Cloning and Expression of the *pepD* Gene of *Escherichia coli*

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Peptidase D of *Escherichia coli*, cleaving the unusual dipeptide carnosine, was found to be encoded by the ColE1 hybrid plasmid pLC44-11. From this plasmid the *pepD* gene was subcloned into small vectors. As shown by successive reduction of the flanking sequences of genomic DNA, the order of genes in the region at 6 min of the *E. coli* K12 map is *phoE*, *pepD*, in the clockwise orientation. Insertional inactivation of the *pepD* gene and expression of recombinant plasmids in maxicells allowed the identification of the *pepD* product as a 52 kDa protein. Comparison with the 100 kDa protein molecular mass determined by gel filtration suggests that active peptidase D is probably a dimer.

INTRODUCTION

The presence of various peptidases in *Escherichia coli* and *Salmonella typhimurium* (Sussman & Gilvarg, 1971) is required for the utilization of exogenously supplied peptides (Simmonds & Fruton, 1949; Gutnick *et al.*, 1969), as well as for the catabolism of intracellular proteins, particularly during starvation of the cells (Pine, 1972; Yen *et al.*, 1980). Peptidases are also involved in the removal of N-terminal methionine from nascent polypeptide chains (Livingston & Leder, 1969), in the processing of secretory proteins (Blobel & Dobberstein, 1975) and in the breakdown of abnormal proteins (Miller & Green, 1981).

As shown by the isolation and characterization of mutant strains, *E. coli* possesses at least five different peptidases, denoted A, B, D, N and Q, with partly overlapping substrate specificities (Miller & Schwartz, 1978). This pattern of peptide-degrading activities is similar to that of the closely related organism *S. typhimurium* (Miller & Mackinnon, 1974; Miller, 1975*a*). One of these enzymes, peptidase D, which is active only on dipeptides, was found to be the only peptidase of *S. typhimurium* that cleaved the unusual dipeptide carnosine (β -alanyl-L-histidine) (Kirsh *et al.*, 1978). The corresponding gene maps at the 6 min position on the circular map of the *S. typhimurium* genome (Miller, 1975*b*).

In order to facilitate a more detailed genetic, biochemical and physiological characterization of peptidase D, we cloned the *pepD* gene of *E. coli*. In this communication we report the detection of *pepD* in a plasmid library, its subcloning into small vectors and its expression in a maxicell system.

METHODS

Bacteria, plasmids and phage. *E. coli* CM17 (Miller & Schwartz, 1978) was obtained from C. G. Miller, Case Western Reserve University, Cleveland, USA, and strain AB1133 (Howard-Flanders *et al.*, 1966) was from B. Bachmann, *E. coli* Genetic Stock Center, New Haven, USA. Strain NK5526 [*hisG*::Tn10 IN(*rrnD*–*rrnE*)] was provided by N. Kleckner, Harvard, Cambridge, USA, and strain CSR603 (Sancar & Rupert, 1978) was from M. Jackson, Leicester University, UK. Strains PC2086 [*thr leu* Δ (*gpt*–*proA*) *his thi argE purH lacY galK xyl ara mtl tsx rpsL supE*] and PC1324 (Low, 1972) as well as the Clarke–Carbon plasmids pLC25-25, pLC32-46, pLC40-18 and pLC44-11 (Clarke & Carbon, 1976) were obtained from the Phabagen Collection, Utrecht, The Netherlands. Plasmid pBR322 (Bolivar *et al.*, 1977) was from Boehringer Mannheim, the vector pUC18 (Norrander *et al.*, 1983) was obtained from U. Rütter, European Molecular Biology Laboratories, Heidelberg, FRG, and plasmid pUC4-K (Vieira & Messing, 1982) was purchased from Pharmacia. The transducing phage P1CMclr100 (Rosner, 1972) was provided by R. Rosner, National Institutes of Health, Bethesda, USA.

Growth conditions. Cells were grown in LB medium, containing 10 g tryptone, 5 g yeast extract and 5 g NaCl per litre, or in Davis minimal medium (Davis & Mingioli, 1950), supplemented with the required amino acids at a concentration of $50 \mu\text{g ml}^{-1}$. Antibiotics used were ampicillin ($100 \mu\text{g ml}^{-1}$), kanamycin ($40 \mu\text{g ml}^{-1}$) and tetracycline ($12 \mu\text{g ml}^{-1}$ in the case of plasmid-encoded resistance or $30 \mu\text{g ml}^{-1}$ in the case of Tn10-encoded resistance).

Construction of strain UK61. The *hisG* : Tn10 marker of strain NK5526 was transferred to the *pepD* strain CM17 by use of the transducing phage P1CME100 according to the method of Rosner (1972). Transductants which had simultaneously acquired resistance to tetracycline and auxotrophy for histidine were named UK61.

