The 65-kDa protein derived from the internal translational start site of the *clpA* gene blocks autodegradation of ClpA by the ATP-dependent protease Ti in *Escherichia coli*

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Abstract **The ATP-dependent protease Ti consists of two different components: ClpA containing ATP-cleaving sites and ClpP having serine active sites for proteolysis. The *clpA* gene has dual translational start sites and therefore encodes two polypeptides with sizes of 84 and 65 kDa (referred to as ClpA84 and ClpA65, respectively). Here we show that ClpA84, but not ClpA65, is degraded in vitro by ClpP in the presence of ATP. The ClpP-mediated hydrolysis of ClpA84 could be prevented by casein, which is an excellent substrate of protease Ti (i.e. ClpA84/ClpP complex). Thus, it appears that free form of ClpA84 competes with casein for the degradation by ClpA/ClpP complex. Furthermore, ClpA65 inhibited the auto-degradation of ClpA84 by the complex. These results suggest that ClpA65 may play an important role in the control of the ClpA84 level and in turn in the regulation of ATP-dependent protein breakdown in *E. coli*.**

Key words: ATP-dependent protease; ClpA; ClpP; Protease Ti; Autodegradation; *E. coli*

1. Introduction

Protease Ti, also called Clp, consists of two different multimeric components, both of which are required for ATP-dependent proteolysis in *E. coli* [1–4]. While ClpA contains the ATP- hydrolyzing sites, ClpP contains the serine-active sites for proteolysis. The purified ClpA behaves as a dimer or trimer of 84-kDa subunits in the absence of ATP and as a hexamer in its presence, while ClpP is comprised of 12–14 subunits of 21 kDa [5,6]. The isolated ClpA shows protein-activated ATPase activity, which in the reconstituted enzyme is linked to protein breakdown.

The *clpA* gene has been shown to contain dual initiation sites for translation and therefore encode two polypeptides with different sizes (i.e. 84- and 65-kDa subunits), of which the smaller polypeptide is derived from the internal start site [5]. Accordingly, mutagenesis of the 5′-end AUG codon results in an exclusive synthesis of the 65-kDa protein (referred to as ClpA65), while mutation at the internal 169th AUG codon (Met) to ACG (Thr) produces only the 84-kDa protein. However, ClpA65 lacking the N-terminal 168 amino acids shows little or no ATPase and is unable to support the proteolytic activity of ClpP, although it retains the intact, two highly conserved sequences for ATP-binding like ClpA84 [7].

ClpA84, but not ClpP, has been shown to be degraded in vivo with a half-life of about 1 h [8]. This rate of degradation is sufficient to remove almost half of ClpA84 from the cell during each generation. Furthermore, ClpA84 has been demonstrated to be stable in a mutant cell lacking ClpP [8]. Therefore, it has been suggested that the level of ClpA84 in *E. coli* is regulated in part by autodegradation of ClpA84 by protease Ti (i.e. ClpA84/ClpP complex). In the present studies, we examined whether ClpA84 can indeed be autodegraded in vitro by ClpA84/ClpP complex in an ATP-dependent manner. We also examined the effect of ClpA65 on the ClpP-mediated ClpA84 hydrolysis in an attempt to clarify the role of the abbreviated form of ClpA84 in the ATP-dependent protein breakdown in the cells.

2. Materials and methods

2.1. Materials

The Bluescript KS$^+$ plasmid carrying *clpA* (called pClpA) was constructed as described previously [5]. The plasmids producing ClpA65 (pClpA65) were generated by site-directed mutagenesis of the 5′-end translational start sites of the *clpA* gene in pClpA as described [5]. ClpA84 and ClpA65 were purified from an *E. coli clpA* null mutant strain SG21118 (*clpA*319::*Δkan*) carrying the plasmids as described [5]. ClpP was purified as described [9]. [^{3}H]Methyl-casein was prepared as described [10].

2.2. Assays

Proteolysis was assayed by incubating the reaction mixtures (0.2 ml) containing 10 μg of [^{3}H]casein, 5 mM ATP, 100 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM EDTA at 37°C [9]. After the incubation, radioactivity released into trichloroacetic acid-soluble products was counted using a scintillation counter. Proteins were assayed by the dye-binding method of Bradford using bovine serum albumin (BSA) as a standard [11].

