Vol. 203, No. 2, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 15, 1994 Pages 798-804

clpX encoding an alternative ATP-binding subunit of protease Ti (Clp) can be expressed independently from *clpP* in *Escherichia coli*

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Received July 26, 1994

Summary: ClpX, an alternative ATP-binding subunit for protease Ti (also called Clp), has been shown to support the ATP-dependent hydrolysis of λO-protein by ClpP. *clpX* has also been reported to be in an operon with *clpP*, and therefore both are co-transcribed in a single mRNA using the promoter proximal to *clpP*. Here, we show that *clpX* can be expressed independently from *clpP* using its own promoter. The cells carrying *clpX* alone on a multicopy plasmid successively produced the 46-kDa ClpX protein. Moreover, *in vitro* translation analysis revealed that the recombinant plasmid containing *clpX* generates the 46-kDa protein that can be immunoprecipitated with anti-ClpX antibody. In addition, it has recently been reported that ClpX, but not ClpP, is required for normal replication of bacteriophage Mu. Thus, it appears that *clpX* can be expressed alone and/or co-expressed with *clpP* in cells depending on physiological conditions.

Escherichia coli contain at least two distinct proteolytic enzymes, proteases La and Ti, that require ATP and Mg^{2+} for activity (1-3). Protease La, the *lon* gene product, is a heat shock protein and plays an essential role in the degradation of most abnormal proteins and certain short-lived regulatory proteins. Protease Ti, also called Clp, consists of two different polypeptide subunits, both of which are required for ATP-dependent proteolysis. ClpA is a protein-activated ATPase, and ClpP is a peptidase containing a serine active site (4,5). However, it has been clear that ClpP can also function in the ATP-dependent degradation of certain proteins in *clpA* mutants but in association with other ATPase regulatory components, homologous to ClpA (6,7).

Recently, such an alternative subunit for the ATP-dependent Clp protease, called ClpX, has been purified and its gene was cloned (8,9). In the presence of ATP, this protein, but not ClpA, supports the hydrolysis of bacteriophage λO-protein by ClpP. The

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0006-291X/94 $5.00

ClpX open reading frame begins 125 bases downstream from the termination codon for ClpP. In addition, it has been reported that *clpX* is in an operon with *clpP* and hence both genes are co-transcribed in a single heat-inducible mRNA with the promoter proximal to *clpP* (9). We also have independently cloned and sequenced *clpX* (Yoo, S.J., Seol, J.H., Goldberg, A.L. and Chung, C.H., unpublished; EMBL accession No. Z-23278). During this study, however, we noticed that the plasmid containing *clpX* alone could successively express the 46-kDa ClpX protein. Furthermore, it has recently been reported that normal replication of a transposable phage Mu requires ClpX but is independent of ClpP (11). In the present study, therefore, we demonstrate that *clpX* by itself can be transcribed and translated independently from *clpP* using *in vitro* translation analysis. We also purified ClpX for further characterization of its biochemical properties.

MATERIALS AND METHODS

Materials: ClpP was purified as described (4). Antisera against the purified ClpX (see below), and ClpP were obtained by injecting the proteins into albino rabbits. IgGs were then purified from the antisera using a protein A-Sepharose column. The *E. coli* S30 Prokaryotic Translation System was obtained from Promega; 8-N_3-[α-^{32}P]ATP from ICN; ^{125}I-protein A and [^{35}S]methionine from NEN-Dupont; protein A-Sepharose from LKB-Pharmacia. All other reagents were purchased from Sigma.

Purification of ClpX: *E. coli* cells transformed with pClpXt (see below) were grown to a density of OD_{600} 1.0 in Luria broth and induced for 2 h by adding isopropyl-thiogalactoside (IPTG) to 1 mM. The cells (3.5 g) were lysed by treating with lysozyme-EDTA as described (10). The lysates were then centrifuged at 500 x g for 10 min to remove cell debris. The supernatants were again centrifuged at 10,000 x g for 30 min. The aggregates in the pelleted fraction were solubilized with 25 mM Tris-HCl buffer (pH 7.8) containing 8 M urea, 1 mM dithiothreitol and 25 mM NaCl. The sample was then loaded on a DEAE-Sepharose column (1.5 x 5 cm) equilibrated with the Tris buffer containing 6 M urea. Proteins bound to the column were eluted with a linear gradient of 25-300 mM NaCl. Elution of the 46-kDa ClpX was assessed by polyacrylamide gel electrophoresis of aliquots of the column fractions in 10% (w/v) slab gels containing sodium dodecyl sulfate (SDS) (12). The fractions enriched with ClpX were pooled and subjected to stepwise dialysis to remove urea as described (10).

