; Table 2, columns 9, 10, and 15). Strains containing the *pepD1* or *pepD4* alleles, for example, grew well on Gly-Leu, but a *pepD3* strain would not use this peptide. Second-step mutants were obtained from a *pepD1* strain by screening for mutants unable to use Gly-Leu (see Table 1, footnote *c*). These mutants no longer grew on Car as a histidine source (Table 2, columns 11 and 12). Strains carrying *pepD1* also grew on Gly-His, and a mutant derivative of a *pepD1* strain unable to use this peptide as a histidine source has been isolated. This strain failed to grow Car also (Table 2, column 16).

All of the *pepD* alleles isolated as Leu-Gly nonutilizers were cotransducible with *proAB*, as were the second-step mutations leading to loss of the ability of a *pepD1* strain to use Gly-Leu or Gly-His. In addition, an extract prepared from TN362 (a strain carrying the *pepD4* allele that allows weak growth on Car) contained levels of Car activity that were 14 to 29% of wild-type activity (Table 6, line 16), whereas extracts of a *pepD3* strain (that is unable to grow on Car) contained even more severely reduced Carnosinase levels (Table 6, line 9). Severely reduced carnosinase levels were also found in extracts of the Gly-Leu nonutilizing strains derived from *pepD1* (Table 6, lines 17-23). These results suggest that mutations in *pepD* frequently result in the production of peptidase D proteins that have altered substrate specificities. The biochemical and genetic results discussed below support this conclusion.

(iii) A series of strains carrying deletions in the *supQ-proAB-gpt-pepD* region of the *Salmonella* chromosome (9) was assayed for carnosinase activity in toluenized extracts and for the ability of phage grown on such strains to transduce TN215 (*pepD1*) to growth on Leu-Gly (*pepD*$^+$) (13). Seven strains had lost carnosinase activity (strains JK1274, 1302, 1305, 1306, 1311, 1313, and 1356), and these seven had been scored *pepD* via the transductional tests. Eleven strains retained significant levels of carnosinase activity (JK1307, 1318, 1319, 1337, 1348, 1357, 1359, 1361, 1371, 1375, and 1384); these strains were *pepD*$^+$.

TABLE 2. *Growth responses to histidyl peptides*[a]

Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	pep$^+$,[b] *hisBH22*[c]	*pep*$^+$, *hisG46*	Δ*D*, SB5401	*D101*, SB3041	*D102*, SB3095	*A1 B1 N10*, SB3797	*A1 B1 N10 D1 D105/D*$^+$, SB3773	*A1 B1 N10 D105*, SB3843	*A1 B1 N10 D4*, SB4045	*A1 B1 N10 D1*, TN277	*A1 B1 N10 D1 D114*, TN432	*A1 B1 N10 D1 D113*, TN431	*A1 B1 N10 D102*, SB3844	*A1 B1 N10 D101*, SB3845	*A1 B1 N10 D3*, SB4044	*A1 B1 N10 D1 D105*, SB3760	*A1 B1 N10* Δ*D*, SB3796
Ala-His	++	++	++	++	++	++	++	++	++	++	+	0	0	0	0	0	0
Asp-His	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Car	++	++	0	0	0	++	++	0	wk	++	0	0	0	0	0	0	0
Gly-His	++	+	++	+	++	++	++	++	++	+	++	0	+	wk	+	0	0
Gly-His-Gly	++	++	++	++	++	0	0	0	0	0	0	0	0	0	0	0	0
Gly-His-Lys	++	++	++	++	++	NT	NT	NT	NT	0	0	0	NT	NT	NT	0	0
His-Ala	++	++	++	++	++	++	++	0	++	0	++	+	+	+	++	wk	0
His-β-Ala	++	++	++	++	++	++	++	0	0	0	0	+	0	wk	wk	0	0
His-Asp	++	++	++	++	++	++	++	++	++	++	+	0	0	0	0	0	0
His-Glu	++	++	++	++	++	++	++	++	++	wk	0	0	0	0	0	0	0
His-Gly	++	++	++	++	++	++	++	++	++	+	+	0	0	0	0	0	0
His-Gly-Gly	++	++	++	+	++	0	0	+	0	0	0	0	0	0	0	0	0
His-Gly-His	wk	++	+	+	+	0	+	0	wk	0	wk	wk	0	0	wk	0	0
His-His	++	++	++	++	++	++	++	++	++	+	+	0	0	0	0	0	0
His-Leu	++	++	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0
His-Lys	++	++	++	++	++	++	++	++	++	++	+	0	0	0	0	0	0
His-Met	++	++	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0
His-Phe	++	++	++	++	++	++	++	++	++	+	0	0	0	0	0	0	0
His-Ser	++	++	++	++	++	++	++	++	++	+	0	0	0	0	0	0	0
His-Trp	++	++	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0
His-Tyr	++	++	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0
His-Val	++	++	++	++	++	++	++	++	0	+	0	0	0	0	0	0	0
Ile-His	++	++	++	++	++	+	++	++	0	0	0	0	0	0	0	0	0
Leu-His	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0	0
Met-His	++	++	+	++	++	++	++	++	++	++	++	0	0	0	0	0	0
Ser-His	++	++	++	+	++	wk	++	++	++	++	++	0	0	0	0	0	0
Tyr-His	++	++	wk	wk	++	wk	+	++	+	wk	+	0	0	0	0	0	0
Val-His	++	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0
His	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

[a] ++, Strong growth response; +, moderate growth response; wk, weak growth; 0, no growth detected; NT, not tested.
[b] *pep* genotype.
[c] Strain.