3. Results and discussion

3.1. ClpP-mediated hydrolysis of ClpA84

In order to determine whether ClpA84 can be degraded in vitro by ClpP, the two purified proteins were incubated together for varying periods in the presence of ATP. After the incubation, the samples were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) followed by staining with Coomassie R-250 [12]. As shown in Fig. 1A, ClpA84 was degraded in a time-dependent manner. More than 80% of ClpA84 was hydrolyzed in about 30 min, when analyzed the density of the protein bands using a densitometer. In addition, little or no intermediate polypeptide products could be seen in the Coomassie-stained gel at any time of the incubation period, indicating that the ClpP-mediated degradation of ClpA84 have occurred extensively.

We have recently demonstrated that ATP hydrolysis is essential for assembly of protease Ti (i.e. ClpA84/ClpP complex) and

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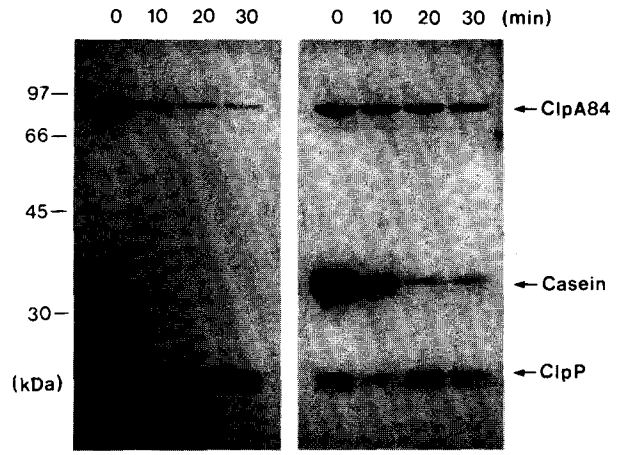

Fig. 1. ClpP-mediated hydrolysis of ClpA84 in the absence (A) and presence of casein (B). ClpA84 (4 μg) was incubated with 1 μg of ClpP and 5 mM ATP at 37°C for varying periods in the presence and absence of 10 μg of casein. After the incubation, the samples were electrophoresed on 13% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol. Proteins in the gels were then visualized by Coomassie-staining. The numerals on top of the gels indicate the incubation period.

that ADP generated upon the ATP cleavage involves in the rapid dissociation of the complex [13]. Therefore, ClpA65 lacking the ATPase activity cannot be assembled with ClpP into an active form of protease Ti. On the other hand, the ClpA84 and ClpP molecules should be in rapid equilibrium with their complex. During this association–dissociation cycle at the expense of ATP, it appears possible that free form of ClpA84 is recognized as a substrate of ClpA84/ClpP complex unless other degradable proteins are available.

To test this possibility, the purified ClpA84 and ClpP were incubated in the presence of casein, which is known to be an excellent substrate for protease Ti. The incubation mixtures were then subjected to electrophoresis as above. As shown in Fig. 1B, hydrolysis of ClpA84 was nearly completely prevented upon the addition of casein. Instead, casein molecules were extensively degraded. This result indicates that casein acts as a competing substrate against ClpA84 for degradation by protease Ti. We then examined whether the remaining ClpA84 after incubation with ClpP only is still capable of supporting the casein hydrolysis. Table 1 shows that the casein-degrading activity of the preincubated mixture of ClpA84 and ClpP is about 20% of that seen without the preincubation. These results again shows that ClpA84 is rapidly autolyzed by ClpA84/ClpP

Table 1
Effect of preincubation of ClpA84 with ClpP on casein hydrolysis

Additions for preincubation	Additions for proteolysis	% relative activity
None	ClpA84, ClpP, Casein	100
ClpA84	ClpP, Casein	86
ClpA84, Casein	ClpP	85
ClpA84, ClpP	Casein	18

The preincubation mixtures were incubated with ATP for 30 min at 37°C. The samples were further incubated for the next 1 h together with the additions for proteolysis. Hydrolysis of [^{3}H]casein was then assayed as described in section 2. The amounts of the proteins used in this study were one-fifth of that used in Fig. 1, except that [^{3}H]casein was 10 μg.

complex and this autodegradation can be prevented when other protein substrates are available.

3.2. *ClpA65 blocks the autodegradation of ClpA84*

To determine whether ClpA65 derived from the internal translational initiation site can also be hydrolyzed by ClpP, the abbreviated form of ClpA84 was incubated with ClpP and ATP. Unlike ClpA84, ClpA65 was not at all cleaved even when the two proteins were incubated for prolonged periods (Fig. 2). This result is accordance with our previous findings that ClpA65 is unable to hydrolyze ATP or to support the ATP-dependent degradation of casein by ClpP [5] and that ATP hydrolysis is essential for assembly of the ClpA and ClpP proteins into an active form of protease Ti.

