Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5998–6001, December 1978
Biochemistry

Escherichia coli mutants defective in dipeptidyl carboxypeptidase

[peptide metabolism/periplasmic enzymes/peptidyl dipeptidase (angiotensin-converting enzyme)/1-(D-3-mercapto-2-methylpropanoyl)-L-proline (SQ 14225, Captopril)]

CHARLES E. DEUTCH AND RICHARD L. SOFFER

Departments of Biochemistry and Medicine, and the Hypertension Research Center, Cornell University Medical College, New York, New York 10021

Communicated by Alton Meister, October 4, 1978

ABSTRACT **Two independent mutants of *Escherichia coli* deficient in dipeptidyl carboxypeptidase activity (Dcp$^-$) were isolated after mutagenesis with ethyl methanesulfonate. Mating experiments and introduction of specific episomes indicated that the responsible gene was located at approximately 27–31 min on the *E. coli* chromosome. The Dcp$^-$ mutants differed from the parental strain in their inability to grow with *N*-acetylalanylalanylalanine as the sole nitrogen source. Revertants selected for growth on this substrate of the enzyme were found to have reacquired the activity. Enzyme activity was highly sensitive to inhibition by 1-(D-3-mercapto-2-methylpropanoyl)-L-proline (SQ 14225), a potent inhibitor of mammalian dipeptidyl carboxypeptidase (angiotensin-converting enzyme, peptidyl dipeptidase, EC 3.4.15.1). This compound also reduced the rate of growth of the wild type with *N*-acetylalanylalanylalanine but not with ammonium sulfate. A fraction of the enzyme was released into the medium by osmotic shock, indicating that its presence in the periplasmic space may account for growth with *N*-acetylated peptides that cannot be taken up by *E. coli*. In addition to providing information about the specific role of this exopeptidase in *E. coli*, the Dcp$^-$ mutants may prove useful for delineating the regulation and cellular function of dipeptidyl carboxypeptidases in higher organisms.**

In 1972 Yaron *et al.* (1) purified a soluble enzyme from *Escherichia coli* B that they called dipeptidyl carboxypeptidase. This exopeptidase catalyzed cleavage of dipeptidyl residues from unblocked COOH-termini of various peptides but did not act on a peptide bond containing the imino group of a prolyl residue. In these and other catalytic properties (2) the enzyme closely resembles mammalian angiotensin-converting enzyme (peptidyl dipeptidase, peptidyldipeptide hydrolase, EC 3.4.15.1). Angiotensin-converting enzyme (reviewed in ref. 3) plays a major role in blood pressure regulation by catalyzing the generation of angiotensin II and the inactivation of bradykinin. Recent experiments with chemical inhibitors of this enzyme (4–6) have suggested that reduction of its activity may ameliorate most human hypertensive disease (7, 8). Because one of these agents, 1-(D-3-mercapto-2-methylpropanoyl)-L-proline (SQ 14225), is already in advanced stages of clinical evaluation (8), it has become increasingly important to determine what other functions converting enzyme may serve in cellular metabolism. Because *E. coli* contains a similar activity, is easily genetically manipulated, and can be grown in simple media, we have chosen to use this organism as a model for examining the physiological functions of dipeptidyl carboxypeptidases. In the present paper we describe the isolation and characterization of mutants deficient in this activity.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. *E. coli* K-12 strains KL16 (Hfr, *thi-1, relA1*), AB2495 (F$^-$, *thi-1, thr-1, leuB6, argE3, his-4, trp-35, proA2, thyA20, thyR13, rpsL31* . . .) and a set of F′ strains carrying different segments of the *E. coli* chromosome were provided by Barbara Bachman of the Coli Genetic Stock Center, Yale University. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), was obtained from Research Plus Laboratories; hippurylglycylglycine (Hip-Gly-Gly), glycylglycine, and L-alanyl-L-alanine were from Vega–Fox Biochemicals. Poly(L-prolyl-glycyl-L-proline) (M_r 2450) was supplied by Miles–Yeda; the *N*-dinitrophenyl derivative was prepared according to Yaron (9). SQ 14225 and SQ 20881 (<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were a gift from Maxim Wilson. All other peptides were purchased from Bachem Fine Chemicals, Torrance, CA. Ethyl methanesulfonate was from Eastman.