RESULTS AND DISCUSSION

Cloning and expression of *clpX*

We have previously cloned an *E. coli* 6.5 kb *Bam*HI/*Hin*dIII DNA fragment containing *clpP* in a multicopy plasmid (Zhu, Y., Wallner, B.P., Tizard, R., Mattaliano, R., Xia, K., Chung, C.H. and Goldberg, A.L., unpublished). Fig. 1A shows a part of the restriction

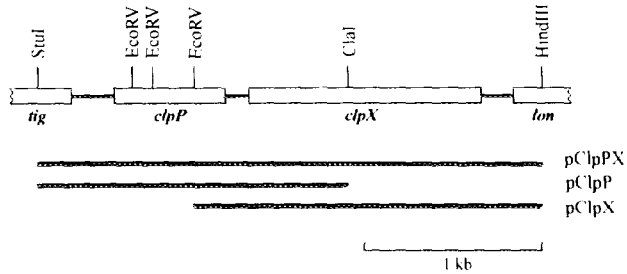

Fig. 1. Restriction map of the *clpX* region of *E. coli*. From this DNA clone containing 3 kb *Stu*I/*Hin*dIII fragment, three different recombinant plasmids (shown by black bars) were constructed as described in the text and were named as pClpPX, pClpP and pClpX.

map of the DNA clone that contains the open reading frames for the C-terminal fragment of trigger factor (13), ClpX and N-terminal fragment of Lon, in addition to that for ClpP. Using this clone, we have constructed three different plamids by ligating *Stu*I/*Hin*dIII, *Stu*I/*Cla*I and *Eco*RV/*Hin*dIII fragments into multicloning sites of Bluescript KS^+ plasmids (Fig. 1). The resulting plasmids, named pClpPX, pClpP and pClpX, respectively, were transformed and propagated in an *E. coli* strain JM109.

In order to determine whether *clpPX*, *clpX* and *clpP* in the plasmids can encode their respective gene products, the transformed cells were cultured, and aliquots of these cultures were then subjected to gel electrophoresis in triplicate in the presence of SDS. One of the gels, that was stained with Coomassie R-250, clearly shows that the cells carrying pClpPX or pClpP can overproduce the 21-kDa ClpP (Fig. 2A). However, the band corresponding to the 46-kDa ClpX could not be seen in the gel lanes corresponding to the cells carrying pClpPX or pClpX (*i.e.*, lane b and c). Therefore, the other two gels were subjected to immunoblot analyses using antibodies raised against the purified ClpP or ClpX (see below). As shown in Fig. 2B and 2C, ClpX could be detected not only in the cells carrying pClpPX but also in the cells with pClpX. These results strongly suggest that *clpX* can be expressed independently from *clpP* for production of the 46-kDa ClpX.

In vitro translation of *clpX*

Because the host JM109 cell has the wild type *clpP* and *clpX* in its chromosome and because the production of ClpX can not be easily detected in the Coomassie-stained gel even in the presence of the multicopy pClpX plasmid, we performed *in vitro* translation analysis to clarify further whether *clpX* can be independently expressed. The purified plasmids, pClpPX, pClpP and pClpX, were incubated with the *E. coli* S30 Prokaryotic