Assay of peptidase D activity. Peptidase D was assayed in sonic extracts of exponentially growing cells. Free histidine released from carnosine (Bachem) was determined fluorimetrically as described by Kirch *et al.* (1978).

Recombinant DNA techniques. Small scale preparations of plasmid DNA and isolation of restriction fragments from polyacrylamide gels were done by the methods of Maniatis *et al.* (1982). Restriction endonucleases and T4 DNA ligase (Boehringer Mannheim) were used as recommended by the supplier. Gel electrophoresis of DNA, and preparation and transformation of competent cells, were done as described previously (Henrich *et al.*, 1982).

Protein labelling in maxicells. CSR603 cells transformed with the appropriate plasmids were UV-irradiated, labelled with [^{35}S]methionine ($1110 \text{ Ci mmol}^{-1}$, $41.1 \text{ TBq mmol}^{-1}$) (New England Nuclear) and processed for gel electrophoresis essentially as described by Sancar *et al.* (1979). Following irradiation, D-cycloserine ($100 \mu\text{g ml}^{-1}$ final concentration) was added to the suspension during a 20 h incubation in order to prevent overgrowth of surviving cells. Total lysates were subjected to 12% (w/v) SDS-PAGE at 150 V for 15 h using the discontinuous buffer system of Laemmli (1970). Labelled proteins were visualized by autoradiography of vacuum-dried gels.

Gel filtration of peptidase D. For the preparation of a crude cell extract, strain PC1324, which carries the *pepD*⁺ gene not only on the chromosome but also on the F' 104 plasmid, was used. Cells from 2 litres of an overnight culture of strain PC1324 in supplemented minimal medium were collected by centrifugation, washed twice with 5 mM-Tris/HCl, pH 7.5, resuspended in 10 ml of the same buffer and subsequently disrupted by ultrasonication. Debris and ribosomes were removed by ultracentrifugation at $100\,000 g$ and 4°C for 1 h. A sample of the supernatant containing 40 mg protein was mixed with 5 mg of each of the marker proteins (egg white lysozyme and horseradish peroxidase) and then applied to a column ($2.5 \times 100 \text{ cm}$) of Ultrogel AcA44 (LKB) equilibrated with 5 mM-Tris/HCl, pH 7.5. Gel filtration was done at 8°C with a flow rate of 0.4 ml min^{-1} ; 2 ml fractions were collected.

To calibrate the column, the activities of different marker enzymes were determined. Peptidase N (87 kDa) and cyclic phosphodiesterase (63 kDa), intrinsic proteins of *E. coli*, were assayed in the presence of the chromogenic substrates L-alanyl-*p*-nitroanilide (Lazdunski *et al.*, 1975) and bis-*p*-nitrophenyl phosphate (Anraku, 1964), respectively. Lysozyme (14 kDa) was detected by its lytic action on *Micrococcus luteus* (Shugar, 1952), and peroxidase (40 kDa) was monitored photometrically by the method of Pütter (1970).

RESULTS AND DISCUSSION

Identification of the pepD gene in the Clarke–Carbon bank

According to the genetic map of the *E. coli* chromosome given by Bachmann (1983), the *pepD* gene is located between min 5 and 6. However, its precise position with respect to the adjacent genes has not been determined. Therefore the well selectable marker *proBA*, mapping at min 5.9, was chosen to search for plasmids of the Clarke–Carbon library (Clarke & Carbon, 1976) which were likely to carry the *pepD* gene.

Four plasmids, pLC25-25, pLC32-46, pLC40-18 and pLC44-11, that are known to contain the *proBA* locus (Hayzer & Leisinger, 1980) were introduced into strain CM17 carrying a *proBA pepD* deletion, and transformants were selected by their proline-independence. Fluorimetric determination of peptidase D activity in cell free extracts of the transformants revealed that the only plasmid bearing a functional *pepD* gene was pLC44-11. This plasmid, 29.7 kb in size, was shown to code for at least eight polypeptides with distinct electrophoretic mobilities (Neidhardt *et al.*, 1983), and it had already served as a source for the cloning of the *E. coli* genes *phoE* (Tomassen *et al.*, 1982) and *proBA* (Hayzer, 1983). Therefore the location of these genes as well as the positions of some restriction sites in plasmid pLC44-11 are known (Fig. 1).