Assays. For screening assays, single colonies were grown overnight in 3 ml of LB broth (10). The cells were harvested by centrifugation, suspended in 200 μl of 50 mM Tris·HCl, pH 7.8, and vigorously mixed for 15 sec with 10 μl of toluene. Hip-His-Leu was then added to a concentration of 5 mM and the release of hippuric acid was measured spectrophotometrically after extraction with ethyl acetate according to Cushman and Cheung (11). To prepare cell extracts, late exponential phase cells grown in LB broth were broken by passage through a French pressure cell [12,000 pounds/inch^2 (83 MPa)] and 105,000 × *g* supernatant fractions were dialyzed against 50 mM Tris·HCl, pH 7.8. Generation of hippuric acid from Hip-His-Leu and Hip-Gly-Gly was determined as described above and cleavage of *N*-dinitrophenyl-poly(Pro-Gly-Pro) was measured according to Yaron (9). Units of activity on these three substrates are expressed as nmol hydrolyzed per min at 37°C. Various dipeptide reaction products were qualitatively identified by comparison with authentic standards after paper electrophoresis in pyridine/acetic acid/water 1:10:89 (vol/vol), pH 3.5, for 2 hr with a potential gradient of 50 V/cm. The dried paper strips were developed with 0.3% ninhydrin. Protein was determined by the method of Lowry *et al.* (12) with bovine serum albumin as the standard.

RESULTS

Isolation and Genetic Characterization of Mutants. Strain KL16 was incubated with 1.4% ethyl methanesulfonate for 45 min at 37°C (10) to give 1% survival. Survivors were screened for activity with Hip-His-Leu; two of the 1650 isolated clones (K79, K93) were found to be deficient. Extracts of these strains (Table 1) exhibited less than 2% of the wild-type activity with this substrate and negligible activity on Hip-Gly-Gly and *N*-

Abbreviations: Hip, hippuryl; Dcp$^-$, dipeptidyl carboxypeptidase-deficient.

Table 1. Specific activities of dipeptidyl carboxypeptidase in *E. coli* strains

Strain	Specific activity, nmol/min per mg soluble protein		
	Hip-His-Leu	Hip-Gly-Gly	*N*-Dinitrophenyl-poly(Pro-Gly-Pro)
KL16	143	1.96	1.36
K79	1.02	<0.06	<0.15
K93	1.09	<0.06	<0.15
K79R1	155	1.34	1.68
K93R1	144	1.23	0.97

K79R1 and K93R1 are spontaneous revertants of K79 and K93 isolated by their ability to grow on Ac-Ala-Ala-Ala as a sole source of nitrogen.

dinitrophenyl-poly(Pro-Gly-Pro). Paper electrophoreses confirmed that His-Leu, Gly-Gly, and Gly-Pro were absent from reaction mixtures incubated with mutant extracts and present in those containing wild-type preparations. Appropriate mixing experiments (not shown) indicated that loss of activity was not due to an inhibitor. The relatively low activity of the enzyme in wild-type extracts on Hip-Gly-Gly compared to Hip-His-Leu is consistent with the observation of Yaron *et al.* (1) that the Gly-Gly bond is poorly hydrolyzed. To determine the approximate location on the *E. coli* chromosome of the gene for dipeptidyl carboxypeptidase, each mutant was mated with a polyauxotrophic F^- strain (AB2495) for 2 hr. Thy^+, His^+, Trp^+, Pro^+, Leu^+, Thr^+, and Arg^+ recombinants were isolated on selective M63 minimal media (13) containing streptomycin (120 μg/ml) and thiamine (10 μg/ml). About 140 of each type were tested for activity in the screening assay. Loss of enzyme activity occurred with greatest frequency among Thy^+, His^+, and Trp^+ recombinants, suggesting that the mutations mapped in the second quadrant of the chromosome. From this experiment, Thr^+ recombinants (AE12 and W8, respectively from matings with K79 and K93) lacking activity were then used as recipients for complementation analysis with F′ factors carrying different segments of the second quadrant. Merodiploids were recovered by plating on minimal agar selective for His^+ or Trp^+. The only F′ factor restoring activity (Table 2) was F123, which contains the portion of the chromosome from *trp* (27 min) to *rac* (31 min). We propose that the structural gene for dipeptidyl carboxypeptidase in *E. coli* be designated *dcp*, and we refer to the two mutations described here as *dcp-1* and *dcp-2* because they are probably in this structural gene.