We have previously shown that ClpA65 inhibits the ability of ClpA84 in supporting the ClpP-mediated casein breakdown but without any effect on its protein-activated ATPase activity [5]. Therefore, it has been suggested that ClpA65 may play an important role in the regulation of the ATP-dependent protein breakdown in *E. coli*. An additional role of ClpA65 could be the prevention of autodegradation of ClpA84 and hence involved in the control of ClpA84 level in the cells. In order to test the latter possibility, ClpA84 and ClpP were incubated in the presence of increasing amounts of ClpA65. As shown in Fig. 3, ClpA65 inhibited the autodegradation of ClpA84 by ClpA84/ClpP complex in a dose-dependent manner. In addition, the inhibitory effect of ClpA65 is not affected by the presence of excess ClpP over ClpA84 and ClpA65 (data not shown), indicating that the inhibition is not due to simple competition between ClpA84 and ClpA65 for interaction with ClpP. This observation is in accord with our finding that ClpA65 lacking the ATPase activity cannot interact with ClpP [5,13]. Therefore, ClpA65 with ClpA84 seems to form a hetero-oligomeric complex, which may in turn interfere the interaction of ClpA84 with ClpP. In summary, we suggest that ClpA65

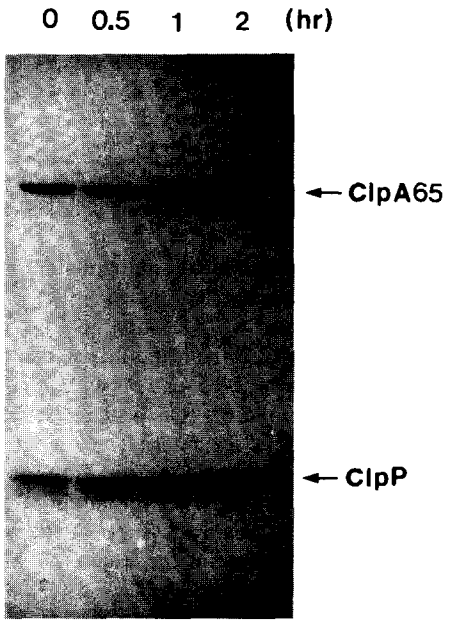

Fig. 2. Hydrolysis of ClpA65 by ClpP. ClpA65 (4 μg) was incubated with 1 μg of ClpP and 5 mM ATP at 37°C for varying periods. The samples were then electrophoresed as in Fig. 1. The numerals on top of the gels indicate the incubation period.

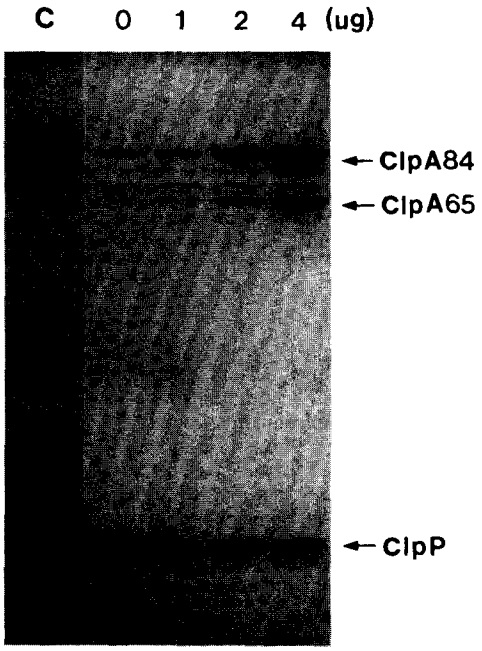

Fig. 3. Dose-dependent effect of ClpA65 on the ClpP-mediated hydrolysis of ClpA84. ClpA84 (4 μg) and ClpP (1 μg) were incubated with 5 mM ATP in the presence of increasing amounts of ClpA65 at 37°C for 30 min. Lane C indicates the mixture of ClpA84 and ClpP, which were electrophoresed without the incubation. The numerals on top of the gel shows the added amounts of ClpA65.

derived from the internal translational initiation site of the *clpA* gene may also play an important role in the regulation of the ClpA84 level and hence in the control of ATP-dependent protein breakdown in *E. coli*.

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