TABLE 5. *Growth responses to leucyl peptides*[a]

Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	pep^{+},[b] *leu-39*[c]	*A1 B1 N10*, TN274	*A1 B1 N10 D1 D105/D*$^{+}$, SB3773	*A1 B1 N10 D105*, SB3843	*A1 N10 D1*, TN213	*A1 N10 D3*, TN360	*A1 N10 D4*, TN362	*A1 B1 N10 D4*, SB4045	*A1 B1 N10 D1*, TN277	*A1 B1 N10 D1 D114*, TN432	*A1 B1 N10 D1 D113*, TN431	*A1 B1 N10 D102*, SB3844	*A1 B1 N10 D101*, SB3845	*A1 B1 N10 D3*, SB4044	*A1 B1 N10 ΔD*, SB3796
Asp-Leu	+	++	++	++	++	++	++	++	+	+	+	+	++	++	+
Gly-Ala-Leu	++	0	wk	wk	wk	+	++	0	0	0	0	0	0	0	0
Gly-Leu	++	++	++	++	++	+	++	++	++	0	0	0	0	0	0
His-Leu	++	++	++	++	++	++	++	++	0	0	0	0	0	0	0
Leu-Ala	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0
Leu-Gly	++	++	++	++	0	0	0	0	0	0	0	0	0	0	0
Leu-Gly-Gly	++	0	0	wk	++	+	wk	0	0	0	0	0	0	0	0
Leu-His	++	++	++	++	wk	+	+	0	0	0	0	0	0	0	0
Leu-Leu	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0
Leu-Leu-Leu	+	0	0	0	++	++	0	0	0	0	0	0	0	0	0
Leu-Met	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0
Leu-Phe	++	+	++	++	++	++	++	0	0	0	0	0	0	0	0
Leu-Pro	++	++	++	++	++	++	++	++	++	++	++	+	++	++	+
Leu-Val	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0
Met-Leu	++	++	++	++	++	++	++	0	0	0	0	0	0	0	0
Pro-Leu	++	++	++	++	++	0	0	0	+	0	0	0	0	0	0
Trp-Leu	+	++	+	++	wk	wk	wk	++	0	0	0	0	0	0	0
Leu	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

[a-c] See Table 1.

TABLE 6. *Carnosinase activity in selected strains*

	Strain and pertinent genotype	Growth medium	Sp act	Percent of wild-type activity
T buffer[a]				
1.	TN274 (*pepD*$^+$)	Nutrient broth	0.036	100
2.	*hisBH22* (*pepD*$^+$)	Nutrient broth	0.046	100
3.	*hisBH22* (*pepD*$^+$)	E + histidine	0.044	100
4.	*hisBH22* (*pepD*$^+$)	E + Car	0.042	100
5.	TN213 (*pepD1*)	Nutrient broth	0.037	88
6.	TN362 (*pepD4*)	Nutrient broth	0.0057	14
7.	SB3041 (*pepD101*)	Nutrient broth	<0.002	<4
8.	SB3095 (*pepD102*)	Nutrient broth	<0.002	<4
9.	TN360 (*pepD3*)	Nutrient broth	<0.002	<4
I buffer[a]				
10.	TN274 (*pepD*$^+$)	Nutrient broth	0.16	
11.	*hisBH22* (*pepD*$^+$)	Nutrient broth	0.12	
I buffer[b]				
12.	*hisBH22* (*pepD*$^+$)	Nutrient broth	2.58	100
13.	TN274 (*pepD*$^+$)	Nutrient broth	2.15	
14.	TN213 (*pepD1*)	Nutrient broth	3.59	156
15.	TN277 (*pepD1*)	Nutrient broth	3.78	
16.	TN362 (*pepD4*)	Nutrient broth	0.68	29
17.	TN430 (*pepD1 pepD112*)	Nutrient broth	0.11	5
18.	TN421 (*pepD1 pepD109*)	Nutrient broth	0.06	3
19.	TN422 (*pepD1 pepD110*)	Nutrient broth	0.06	3
20.	TN429 (*pepD1 pepD111*)	Nutrient broth	0.03	1
21.	TN432 (*pepD1 pepD114*)	Nutrient broth	0.02	1
22.	TN493 (*pepD1 pepD115*)	Nutrient broth	0.02	1
23.	TN417, -419, -420, -431, -494, -495, -496, -497, -498, -516 (*pepD1 pepD*$^-$)	Nutrient broth	<0.02	<1

[a] Plus 0.442 mM Car 1.0 mM Mn^{2+}.

[b] Plus 0.884 Car and 1.0 mM Co^{2+}. Assays with all buffers were on toluenized cell suspensions as described in the text.