Physiological Characteristics of Dipeptidyl Carboxypeptidase. The dipeptidyl carboxypeptidase-deficient (Dcp^-) mutants grew normally in enriched medium or minimal medium containing glucose or glycerol as the carbon source. However, they differed from the wild type in their ability to grow in minimal medium with either of these carbon sources and 10 mM Ac-Ala-Ala-Ala as the sole nitrogen source (Fig. 1).

Table 2. Complementation of *dcp* mutation by F factors

Recipient	Episome	Genome segment, min	Selected phenotype	Complementation
AE12	F123	27–31	Trp^+	20/20
W8	F123	27–31	Trp^+	20/20
W8	F148	37–44	His^+	0/20
W8	F129	44–50	His^+	0/20

AE12 and W8 lack dipeptidyl carboxypeptidase activity and are Thr^+ recombinants of matings between AB2495 and the mutants K79 and K93. AE12 thus carries the mutation we have designated *dcp-1* and W8 carries that termed *dcp-2*. Complementation analyses were performed on isolates from each F-duction by the standard screening assay. The genome segments are taken from Low (14).

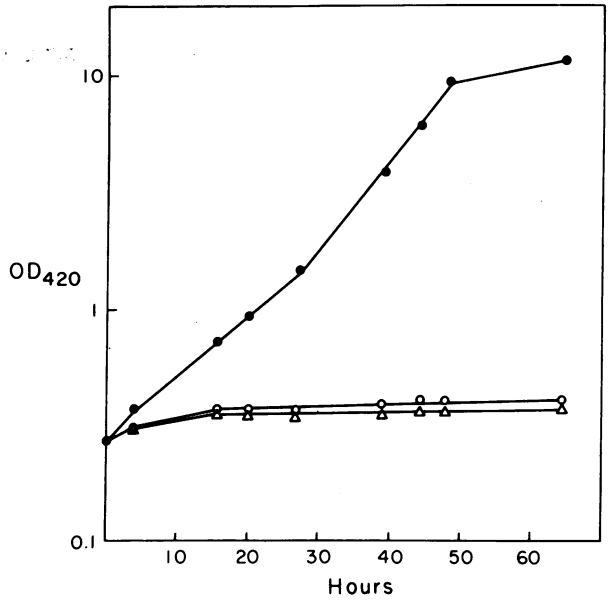

FIG. 1. Growth of *E. coli* strains in minimal medium containing 10 mM Ac-Ala-Ala-Ala as the nitrogen source and 0.5% glycerol. ●, KL16; ○, K79; △, K93.

The generation time for the parental strain in this medium with glycerol was approximately 8 hr, whereas the mutants failed to grow. The mutants were able to multiply normally with L-alanine, Ala-Ala, or Ala-Ala-Ala as nitrogen sources, indicating that their defect was unrelated to catabolism of alanine or to uptake and degradation of alanine-containing peptides. Extracts of the wild type catalyzed the generation of Ala-Ala from Ac-Ala-Ala-Ala as detected by paper electrophoresis, while those from the mutants did not. Spontaneous revertants were isolated at a frequency of about 10^{-9} by growth on minimal agar containing Ac-Ala-Ala-Ala. All nine of these had regained the ability to hydrolyze Hip-His-Leu as detected by the screening assay. Extracts were prepared from two of these and shown to have reacquired activity for all three standard substrates (Table 1). In contrast to the results with Ac-Ala-Ala-Ala, the wild type failed to multiply on Ac-Ala-Ala, consistent with the finding that tripeptides with protected NH_2-termini are the smallest substrates for dipeptidyl carboxypeptidase (9).

