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Site-directed mutagenesis of La protease

A catalytically active serine residue

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Comparative sequence analysis of *Escherichia coli* ATP-dependent La protease led to the suggestion that Ser^{679} is the catalytically active enzyme residue. Site-directed mutagenesis $Ser^{679} \rightarrow Ala$, investigation of the cells containing the mutant plasmid, and study of the partially purified mutant protein produced results in favour of this suggestion.

La protease; Active site; Site-directed mutagenesis; Sequence comparison; Chymotrypsin family; *E. coli*

1. INTRODUCTION

In procaryotic cells abnormal proteins and peptides are degraded mainly by ATP-dependent proteolytic enzymes. First, ATP-dependent protease, termed La protease, was found in *E. coli* cells by A. Goldberg et al. [1]. This enzyme is coded by the *lon* gene, which was cloned [2] and its structure was determined by two research groups [3–5] independently, however, the results differed to a certain extent.

La protease was assigned to the serine proteases family [2], but location of catalytically active residues remains unclear, since the enzyme amino acid sequence showed no similarity with sequences of classic serine proteases.

This paper deals with the examination of the amino acid sequence of La protease in comparison with other serine proteases and presents a suggestion on the location of the catalytically active serine residue. Experiments on site-directed mutagenesis support this conclusion.

2. MATERIALS AND METHODS

Enzymes for DNA restriction and modification were from Amersham (UK) and Boehringer Mannheim (Germany). [γ-^{32}P]ATP and [α-^{32}P]dNTP with specific activities 5000 and 3000 Ci/mmol, respectively, were purchased from Amersham. For mutagenesis, use was made of strain *E. coli* HB 101; native and mutant forms of La protease were expressed in *lon*$^-$ *E. coli* cells AB 1899. *E. coli* competent cells were obtained by the standard procedure with $CaCl_2$ [6].

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Oligonucleotide synthesis was performed by I. Chernov. [^{14}C]Acetyl-α-casein (30–50 mCi/mmol) was obtained by a modified technique [7]. Antibodies against purified La protease were produced as in [8].

The mutagenesis procedure in the plasmid variant was employed to introduce the mutation [9]. The DNA nucleotide sequence was determined by the solid phase Maxam–Gilbert method [10]. ATP-dependent proteolytic activity was measured with [^{14}C]acetyl-α-casein substrate as in [4]. Interaction of cell proteins with antibodies against La protease was checked by the method of double radial diffusion [11].

The amino acid sequence of La protease was analyzed by a set of computer programs GENEBEE [12]. The enzyme sequence was compared with those of the SWISSPROT data base by means of programs QUICK and SMART [12]. Program SITE was applied to analyze the La protease sequence in order to reveal the sites similar to the known sequence motifs [13]. A detailed comparison between the pairs of sequences was performed by program DOTHELIX [14] – an improved version of the dot matrix method.

3. RESULTS AND DISCUSSION

3.1. *Prediction of the possible catalytically active serine residue of La protease*

Analysis of the amino acid sequence of ATP-dependent La protease performed earlier, allowed identification of the ATPase-like domain covering about 200 amino acid residues and located in the central part of the protein [4,5,15]. Bearing these results in mind we suggested that either the N- or the C-terminal enzyme region containing about 330 and 250 amino acid residues, respectively, is a potential proteolytic domain. The amino acid sequences of these regions were thoroughly compared with those of serine proteases pertaining to three well characterized families of cell and virus enzymes [16–20]. As a result, a remote resemblance between the N-terminal part of the so-called 'serine loop' of chymotrypsin-like enzymes and the

Table I

The alignment of the 'serine loop' region of chymotrypsin-like proteases and the Ser^{679} region of the protease La.