(iv) As the data depicted in the top cross in Fig. 2 demonstrate, the genetic lesions engendering the Car$^-$ phenotype to strains SB3041 (*pepD101*) and SB3095 (*pepD102*) were each about 82% cotransducible with *pro*$^+$ when the *supQ357-proAB-gpt* deletion mutant strain SB3761 served as the recipient. These data also indicate that the *pep* mutations in the Car$^-$ strains were closely linked to *pepD1*. As pointed out for SB3845 and SB3844 in (ii), the phenotype of the recombinants carrying the "Car$^-$" mutations, now in *pepA pepB pepN* genetic background, was similar to the phenotype of some of the other independently isolated *pepD* mutations in this genetic background.

A second pair of crosses was made by using strains SB3041 and SB3095 as donors (bottom cross, Fig. 2). Strain SB3804 (*pepD1 pepD105 pro-520*) served as recipient, and selection was exerted for transductants on minimal medium lacking proline and containing His-His and Gly-Leu (i.e., transductants must be *pro*$^+$ and not carry *pepD101* or *D102*, or both *D1* and *D105*). Thus, recombinational events that were detected took place in a short chromosomal region involving closely linked *pepD* mutations. An interpretation of these data is that genetic lesions engendering the Car$^-$ phenotype (*pepD101*, *D102*, and *D105*) span *pepD1* (Car$^+$ phenotype).

(v) Polyacrylamide gels run on extracts prepared from strain *leu-485* (*pep*$^+$) and stained for carnosinase activity exhibited a single prominant band (data not shown). This band had the same R_f value as a band that stained positively when Gly-Leu or Leu-Gly was used as substrate. Extracts of TN215 (*pepD1*) exhibited a band that possessed activity toward Car and Gly-Leu, but not Leu-Gly. Extracts of TN431 (*pepD1 pepD113*) showed no detectable activity toward these three substrates.

(vi) Gly-Leu-, Leu-Gly-, and Car-hydrolyzing activities in extracts prepared from TN274 (*pepA pepB pepN*) coeluted from both diethylaminoethyl-cellulose and Bio-Gel P-300 columns (see Materials and Methods). Within the limits of our assays, the ratios of the three activities

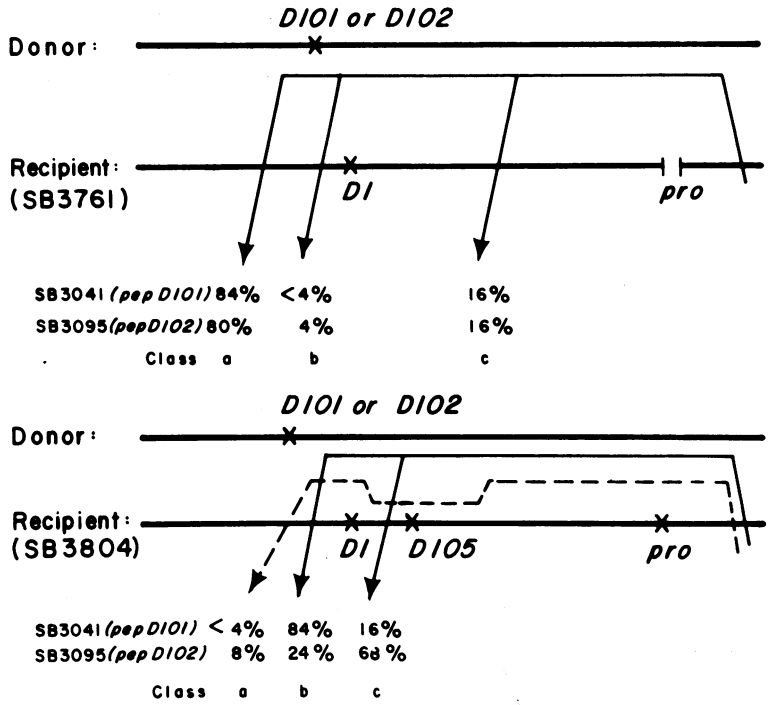

FIG. 2. *P22-mediated transduction tests involving Car⁻ mutants. In each cross, 25 pro⁺ transductants were tested for ability to utilize Leu-Gly and Gly-Leu as leucine sources and Car and His-His as histidine sources. (Upper cross) Linkage between pepD1 and mutations conferring the Car⁻ phenotype (pepD101 and pepD102). The three classes of phenotypes of the transductants were: (a) Leu-Gly⁻ Gly-Leu⁻ Car⁻ His-His⁻; (b) Leu Gly⁺ Gly Leu⁺ Car⁺ His-His⁺; and (c) Leu-Gly⁻ Gly-Leu⁺ Car⁺ His-His⁺. Tight linkage is exhibited between pepD1 and each of the two markers conferring the Car⁻ phenotype. (Lower cross) Selection of crossovers within the pepD gene. Selection was for Pro⁺ and both Gly-Leu⁺ and His-His⁺ (i.e., pepD101, pepD102, or pepD1 pepD105 could not grow). The three classes of transductants were: (a) Leu-Gly⁺ Car⁻; (b) Leu-Gly⁺ Car⁺; and (c) Leu-Gly⁻ Car⁺.*

across the peaks remained constant (data not shown).