Growth of the wild type on Ac-Ala-Ala-Ala was somewhat unexpected because *N*-acetylated peptides are generally considered not to be taken up by *E. coli* as measured by their ability to satisfy auxotrophic requirements (15, 16). It therefore seemed appropriate to determine whether some of the dipeptidyl carboxypeptidase was located in the periplasmic space between the cell wall and the cell membrane and could be released by osmotic shock. Almost 10% of the exopeptidase activity was found in the shock fluid (Table 3) compared with <0.3% of glutamate dehydrogenase, an enzyme known to be localized inside the cell membrane (17). Clearly, only a fraction of the enzyme is periplasmic, because marker enzymes for this compartment, such as 5′ nucleotidase and acid phosphatase (17), were almost quantitatively released.

Because potent inhibitors of angiotensin-converting enzyme are now available (4–6), it was of interest to determine their action on this similar microbial exopeptidase. Table 4 shows the reduction in activity caused by these and other agents whose direct effect on the reaction has not been previously examined. Inhibition by direct addition of EDTA and by relatively low concentrations of thiols is similar to that found for angiotensin-converting enzyme (11), which contains a molar equivalent of zinc (20). The concentration of the venom nonapeptide, SQ

Table 3. Release of dipeptidyl carboxypeptidase by osmotic shock

Enzyme	Enzyme activity, units: Shock fluid	Shocked cells	Release, %
Dipeptidyl carboxypeptidase	220	2371	8.5
Glutamate dehydrogenase	<0.005	2.04	<0.3
5′-Nucleotidase	307	0.6	>99
Acid phosphatase	43.2	25.8	63

E. coli KL16 was grown to mid-logarithmic phase in 1 liter of minimal medium containing 0.5% glycerol as the carbon source. The cells were harvested, washed, and suspended in 10 ml of 30 mM Tris·HCl, pH 7.8, containing 20% sucrose (17). EDTA was added to a concentration of 0.1 mM. The cells were harvested and rapidly suspended in 10 ml of cold 0.5 mM $MgCl_2$. After 10 min, the cell suspension was centrifuged and the supernatant (shock fluid) was decanted and saved. The shocked cells were suspended in 10 ml of 30 mM Tris·HCl, pH 7.8, and an extract was prepared by French pressure cell. Both the shock fluid and the cell extract were dialyzed against 50 mM Tris·HCl, pH 7.8. Dipeptidyl carboxypeptidase was assayed with Hip-His-Leu. Glutamate dehydrogenase was determined by the method of Miller and Stadtman (18). 5′-Nucleotidase and acid phosphatase were measured as described by Neu and Heppel (19). Units for these enzymes are defined as specified in each reference.

20881, required to yield 50% inhibition was considerably higher than that reported for the mammalian enzyme (21); however, this may reflect its degradation by other peptidases in the crude extract. The most striking result was that obtained with SQ 14225, a compound whose structure was designed to interact specifically with the active site of angiotensin-converting enzyme (5, 6). *E. coli* dipeptidyl carboxypeptidase exhibited a comparable exquisite sensitivity to this reagent. It should be noted that the residual activity in the Dcp^- mutants was inhibited identically to the wild-type activity by the agents shown in Table 4, suggesting that *E. coli* contains only a single dipeptidyl carboxypeptidase.