Streptomyces griseus protease A	(131–141)	*P* **G** *D* **S** **G** **G** *S* *L* *F* *A* *G*
Streptomyces griseus protease B	(135–145)	*P* **G** *D* **S** **G** **G** *P* *L* *Y* *S* *G*
Bovine α-chymotrypsin	(176–186)	*M* **G** *D* **S** **G** **G** *P* *L* **V** **C** *K*
Bovine α-trypsin	(173–183)	**Q** **G** *D* **S** **G** **G** *P* *V* **V** **C** *S*
Porcine elastase	(184–194)	**Q** **G** *D* **S** **G** **G** *P* *L* *H* **C** *I*
Staphylococcus aureus V8 protease	(166–176)	*G* **G** *N* **S** **G** *S* *P* *V* *F* *N* *K*
Achromabacter protease I	(196–206)	*P* **G** *S* **S** **G** *S* *P* *I* *Y* *S* *P*
		. : : . . . . .
La Protease	(676–686)	**D** **G** *P* **S** **A** **G** *I* *A* **M** **C** **T**

The primary structures are taken from the SWISSPORT data base [12]; the data for *Achromabacter* protease I structure from [21]. Identical and similar residues of La protease and other enzymes are denoted in bold type. The catalytically active serine residue is indicated. Colons show identical residues in all sequences. Dots indicate identical or similar residues in the La protease sequence and, at least, in one of the sequences presented.

short stretch located in the C-terminal domain of La protease was demonstrated (Table I). The main point here is that Ser^{679} of La protease accords to the catalytically active serine residues of other proteases. No other serine residue of La protease is surrounded by sequences similar to the catalytic region of the known serine proteases.

Thus, Ser^{679} of La protease might be a catalytically active residue and the C-terminal domain is serine protease itself. It seems reasonable that characterization of the point mutants of the *lon* gene at this corresponding position might contribute much to the elucidation of the residues responsible for the enzyme's catalytic activity.

3.2. *Site-directed mutagenesis and mutant characterization*

To replace Ser^{679} by Ala we used a plasmid variant of the directed mutagenesis [9]. The 33-mer nucleotide 5′CCGAAAGATGGTCCGGCCGCCGGTATTGCTATG 3′ providing the substitution of the serine codon AGT by the alanine codon GCC was synthesized, while the mutation yielded restriction site *Xma*III (CGGCCG) that much facilitated a selection of the desired clones. The scheme for mutagenesis is shown in Fig. 1; fragment *Pst*I/*Pst*I of the *lon* gene cloned in the plasmid vector pSP64 was used as the starting material. Restriction analysis and nucleotide sequence determination were applied to the plasmid DNA of clones with the positive hybridization signal.

To reconstruct the full-size mutant *lon* gene the *Sal*I/*Sal*I fragment containing the 3′-terminal part of the gene was cloned in the corresponding restriction site of plasmid pBR327 lon_m earlier described [3]. This plasmid carries the *Eco*RI/*Sph*I fragment of bacterial DNA, including the major part of the coding region of the *lon* gene and its regulatory sequences. The clones containing recombinant plasmids with insertions in the required orientation were used in subsequent experiments. The correctness of the full-gene reconstruction and the presence of the Ala for Ser substitution were confirmed by restriction analysis and nucleotide sequencing.

The *lon* gene mutant was characterized in different ways: (1) its influence on the phenotype of transformed *E. coli* cells defective in the *lon* gene was monitored; (2) its expression was checked by SDS-electrophoresis and by immunoprecipitation of the transformed *E. coli* extracts; (3) the protease activity of its partially purified product was estimated in the presence and absence of ATP.