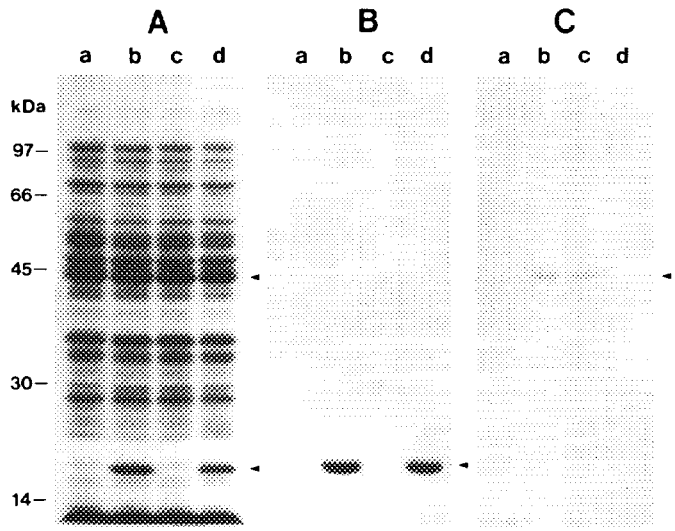

Fig. 2. Electrophoretic analyses for the expression of *clpP* and *clpX*. *E. coli* cells harboring Bluescript KS^+ plasmid (lane a), pClpPX (b), pClpX (c) and pClpP (d) were grown to late-log phase. Aliquots of these cultures were then added with equal volumes of 2% SDS and 2% (v/v) 2-mercaptoethanol, bolied, and electrophoresed on 12.5% polyacrylamide slab gels in triplicates in the presence of SDS. One of the gels was stained with Coomassie R-250 (A). The other two gels were transferred to nitrocellulose membranes and incubated with anti-ClpP IgG (B) or anti-ClpX IgG (C) and then with ^{125}I-labeled protein A. The membranes were then dried and exposed to X-ray films.

Translation System and [^{35}S]methionine. The incubation mixtures were then subjected to electrophoresis in the presence of SDS followed by autoradiography. As shown in Fig. 3A, *in vitro* translation of pClpPX generated both the 21- and 46-kDa proteins while that of pClpP or pClpX produced only the 21- or 46-kDa protein, respectively. In order to confirm that the radio-labeled protein bands indeed represent ClpP and ClpX, aliquots of the same incubation mixtures were also subjected to immunoprecipitation using the mixture of anti-ClpP and anti-ClpX IgGs. The precipitates were then electrophoresed and autoradiographed as above. Fig. 3B (lane b) again shows that pClpX can produce the 46-kDa ClpX protein. Nearly identical data were obtained when isolated, linear *clpX* DNA (*i.e.*, the *Eco*RV/*Hin*dIII fragment from pClpX; see Fig. 1) was subjected to *in vitro* translation analysis as above (data not shown). Thus, it appears clear that *clpX* can be expressed independently from *clpP* using its own promoter.

Recently, it has been demonstrated that the two enteric transposable phages Mu and D108 do not multiply in the absence of ClpX while they grow normally in a *clpP* null mutant strain (11). Furthermore, it has also been reported that *clpX* mutant strains are

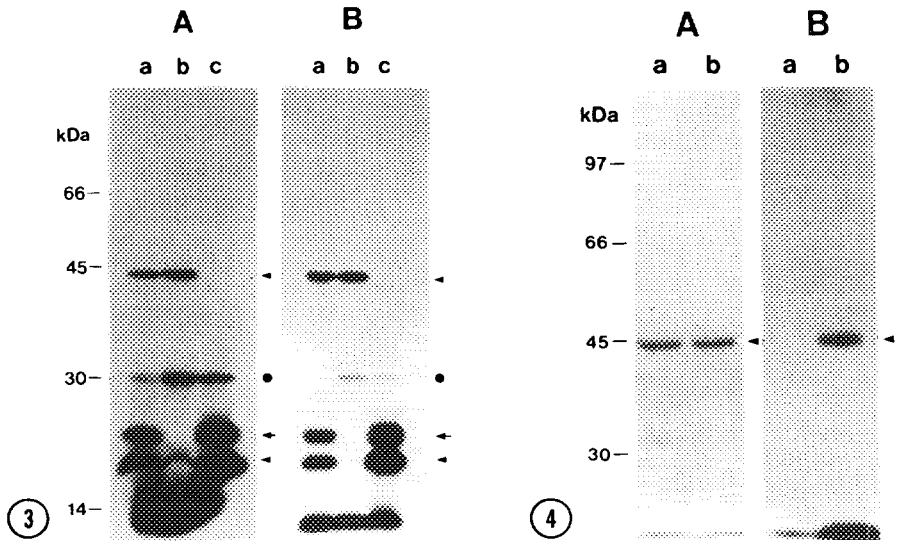

Fig. 3. Electrophoretic analyses of *in vitro* translated products from the plasmids containing *clpP* and *clpX*. The purified plasmids (2 μg each), pClpPX (lane a), pClpX (b) and pClpP (c), were incubated for 2 h at 37 °C with 15 μl of *E. coli* S30 Prokaryotic Translation System and 2 μCi of [^{35}S]methionine in final volumes of 50 μl. (A) Aliquots (10 μl) of the incubation mixtures were electrophoresed as described in Fig. 2. The resulting gel was then dried and autoradiographed. (B) The incubation mixtures (20 μl each) were also subjected to immunoprecipitation by adding with the mixture of anti-ClpP and anti-ClpX IgGs (50 μg total) and then with protein A-Sepharose. The precipitates were dissolved in 2% SDS and 2% 2-mercaptoethanol, electrophoresed, and autoradiographed. The dots indicate β-lactamase originated from the Bluescript plasmids.