In order to locate the *pepD* gene on the pLC44-11 map, the three fragments generated by *SalI* cleavage of the plasmid were cloned into the *SalI* site of pBR322 (Bolivar *et al.*, 1977). Fluorimetric assays of peptidase D showed that none of the three types of recombinant plasmids which were detected in tetracycline-sensitive transformants of strain CM17 contained a functional *pepD* gene. This suggested that the *pepD* gene itself or its promoter had been

Fig. 1. Subcloning of the *pepD* gene of *E. coli* from pLC44-11. The fragments generated by *Bam*HI digestion of plasmid pLC44-11 were ligated with *Bam*HI-linearized pBR322; strain UK61 was transformed with the mixture. All of the carnosine-utilizing clones obtained were found to contain the largest (20.9 kb) *Bam*HI fragment in a unique orientation. In the recombinant plasmid pJK6 a 9.5 kb *Eco*RI fragment, containing the *phoE* and *proBA* genes, was deleted by *Eco*RI digestion and religation. The resulting plasmid, pJK7, was still able to confer peptidase D activity to strain UK61. In contrast, elimination of the 9.1 kb *Sal*I fragment from pJK7, giving rise to pJK10, resulted in loss of *pepD*-complementing activity. Plasmid pJK9 arose from pJK7 by a spontaneous deletion which eliminated a 1.8 kb fragment including the unique *Bam*HI site and the adjacent *Sal*I site without affecting the functional integrity of the *pepD* gene. Plasmid sizes and positions of restriction sites are drawn to scale. Hatched bars represent sequences originating from pBR322; portions of the ColE1 vector are marked by open bars; inserts of chromosomal *E. coli* DNA are drawn as heavy lines. The approximate positions of the genes *phoE* (Tommassen *et al.*, 1982) and *proBA* (Hayzer, 1983) are indicated. Recognition sites for restriction enzymes are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; P, *Pst*I; S, *Sal*I. The restriction map of pLC44-11 was originally published by Hayzer (1983). It contains the positions of only four of altogether nine *Pst*I sites.

destroyed by *Sal*I cleavage of pLC44-11. Two of the *Sal*I sites of pLC44-11 lie within the *phoE* and *proBA* coding regions (Fig. 1), which have been shown to be closely linked (Tommasen & Lugtenberg, 1981). Therefore the third *Sal*I site, flanking the ColE1 vector sequence, appeared to be the *pepD* inactivating site.

Fig. 2. Maps of the pUC18 hybrid plasmids pJK11 and pJK13. The vector pUC18 was cleaved at its unique *Pst*I and *Eco*RI sites and ligated with *Pst*I/*Eco*RI-digested plasmid pJK9 (see Fig. 1). After transformation of strain UK61 with the mixture the recombinant plasmid pJK11, containing the 4.5 kb *Pst*I-*Eco*RI fragment of pJK9, was detected in carnosine-utilizing clones. Ligation of *Pst*I/*Bgl*II cut pJK9 with *Pst*I/*Bam*HI-digested pUC18 yielded the hybrid plasmid pJK13, which includes the 2.4 kb *Pst*I-*Bgl*II fragment of pJK9 and still carries a functional *pepD* gene. Inserts of chromosomal DNA, portions of the ColE1 plasmid and restriction sites are marked as in Fig. 1. Hatched bars represent plasmid sections originating from the vector pUC18. The maximum *pepD* coding region is indicated. Of this region, a segment of about 0.6 kb, marked by an uninterrupted line, can be assigned definitely to the *pepD* gene, assuming a 52 kDa molecular mass for peptidase D (see Results and discussion). The scale given outside the maps is in kb.

Subcloning of the *pepD* gene

In order to facilitate subsequent cloning experiments, strain UK61, which carries a deletion of the *pepD* gene and, in addition, a *Tn10* insertion in the gene *hisG*, was constructed. Since, as in *S. typhimurium*, peptidase D proved to be the only peptidase able to cleave carnosine in *E. coli*, the presence of plasmid-encoded peptidase D in strain UK61 can be detected by simple complementation.

A *pepD* containing fragment of plasmid pLC44-11 was subcloned in the vector pBR322 and reduced in size as outlined in Fig. 1. The resulting plasmid, pJK9, was used to construct pJK11 and pJK13 (Fig. 2), which are *pepD*⁺ derivatives of the small vector pUC18 (Norranders *et al.*, 1983). Plasmid pJK13 bears only a 2.2 kb segment of genomic DNA, which corresponds to a coding capacity for approximately 730 amino acids. The localization of the *pepD* gene in the newly constructed plasmids was finally confirmed by insertional inactivation. A 1.45 kb *Sal*I fragment, carrying the kanamycin resistance gene from *Tn903* (Oka *et al.*, 1981), was isolated from plasmid pUC4-K (Vieira & Messing, 1982) and inserted into the unique *Sal*I site of pJK13, which was suggested to lie within the *pepD* gene or its promoter. As expected, the resulting plasmid, pJK13-K, did not confer peptidase D activity on strain UK61.