(vii) In partially purified preparations of peptidase D, Gly-Leu- and Car-hydrolyzing activities were inactivated rapidly and at similar rates upon incubation at 42°C (Fig. 3). Activity toward Leu-Gly, Gly-Leu, and Car decreased on storage at 4°C, and the half-life of all three activities was about 1 day. The addition of either 1 mg of bovine serum albumin per ml or 50% glycerol (vol/vol) increased the half-life of each of the three enzymatic activities to about 1 week (data not shown). Storage in glycerol was preferable because the addition of bovine serum albumin at a concentration sufficient to preserve enzymatic activity caused high blanks and precluded the use of the spectrophotometric peptidase assay.

Some biochemical parameters of peptidase D. In toluenized extracts, the carnosinase activity of peptidase D measured in I buffer had a broad optimum between pH 6.8 and 7.5 (at 25°C). No activity was observed toward Car in the absence of exogenously added divalent cation. The optimum metal ion concentrations for activation were 1.0 and 0.1 mM for Mn^{2+} and Co^{2+}, respectively. Measured at optimum concentrations, activity was about seven times higher with Co^{2+} than with Mn^{2+}. The addition of both cations resulted in slightly less activity than with Co^{2+} alone. The presence of 1.0 mM Zn^{2+}, either alone or in combination with Mn^{2+} and/or Co^{2+}, completely abolished activity toward Car. Similarly, no activity toward Gly-Leu was observed in purified peptidase D preparations in the absence of added cation. The optimum Co^{2+} concentration for activity toward this substrate was 0.5 mM. Exogenously supplied divalent cation was not required for Leu-Gly hydrolysis, however; the reaction rate in the absence of added metal ion was about 50% of that exhibited by the enzyme in the presence of an optimal (0.5 mM) concentration of Mn^{2+}.

Linear Lineweaver-Burk plots were observed for Car, Gly-Leu, and Leu-Gly activities for the range of substrate concentrations greater than

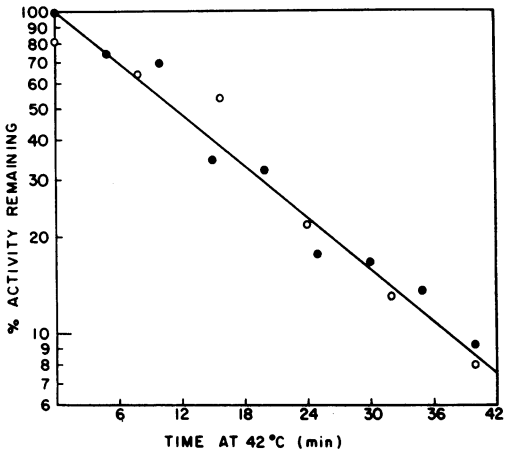

FIG. 3. *Thermal inactivation of Gly-Leu- and Car-hydrolyzing activities in peptidase D preparations. Gly-Leu (●): 5 ml of peptidase D preparation was placed at 42°C at time zero. At 5-min intervals, 0.5-ml samples were withdrawn and added to 1 ml of cold, standard Gly-Leu reaction mixture and kept on ice until all samples had been taken. Reaction mixtures were then allowed to warm to room temperature for 10 min, and the optical density at 228 nm was monitored. Car (○): 7 ml of peptidase D preparation was placed at 42°C at time zero. At 8-min intervals, 1 ml of enzyme was added to 9 ml of standard Car reaction mix (0.884 mM Car, 0.1 mM Co^{2+}, I buffer) prewarmed to 37°C. Samples were taken and assayed as described in Materials and Methods. A least-squares fit to the data points was performed, and the y intercept of this line was normalized to 100%.*

0.1 mM (Fig. 4). K_m values for Co^{2+}-activated cleavage of these three substrates were 0.25, 0.7, and 1.5 mM, respectively. The K_m for Mn^{2+}-activated cleavage of Leu-Gly was identical to that obtained in the presence of Co^{2+}. With Car as a substrate, velocities measured at still lower substrate concentrations (down to 0.02 mM) appeared to give the double-reciprocal plot an upward slope (Fig. 5). This phenomenon was observed in assays of toluenized cells, of sonically treated extracts, and of partially purified peptidase D preparations. It was not possible to test for this phenomenon in Mn^{2+}-activated hydrolysis due to lower reaction rates with Mn^{2+} as noted previously. Leu-Gly and Gly-Leu kinetics could not be extended to these very low substrate concentrations due to limitations in the sensitivity of the assay used to measure their hydrolysis. It is not clear whether the sigmoidal kinetics of Car cleavage reflects some property of the enzyme or is an artifact of the assay (e.g., substrate-dependent Co^{2+} quenching of fluorescence).

Peptidase D activity eluted from Bio-Gel P-300 columns at a V_e/V_0 ratio of approximately 1.35. This is roughly the position at which aldolase (158,000 daltons) eluted during a standardization run.