SQ 14225 appeared to inhibit dipeptidyl carboxypeptidase activity *in vivo*, although at higher concentrations than expected. This was indicated by the fact that it reduced the growth rate of the wild type on Ac-Ala-Ala-Ala but did not significantly alter the generation time with ammonium sulfate as the nitrogen source (Table 5).

DISCUSSION

The role of specific exopeptidases in *E. coli* is poorly understood because mutants lacking such defined activities have not been available. Our isolation of a class of mutants specifically deficient in dipeptidyl carboxypeptidase has enabled us to establish that this enzyme plays an essential role in the hydrolysis of *N*-acetylated peptides that cannot pass through the cell membrane. The data suggest that a fraction of the dipeptidyl carboxypeptidase is present in the periplasmic space and accounts

Table 4. Inhibitors of *E. coli* dipeptidyl carboxypeptidase

Addition	I_{50}, μM
EDTA	1000
Dithiothreitol	30
SQ 20881	100
SQ 14225	0.04

Reaction mixtures (250 μl) contained 50 mM Tris·HCl at pH 7.8, 5 mM Hip-His-Leu, and 38 μg of soluble protein. Incubation was for 30 min at 37°C. In the absence of additions, 150 nmol of hippuric acid was enzymatically generated, as determined by the method of Cushman and Cheung (11). I_{50} is defined as the concentration of inhibitor required to reduce this activity by 50%.

Table 5. Effect of SQ 14225 on growth of *E. coli* KL16

Nitrogen source	SQ 14225, mM	Generation time, hr
Ac-Ala-Ala-Ala	0	7
	0.1	12
	1	24
	10	No growth
$(NH_4)_2SO_4$	0	1.8
	0.1	1.8
	1	1.8
	10	1.8*

E. coli KL16 was grown to mid-logarithmic phase in minimal medium containing 0.5% glycerol, washed once with minimal salts lacking $(NH_4)_2SO_4$, and suspended in the same solution. Glycerol (0.5%) was added as the carbon source, and either 10 mM Ac-Ala-Ala-Ala or 0.2% $(NH_4)_2SO_4$ was added as the nitrogen source. SQ 14225 was included where indicated, and growth of the cultures was monitored spectrophotometrically at 420 nm. Generation times were calculated directly from the growth curves.

* Cultures containing $(NH_4)_2SO_4$ and 10 mM SQ 14225 exhibited a lag of about 5 hr before resuming growth at the normal rate. No lag was observed at the lower concentrations.

for utilization of Ac-Ala-Ala-Ala as a nitrogen source. Conceivably, this enzyme may be responsible for the slight but unexpected effectiveness of *N*-acetylated arginine and lysine tripeptides in satisfying auxotrophic requirements (15). Localization of the enzyme in the periplasmic space may also explain the relatively high concentration of SQ 14225 required for inhibition *in vivo*, because this compound must pass through the outer membrane in order to be effective. Because only a fraction of the dipeptidyl carboxypeptidase was released by osmotic shock, it seems likely that the enzyme performs other unrelated functions involving intracellular peptide hydrolysis. We have also detected dipeptidyl carboxypeptidase activity in soluble extracts of *Caulobacter crescentus*, *Sarcina lutea*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* and have found that at least the latter two organisms can grow with Ac-Ala-Ala-Ala as a nitrogen source.

The catalytic properties of dipeptidyl carboxypeptidase (9) and angiotensin-converting enzyme (3) are impressively similar. In addition to the characteristics mentioned in the introduction, both appear to be metalloenzymes whose activity is stimulated by Co^{2+} and inhibited by low concentrations of thiols. Each is also inhibited by nonsubstrate peptides, including dipeptides, among which those containing a basic residue are particularly potent. Neither is active with unprotected tripeptides, but both will degrade NH_2-blocked tripeptides as well as free tetra- and higher peptides. Most strikingly, each is inhibited by extraordinarily low concentrations of SQ 14225. Despite these catalytic similarities, we have found no effect on the bacterial activity of inhibitory goat antibodies developed against pure rabbit pulmonary angiotensin-converting enzyme (22). This suggests a lack of structural homology with antigenic determinants that influence catalytic activity in the mammalian enzyme. In addition to the resemblance of their catalytic properties, it is interesting that an analogy can be drawn with respect to the subcellular localization of these enzymes. Angiotensin-converting enzyme is located on the luminal surface of vascular endothelial cells in anatomic juxtaposition to the circulating medium (i.e., the blood) and a fraction of the *E. coli* dipeptidyl carboxypeptidase is also located in close approximation to the medium (i.e., in the periplasmic space).