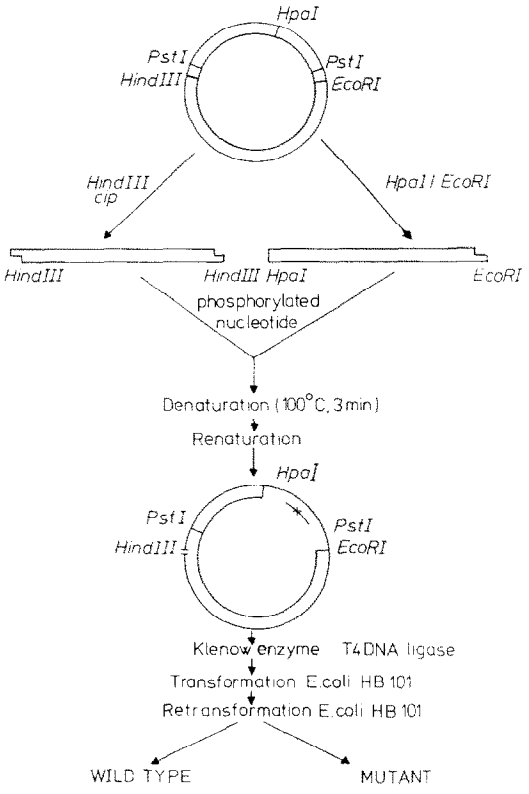

Fig. 1. Scheme of oligonucleotide directed mutagenesis of the *Pst*I/*Pst*I fragment of the *lon* gene of *E. coli* K12.