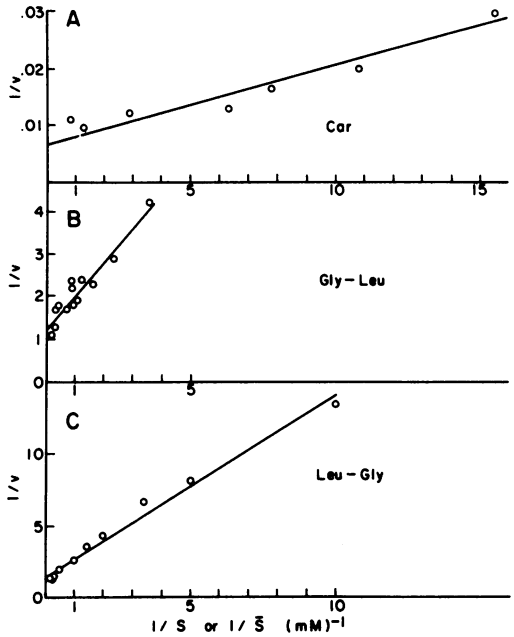

FIG. 4. *Kinetics of peptidase D activity. Carnosine (A): A reaction mixture containing substrate and $CoCl_2$ in 10 mM I buffer was prewarmed to 37°C, and a sample of a toluenized cell suspension of strain TN274 (pepD⁺) was added at time zero (final concentrations: 0.1 mM $CoCl_2$; 0.48 mg of protein per ml). Samples were taken and assayed as described in Materials and Methods. Since about 50% hydrolysis had occurred during the course of the reaction, $1/[\bar{S}]$ ($=1/2[1/[S_0] + 1/[S_t]]$) was used for the ordinate (10). No product inhibition was observed, and the reaction was essentially irreversible. Gly-Leu (B): To 1.25 ml of reaction mixture containing substrate, $CoCl_2$ in 50 mM Tris, pH 7.5, prewarmed to room temperature was added 0.25 ml of a purified peptidase D preparation (final concentrations: 0.1 mM $CoCl_2$; 0.022 mg of protein per ml). Optical density at 228 nm was monitored as described in Materials and Methods. Cleavage of Gly-Leu proceeded after a variable lag period which appeared to be dependent upon enzyme concentration and which was not reduced by prior incubation with divalent cation (data not shown). The maximum velocity recorded during each reaction was used to calculate 1/v, and the [S] at the time of maximum velocity was used to calculate 1/[S]. Leu-Gly (C): Peptidase D preparation (0.3 ml) was added to 1.2 ml of reaction mixture prewarmed to room temperature (final concentrations: 0.1 mM $CoCl_2$; 0.025 mg of protein per ml). Optical density at 225 nm was monitored as described in Materials and Methods. Initial velocity was used in the calculation of 1/v.*

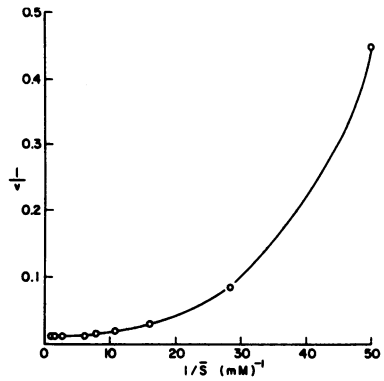

FIG. 5. *Kinetics of Car cleavage. Data from Fig. 3A and two additional assays performed at low substrate concentrations are shown.*

Incubation of "old" enzyme preparations (1 week at 4°C without bovine serum albumin or glycerol) with 2-mercaptoethanol did not restore activity toward Leu-Gly.

Regulation of Carnosinase activity. Carnosinase appears to be a constitutive enzyme. The specific activity of carnosinase was not significantly different in a *pepD*$^+$ auxotroph grown in nutrient broth, in minimal medium containing glucose and L-histidine, or in minimal medium containing glucose and Car (lines 2 to 4 in Table 6). Furthermore, neither a *rel*$^+$ strain (SB3436) nor a *rel* strain (SB3437) showed an increase in carnosinase activity after transfer from a rich medium (see Materials and Methods) to minimal + glucose medium (data not shown). The *rel*$^+$ and *rel* cultures were assayed for one of the histidine biosynthetic enzymes and did exhibit a *rel*$^+$-dependent stimulation in enzyme specific activity (26). Guanosine 5'-diphosphate 3'-diphosphate at 0.2 mM had no observable effect on activity of carnosinase that had been partially purified by chromatography on Bio-Gel and DE-52 (data not shown).

Car in beef extract. The amount of growth of histidine-requiring mutants on limiting amounts of Car was approximately proportional to Car concentration (data not shown). Histidine-requiring strain *hisBH22* grew to saturation in minimal medium containing glucose and a 1:16 dilution of nutrient broth (Fig. 6). On the other hand, a carnosinase-negative mutant, strain SB3041 (*hisBH22 pepD101*), ceased to grow when constituents of broth became limiting. That the growth limitation was due to the lack of utilizable derivatives of histidine was demonstrated by resumption of growth upon addition of L-histidine (arrow in Fig. 6). Carnosinase-negative mutants (*pepD*) wild type for other peptidase genes could not grow on Car but could grow on each of 27 other histidine-containing peptides tested (Table 2, columns 3–5). Therefore, we assumed that the differences in growth response between otherwise isogenic histidine-requiring *pepD*$^+$ and *pepD* strains was an estimate of Car concentration.