These considerations suggest that studies with *E. coli* may be useful for examining certain properties of dipeptidyl carboxypeptidases that cannot easily be investigated for a mammalian enzyme, such as the influence of genetic and environ-

mental factors on enzyme activity. Moreover, the ease with which revertants regaining enzyme activity can be selected from Dcp$^-$ mutants suggests that the *dcp* mutation may ultimately be useful for constructing a recipient in which to clone the gene for mammalian angiotensin-converting enzyme, should this become a desirable objective. In view of the increasing likelihood that a large number of human beings will soon be taking antihypertensive therapy that almost completely abolishes dipeptidyl carboxypeptidase activity, it seems imperative to learn as much as possible concerning the biological role played by this type of enzyme.

This work was supported by Grant HL 21395 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

1. Yaron, A., Mlynar, D. & Berger, A. (1972) *Biochem. Biophys. Res. Commun.* **47,** 897–902.
2. Yaron, A. (1974) *Isr. J. Chem.* **12,** 651–662.
3. Soffer, R. L. (1976) *Annu. Rev. Biochem.* **45,** 73–94.
4. Ondetti, M. A., Williams, N. J., Sabo, E. F., Pluscek, J., Weaver, E. R. & Kocy, O. (1971) *Biochemistry* **10,** 4033–4039.
5. Ondetti, M. A., Rubin, B. & Cushman, D. W. (1977) *Science,* **196,** 441–444.
6. Cushman, D. W., Cheung, H. S., Sabo, E. F. & Ondetti, M. A. (1977) *Biochemistry* **16,** 5484–5491.
7. Case, D. B., Wallace, J. M., Keim, H. J., Weber, M. A., Sealey, J. E. & Laragh, J. H. (1977) *N. Engl. J. Med.* **296,** 641–646.
8. Gavras, H., Brunner, H. R., Turini, G. A., Kershaw, G. R., Tifft, C. P., Cuttelod, S., Gavras, I., Vukovich, R. A. & McKinstry, D. N. (1978) *N. Engl. J. Med.* **298,** 991–995.
9. Yaron, A. (1976) *Methods Enzymol.* **45,** 599–610.
10. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
11. Cushman, D. W. & Cheung, H. S. (1971) *Biochem. Pharmacol.* **20,** 1637–1648.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
13. Herzenberg, L. (1959) *Biochim. Biophys. Acta* **31,** 525–538.
14. Low, K. B. (1972) *Bacteriol. Rev.* **36,** 587–607.
15. Gilvarg, C. & Katchalski, E. (1965) *J. Biol. Chem.* **240,** 3093–3098.
16. Payne, J. W. (1971) *Biochem. J.* **123,** 245–253.
17. Nossal, N. G. & Heppel, L. A. (1966) *J. Biol. Chem.* **241,** 3055–3062.
18. Miller, R. E. & Stadtman, E. R. (1972) *J. Biol. Chem.* **247,** 7407–7419.
19. Neu, H. C. & Heppel, L. A. (1965) *J. Biol. Chem.* **240,** 3685–3692.
20. Das, M. & Soffer, R. L. (1975) *J. Biol. Chem.* **250,** 6762–6768.
21. Cheung, H. S. & Cushman, D. W. (1973) *Biochim. Biophys. Acta* **293,** 451–463.
22. Das, M. & Soffer, R. L. (1976) *Biochemistry* **15,** 5088–5094.