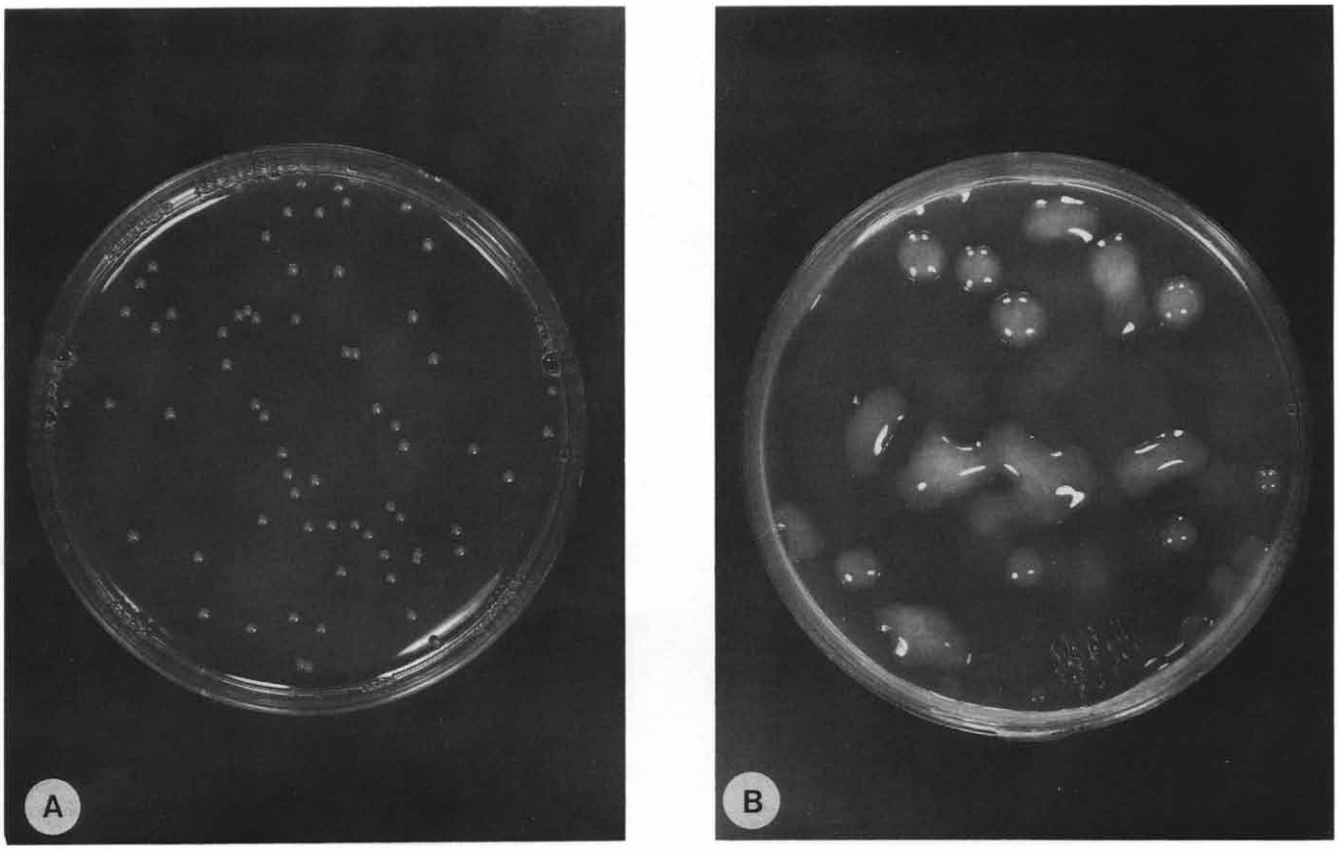

Fig. 2. Phenotypical changes of *E. coli* AB 1899 cells upon transformation by the plasmid bearing the functionally active *lon* gene (pBR 327*lon*) (A) and by the plasmid bearing the mutant *lon* gene (pBR327*lon* $Ser^{679} \rightarrow Ala$) (B).

E. coli cells defective in the *lon* gene (AB 1899) appear to have several particular properties: increased cell sensitivity to the action of nitrofurantoin and UV irradiation, slimming of bacteria colonies, typical elongated structures and decreased levels of intracellular proteolysis [22]. Transformation of *E. coli* AB 1899 cells by the plasmid containing the native full-size *lon* gene led to the complete disappearance of mutant phenotype. Contrary, the mutant plasmid, after introduction into the *E. coli* AB 1899 cells, failed to restore the usual colony size (Fig. 2) and other features intrinsic to wildtype cells.

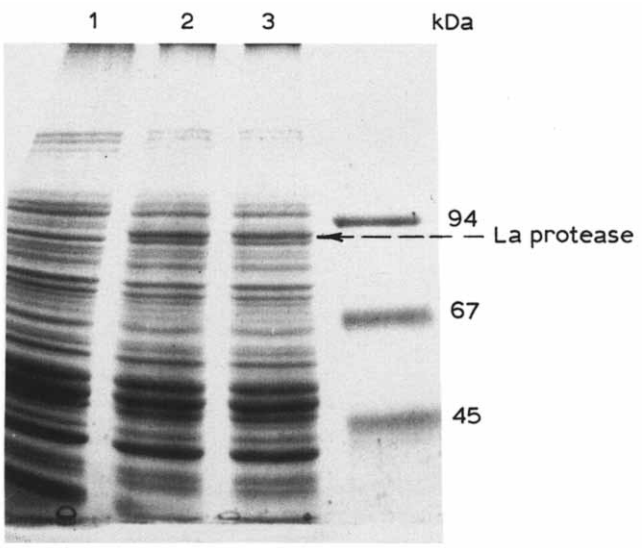

Fig. 3. SDS-electrophoresis of *E.coli* cell extracts: strain AB 1899/pBR327 (1); AB 1899/pBR327*lon* (2); AB 1899/pBR327*lon* $Ser^{679} \rightarrow Ala$ (3).

Electrophoresis of bacterial proteins of cells containing plasmids with the native or mutant gene (Fig. 3) showed that the replacement described did not affect the efficiency of the La protease expression and its electrophoretic mobility. Moreover, immunoprecipitation

Table II

Influence of site-directed mutagenesis of Ser^{679} on La protease activity

E. coli strains	Proteolytic activity*, (U/mg·h**)	
	− ATP	+ ATP
AB 1899/pBR327*lon*	0.11	1.25
AB 1899/pBR327	0.230	0.007
AB 1899/pBR327*lon* ($Ser^{679} \rightarrow Ala$)	0.11	0.015

*After phosphocellulose P-11 chromatography
**U corresponds to a part of the total amount of the substrate converted at standard conditions

demonstrated that extracts of cells bearing the *lon* gene and its mutants contained proteins giving cross reactions with antibodies against pure active La protease.

At the same time, no ATP-dependent proteolytic activity intrinsic to the enzyme was found in extracts of cells transformed by the mutant *lon* gene (Table II). That implied that the enzyme inactivation resulted from the substitution of Ser^{679} by the alanine residue.

Thus, all data presented show that the Ser^{679} residue is sufficient for protease La normal functioning. The catalytic role of the Ser^{679} residue seems to be very plausible. But the three-dimensional structure of the protease La should be resolved to prove it unequivocally. At present, experiments are in progress to delineate other residues of the protease catalytic triad and the residues involved in ATP-binding.

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