Data in Fig. 7 show the growth responses of the *pep*$^+$ histidine-requiring strain *hisBH22* and its *pepD* derivative (strain SB3041) in nutrient broth, in nutrient broth diluted 1:8, and in nutrient broth diluted 1:16 into minimal medium containing glucose as carbon source. From data obtained in titration experiments such as those shown in Fig. 7, we estimated that the diluted beef extract component as found in liquid nutrient broth was approximately 0.85 mM with respect to Car and contained little other source of histidine suitable for growth of *Salmonella*. In contrast, there appeared to be little Car in the peptone component of nutrient broth; instead, free histidine and utilizable histidine-containing peptides were present at a concentration in prepared nutrient broth of about 0.15 mM (L-histidine content).

DISCUSSION

Peptidase D as the sole peptidase active on Car. In a *pep*$^+$ genetic background, certain single-site mutations lead to loss of carnosinase activity, and hence inability to utilize Car as a histidine source (strains SB3041 and SB3095: Table 2, columns 4 and 5; Table 6, lines 7 and 8). Thus, it appears that only one *Salmonella* pep-

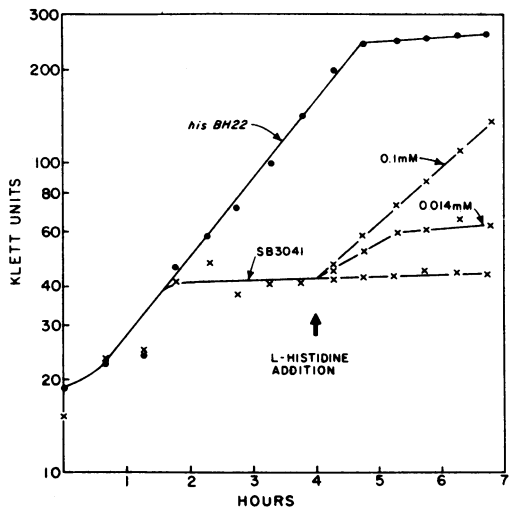

FIG. 6. *Growth of hisBH22 and strain SB3041 (hisBH22 pepD102) on nutrient broth diluted 1:16 into minimal medium plus glucose. The arrow indicates addition of L-histidine at the concentrations noted in the figure.*

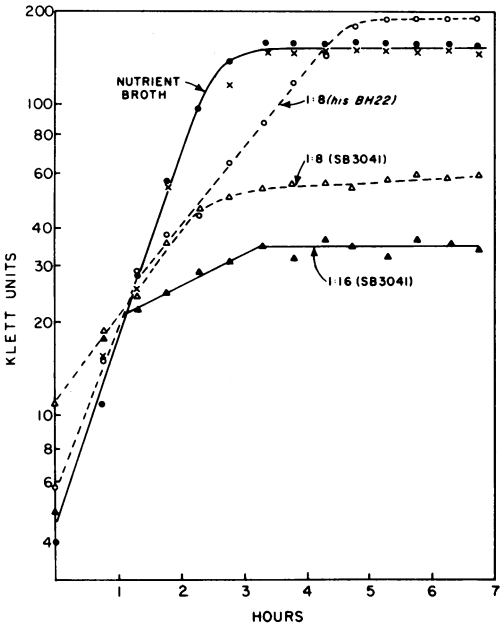

FIG. 7. *Growth of hisBH22 and strain SB3041 (hisBH22 pepD102) on nutrient broth and on broth diluted 1:8 and 1:16 into minimal medium containing glucose.*

tidase is capable of cleaving Car. Genetic lesions conferring the Car$^-$ phenotype, when transduced into a *pepA pepB pepN* genetic background, lead to loss of ability to utilize a large array of dipeptides, including the peptidase D substrate Leu-Gly (strains SB3844 and SB3845: Table 2, columns 13 and 14; Table 5, columns 12 and 13). Similarly, selection for *pepD* mutants through inability to utilize Leu-Gly as a source of leucine can lead to strains with low or absent carnosinase activity (strains TN362 and TN360: Table 6, lines 6, 9, and 16). Certain mutations conferring the Car$^-$ phenotype have been shown to flank *pepD1* (Fig. 1). Gel electrophoresis of extracts of a *pep*$^+$ strain showed a single band of activity toward Car, and this band was in the identical position as the peptidase D band when Leu-Gly was used as substrate. Based on these and additional data (see Results), we have concluded that carnosinase activity resides in peptidase D, the product of gene *pepD*.

Effects of *pepD* mutations on substrate specificity of peptidase D. Many of the *pepD* mutations do not lead to the production of a completely inactive peptidase D. Instead, strains with lesions in *pepD* frequently contain variants of peptidase D with strikingly altered substrate specificities. Strains containing the three *pepD* alleles isolated as Leu-Gly nonutilizers (*pepD1,-D2,-D3*) differ in their patterns of utilization of both leucine and histidine peptides (Tables 2 and 5). Even the strains containing two mutations in *pepD* can still grow on some peptidase D substrates (Table 2). Based on the phenotypic diversity already observed, it seems likely that other peptidase D variants could be obtained simply by selection for failure to use dipeptides other than Leu-Gly, Gly-Leu, Gly-His, and Car.