Expression of the cloned *pepD* gene

Transformation of the *pepD*⁺ strain AB1133 with the multicopy plasmid pJK7 increased the intracellular activity of peptidase D about 50-fold. Because of this effect of gene dosage, peptidase D was expected to be detectable among the proteins of cells harbouring *pepD*-containing plasmids.

Therefore plasmids pJK11 and pJK13 were expressed in the maxicell strain CSR603. As shown in Fig. 3, the pJK11 specific pattern contains three major proteins with molecular masses of 52, 44 and 17 kDa which are not encoded by the vector pUC18. Of these three additional bands, only the 52 kDa band is also visible in the pattern of pJK13-encoded proteins.

Fig. 3. Plasmid-directed protein synthesis in maxicells. ^{35}S -Labelled proteins synthesized in the maxicell strain CSR603 carrying the plasmids indicated were separated by SDS-PAGE. The pUC18-K-encoded proteins are β -lactamase (Ap^{R}) and aminoglycoside phosphotransferase (Km^{R}). Polypeptides with unknown or uncertain origin are denoted X and Y. The sizes of protein standards are given.

Consequently, this 52 kDa protein is likely to represent peptidase D, particularly because pJK13, despite the small size of its insert, is still able to confer the PepD^+ phenotype on UK61 cells. Final evidence for this suggestion comes from the observation that insertion of the kanamycin resistance gene into the putative *pepD* coding region not only prevents complementation of *pepD* deletions by pJK13-K, but also eliminates the 52 kDa band from the pattern of pJK13-encoded proteins. Instead, a polypeptide with the electrophoretic mobility of Tn903-encoded aminoglycoside phosphotransferase (27 kDa; Meagher *et al.*, 1977) appears in the pJK13-K specific pattern. This was confirmed by maxicell expression of pUC18-K, which contains the pUC4-K derived kanamycin resistance gene inserted between the *SalI* sites of pUC18.

In addition to peptidase D and β -lactamase, plasmid pJK11 codes for at least two other polypeptides, called X and Y, with molecular masses of about 17 and 44 kDa. These polypeptides are absent from the pJK13 specific protein pattern. Therefore, in combination with the mapping data of Hayzer (1983), the order of genes around the 6 min position of the *E. coli* map can be established to be *proB-proA-phoE-xy-pepD*.

One of the genes *x* and *y* appears to be *gpt*, which is closely linked with the *proA* locus (Holden *et al.*, 1976). Indeed, plasmid pJK11 was found to confer the ability to utilize guanine to the *gpt* strain PC2086, in which *de novo* purine synthesis is blocked by a mutation in the *purH* gene. A definitive identification of the genes *x* and *y* might be facilitated with the aid of plasmid pJK10 (Fig. 1), which carries no chromosomal genes except *x* and *y*.

Molecular mass of peptidase D

The molecular mass of the *S. typhimurium* peptidase D has been estimated to be 158 kDa (Kirsh *et al.*, 1978). To examine whether this value also applies to the *E. coli* enzyme, a cell free extract of strain PC1324 was subjected to gel filtration through Ultrogel AcA44. Active peptidase D eluted from this column with an apparent molecular mass of 100 kDa. In contrast, the molecular mass of denatured peptidase D as determined by SDS-PAGE of proteins synthesized in pJK13-containing maxicells is 52 kDa (Fig. 3). This value agrees well with the 50 kDa molecular mass of the largest pLC44-11-encoded polypeptide given in Neidhardt's gene-protein index (Neidhardt *et al.*, 1983). As the coding of a protein of this size requires a nucleotide sequence of at least 1.4 kb, at least 63% of the segment of genomic DNA included in the recombinant plasmid pJK13 (Fig. 2) is occupied by the *pepD* gene.

The discrepancy between the molecular masses of native and denatured peptidase D seems too large to be ascribed to inadequate calibration of columns or gels. Therefore, active peptidase D is possibly a dimer of about 100 kDa. As an alternative explanation, plasmid pJK13 might carry only a functional fragment of the original *pepD* gene present on the *E. coli* chromosome.

A suitable explanation for the considerable difference in the sizes of the *E. coli* and *S. typhimurium* peptidase D might have to await the elucidation of the primary structures of these enzymes or of the corresponding genes. The isolation of the functional *pepD* gene from the *E. coli* chromosome and its integration into a convenient multicopy vector should now facilitate the sequencing of the gene as well as the purification and further characterization of its protein product. This might contribute to a more detailed understanding of the enzymology of peptidase D and of its role in peptide metabolism.

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