Fig. 4. Purification and affinity-labeling of ClpX using azido-[α-^{32}P]ATP. To over-produce ClpX, the reading frame for ClpX in pClpX was put under *tac* promoter as described in the text. ClpX was then purified from *E. coli* cells harboring the mutated plasmid (pClpXt) as described in Materials and Methods. The purified ClpX (3 μg each) was incubated with 2 μCi of 8-N_3-[α-^{32}P]ATP for 30 min on ice and irradiated for 1 min at room temperature with UV-light using a UVGL-58 lamp (260 nm, Ultra-Violet Products, Inc.). The resulting samples were electrophoresed in the presence of SDS (lane b). The gels were then visualized by staining with Coomassie R-250 (A) or exposing to X-ray films (B). Lanes a contain the protein samples prepared as above but without UV-irradiation.

temperature sensitive for growth under certain conditions, such as stationary-phase cultures, which do not affect temperature resistance of *clpP* mutants (11). In these *clpP* mutant cells, ClpX must have been generated from the *clpX* gene itself but not from the *clpPclpX* operon. Therefore, we suggest that *clpX* can be expressed alone and/or co-expressed with *clpP* in cells depending on physiological conditions.

Noteworthy was the finding that the translational products derived from pClpPX and pClpP contained an additional major band of about 23 kDa (Fig. 3, indicated by arrows).

Furthermore, this protein strongly interacted with the anti-ClpP IgG. It has been reported that ClpP is synthesized as a precursor and its N-terminal 14 amino acids are cleaved off during assembly of the protein by an autocatalytic process (14). Therefore, it appears likely that the 23-kDa protein is an unprocessed precursor form of ClpP.

Upon analyses by transformation of *E. coli* cells with pClpPX, pClpX and pClpP and by *in vitro* translation of the plasmids, amount of ClpX generated was found to be much lower than that of ClpP. Furthermore, *clpX* mRNA was produced to a much lower extent than *clpP* mRNA as analyzed by run-off transcription experiments (data not shown). These results clearly suggest that the strength of the *clpX* promoter in transcription must be very weak compared to that of others, such as the heat-inducible *clpP* promoter. In fact, the upstream region of the open reading frame for ClpX has a putative -10 sequence (TGAGAATGG) for transcription and a potential Shine-Dalgarno sequence (GAAGAGG) for ribosome-binding but it lacks canonical -35 promoter sequence. Therefore, it is possible that low level of the transcripts from the chromosomal *clpX* might have not been easily detected by Northern analysis (9).

Purification and affinity-labeling of ClpX

In order to overproduce ClpX, a *Bam*HI restriction site was created immediately upstream to the initiation codon for ClpX in pClpX by site-directed mutagenesis as described (15). A *tac* promoter was then introduced into the restriction site and the resulting plasmid was referred to as pClpXt. *E. coli* cells harboring pClpXt were grown and induced with IPTG, and their lysates were prepared. ClpX produced under these conditions was found to be in highly aggregated state in cell lysate in accord with the earlier report (9). Therefore, ClpX was purified from the aggregates by solubilization with 8 M urea followed by stepwise dialysis for renaturation (10). Fig. 4A shows that the purified ClpX consists of a single band of 46 kDa upon analysis by gel electrophoresis in the presence of SDS.

ClpX has been shown to support the degradation of λO-protein by ClpP in an ATP-dependent manner (8). However, it was unknown whether ClpX can bind and/or hydrolyze ATP. Therefore, we first examined whether ClpX can bind with ATP by incubation with azido-[α-^{32}P]ATP. As shown in Fig. 4B, the purified ClpX could efficiently be cross-linked with the ATP analog upon UV-irradiation but not without it. This result is in consistent with the fact that ClpX contains a highly conserved sequence for adenine nucleotide binding (16). However, little or no ATP hydrolysis was observed upon incubation of our purified ClpX with ATP (data not shown). Therefore, it remains

unclear whether the purified ClpX may be dissociated and inactivated during solubilization with urea or whether its binding to ATP may be sufficient in supporting the proteolytic function of ClpP, unlike ClpA.

Acknowledgments: We are grateful to Dr. Alfred L. Goldberg (Harvard Medical School) for his critical discussion. This work was supported by grants from Korea Science and Engineering Foundation through SRC for Cell Differentiation and The Ministry of Education.

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