Properties of peptidase D. The Gly-Leu- and Car-hydrolyzing activities of peptidase D had an absolute requirement for divalent cations. Maximum carnosinase activity was roughly seven times higher in the presence of Co^{2+} than with Mn^{2+} at equal substrate concentrations. The Leu-Gly-hydrolyzing activity of peptidase D did not require exogenously supplied cation; however, the presence of 0.5 mM Mn^{2+} increased the rate of the reaction about twofold. K_m values of the enzyme toward Car, Gly-Leu, and Leu-Gly were found to be 0.25, 0.7, and 1.5 mM, respectively. Aberrant kinetics for Car hydrolysis were observed at very low substrate concentrations. Peptidase D has a molecular weight of approximately 160,000, as determined via molecular exclusion chromatography, and appears to be a constitutive enzyme.

Possible homologous dipeptidases. Two broad-specificity *E. coli* dipeptidases have been described: dipeptidase DP (21), purified from strain K-12, and a Zn-metalloenzyme from strain B (6, 17). Both enzymes exhibit divalent cation requirements for activity toward at least some dipeptides. The Zn-metalloenzyme has been more extensively studied in terms of substrate specificity. Its substrate specificity profile can change drastically, depending on the divalent cation present (6). At fairly low substrate concentrations, Co^{2+}, in general, activates better than Mn^{2+}. The degree of activation depends on the substrate in question; indeed, some dipeptides are cleaved at equal rates in the presence or the absence of divalent cations. Although the purified enzyme contains tightly bound Zn^{2+}, addition of Zn^{2+} to the reaction mixture was found to be inhibitory (6). Thus, the pattern of divalent cation requirements is similar to that found for peptidase D on the three substrates we have examined. Both the Zn-metalloenzyme (17) and dipeptidase DP (22) have molecular weights in the range of 100,000 to 112,000. This contrasts with the molecular-weight estimate of about 160,000 that we observed for peptidase D. Dipeptidase DP activity in aged preparations can be reactivated by the addition of β-mercaptoethanol (21). In contrast, we could show no such reactivation with aged peptidase D preparations, and the metalloenzyme (17) also was shown not to be stabilized by β-mercaptoethanol.

Enzyme activity and growth response.

Strains that were *pepD*$^+$ or had altered peptidase D but retained high carnosinase activity (e.g., strain TN277 in line 15 of Table 6) could utilize Car as a histidine source. In contrast, strain SB4045 (Table 2, column 9) could grow slowly on Car, and its parent strain, TN362, retained some 14 to 29% of carnosinase activity in toluenized cells (lines 6 and 16 in Table 6). All mutants with still lower activities failed to grow on Car although some had detectable carnosinase activity (Table 6).

The *pepD* mutant strain TN277 was able to grow on His-Leu as a source of histidine (Table 2, column 10), but not as a source of leucine (Table 5, column 9). His-Leu did not inhibit growth of TN277 on leucine (data not shown). This anomalous growth response may reflect differential requirements by the cell for these two amino acids.

Many peptides of the configuration Pro-*X* can be hydrolyzed by peptidase D (Table 3). Both peptidase D and peptidase N are required to cleave Pro-Trp rapidly enough to support the growth of a proline auxotroph. Other Pro-*X* peptides can be hydrolyzed by peptidase N or A as well as by peptidase D (Table 3).

Other *Salmonella* peptidases. Multiple mutants defective in the *pepA*, *pepB*, *pepD*, and *pepN* genes could still grow on dipeptides of the configuration *X*-Pro, where *X* can be any of the common amino acids including proline (Tables 3-5). McHugh and Miller (12) showed that *S. typhimurium* contains a separate peptidase (peptidase Q) highly active on prolyl peptides of this configuration. Mutants with lesions in the *pepD* gene utilized Gly-Pro-Ala as a proline source weakly (Table 3). A peptidase active on prolyl peptides, peptidase P, cleaves Gly-Pro-Ala to Gly and Pro-Ala (12). Peptidases A and N cleave Pro-Ala slowly, whereas peptidase D cleaves Pro-Ala rapidly, accounting for the growth pattern observed (Table 3).

Growth on Asp-Pro (Table 3) could reflect activity of peptidase Q (12); however, existence of an additional peptidase was indicated with activity on other peptides of the configuration Asp-*X* (Tables 2 and 5). A peptidase has been purified from *E. coli* with absolute specificity for β-Asp-*X* peptides (14), but the Asp-*X* peptides used here contained α-aspartate. Finally, growth of strain SB3803 on Gly-Met and Met-Ala (Table 4) suggests the presence of still another peptidase.

Car in broth. Mutants unable to grow on Car are very prevalent in aged nutrient agar stocks of histidineless mutants (unpublished data). In fact, one of our *pepD* mutants (strain SB3095) was detected after single-colony isolation from an aged stock. Most of the mutants detected in old stocks are "leaky" or unstably Car$^-$. We show here that nutrient broth, due to its content of beef extract, is about 0.85 mM with respect to Car and only about 0.15 mM with respect to histidine and all other utilizable peptides (23). *Salmonella* histidine-requiring mutants derepress histidine biosynthetic enzyme levels when grown on Car. We believe that this derepression lessens viability of *pepD*$^+$ bacteria in cultures stored for long periods at room temperature, allowing preferential survival of double mutants lacking the constitutive enzyme, carnosinase.

ACKNOWLEDGMENTS

Jost Kemper and John R. Roth kindly provided phage and bacterial strains. Ludwig Brand generously allowed constant access to a spectrofluorometer.

This work was supported in part by Public Health Service grants AI01650 (to P.E.H.) and AI10333 (to C.G.M.) from the National Institute of Allergy and Infectious Diseases and Public Health Service research career development award GM70321 from the National Institute of General Medical Sciences (to C.G.M.).

LITERATURE CITED

1. **Benson, J. R., and P. E. Hare.** 1975. *o*-Phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. Proc. Natl. Acad. Sci. U.S.A. **72**:619-622.
2. **Ely, B., R. M. Weppelman, H. C. Massey, Jr., and P. E. Hartman.** 1974. Some improved methods in P22 transduction. Genetics **76**:625-631.
3. **Gots, J. S., C. E. Benson, and S. R. Shumas.** 1972. Genetic separation of hypoxanthine and guanine-xanthine phosphoribosyltransferase activities by deletion mutations in *Salmonella typhimurium*. J. Bacteriol. **112**:910-916.
4. **Hais, I. M., and K. Macek. (ed.).** 1963. Paper chromatography: a comprehensive treatise, 3rd ed, p. 498-499. Academic Press Inc., New York.
5. **Hartman, P. E.** 1956. Linked loci in the control of consecutive steps in the primary pathway of histidine synthesis in *Salmonella typhimurium*, p. 35-62 *In* Genetic studies with bacteria, Carnegie Institute of Washington Publ. no. 612. Carnegie Institute of Washington, Washington, D.C.
6. **Hayman, S., J. S. Gatmaitan, and E. K. Patterson.** 1974. The relationship of extrinsic and intrinsic metal ions to the specificity of a dipeptidase from *Escherichia coli* B. Biochemistry **13**:4486-4494.
7. **Holzer, H., H. Betz, and E. Ebner.** 1975. Intracellular proteinases in microorganisms. Curr. Top. Cell. Regul. **9**:103-156.
8. **Hoppe, I., and J. Roth.** 1974. Specialized transducing phages derived from Salmonella phage P22. Genetics **76**:633-654.
9. **Kemper, J.** 1974. Evolution of a new gene substituting for the *leuD* gene of *Salmonella typhimurium*: characterization of *supQ* mutations. J. Bacteriol. **119**:937-951.
10. **Lee, H. J., and I. B. Wilson.** 1971. Enzyme parameters: measurement of V and K_M. Biochim. Biophys. Acta **242**:519-522.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. **McHugh, G. L., and C. G. Miller.** 1974. Isolation and characterization of proline peptidase mutants of *Salmonella typhimurium*. J. Bacteriol. **120**:364-371.
13. **Miller, C. G.** 1975. Genetic mapping of *Salmonella typhimurium* peptidase mutations. J. Bacteriol. **122**:171-176.

14. **Miller, C. G.** 1975. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. Annu. Rev. Microbiol. **29**:485-504.
15. **Miller, C. G., and K. MacKinnon.** 1974. Peptidase mutants of *Salmonella typhimurium*. J. Bacteriol. **120**:355-363.
16. **Murphey, W. H., L. Patchen, and D. G. Lindmark.** 1972. Carnosinase: a fluorometric assay and demonstration of two electrophoretic forms in human tissue extracts. Clin. Chim. Acta **42**:309-314.
17. **Patterson, E. K., J. S. Gatmaitan, and S. Hayman.** 1973. Substrate specificity and pH dependence of dipeptidases purified from *Escherichia coli* B and from mouse ascites tumor cells. Biochemistry **12**:3701-3709.
18. **Payne, J. W.** 1972. Variations in the peptidase activities of *Escherichia coli* in response to environmental changes. J. Gen. Microbiol. **71**:281-291.
19. **Schmieger, H.** 1972. Phage *P22*-mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119**:75-88.
20. **Schmitt, A., and G. Siebert.** 1961. Optischer Test zur Bestimmung der Peptidaseaktivitat. Biochem. Z. **334**:96-107.
21. **Simmonds, S., K. S. Szeto, and C. G. Fletterick.** 1976. Soluble tri- and dipeptidases in *Escherichia coli* K-12. Biochemistry **15**:261-271.
22. **Sussman, A. J., and C. Gilvarg.** 1970. Peptidases in *Escherichia coli* K12 capable of cleaving lysine homopeptides. J. Biol. Chem. **245**:6518-6524.
23. **Sykes, G. (ed.).** 1956. Constituents of bacteriological culture media. Society for General Microbiology Special Report. Cambridge University Press, Cambridge.
24. **Taylor, S., and A. L. Tappel.** 1973. Lysosomal peptidase measurement by sensitive fluorometric amino acid analysis. Anal. Biochem. **56**:140-148.
25. **Vogel, H. J., and D. M. Bonner.** 1965. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97-106.
26. **Winkler, M. E., D. J. Roth, and P. E. Hartman.** 1978. Promoter- and attenuator-related metabolic regulation of the *Salmonella typhimurium* histidine operon. J. Bacteriol. **138**:830-843.