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A point mutation within the ATP-binding site inactivates both catalytic functions of the ATP-dependent protease La (Lon) from *Escherichia coli*

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Abstract A point mutant in the ATP-binding motif (GPPGVGK[362]T) of the ATP-dependent protease La from *Escherichia coli* was investigated in which the lysine at position 362 was replaced by an alanine. The catalytic efficiency of the K362A mutant is at least two orders of magnitude lower than that of wild-type protease La due to a decreased V_{max} and an increased K_M for ATP. Simultaneously, the peptidase activity of La K362A is almost completely eliminated. Since selective inactivation of the peptidase activity of La does not affect its intrinsic ATPase activity, coupling of proteolysis with ATP hydrolysis is only uni-directional in this energy-dependent protease.

Key words: Protease La (Lon); ATP-dependent proteolysis; Walker A sequence; ATPase-deficient mutant

1. Introduction

Proteolytic breakdown in prokaryotic and in eukaryotic cells requires energy-dependent proteases, to which the majority of cellular protein degradation can be attributed [1]. This energy is provided by ATP, which is hydrolyzed and enables concomitant cleavage of polypeptides. The reason for this necessity of ATP hydrolysis during proteolysis is as yet unknown, although several ATP-dependent proteases have been intensively characterized ([2], and references therein). There are two major ATP-dependent proteases that play a crucial role in the degradation of abnormal proteins in *E. coli*: the ATP-dependent proteases La (Lon) and Ti (Clp). Protease La is a homotetramer of 87 kDa subunits. Each subunit contains an ATPase and a protease domain. La specifically degrades denatured proteins that stimulate the ATPase activity of the enzyme by interacting with a regulatory binding site [3]. The proteolytic activity of La is, however, not essential for its ability to hydrolyze ATP. Exchange of the catalytically active serine at position 679 by alanine does not alter the ATPase activity of La [4]. In contrast, binding and subsequent hydrolysis of ATP by protease La has been suggested to be necessary for its proteolytic function both in vivo and in vitro [5,6]. ADP, the product of ATP hydrolysis by La, binds tighter than ATP to the acitve site and inhibits the ATPase activity [5]. This mechanism is presumably accompanied by a conformational change affecting the active site of the protease domain. Therefore, the identification of the ATP-binding site and the characterization of an ATPase-deficient mutant would provide further insight into the catalytic mechanism of protease La.

The amino acid sequence of protease La contains a typical ATP-binding motif, the so-called Walker A sequence [7], that is commonly found in many nucleotide-binding proteins such as ATP synthases, kinases, elongation factors, myosin, and Ras proteins [8]. The Walker A sequence comprises a phosphate-binding loop (P-loop) that has been shown to be essential for the binding of the nucleotide triphosphates, which then mediate the specific functions of these proteins [8]. The P-loop typically consists of a glycine-rich sequence followed by a conserved lysine and a serine or a threonine residue. Using site-directed mutagenesis, we have replaced the conserved lysine at positon 362 in the Walker A motif of protease La (GPPGVGKT) by an alanine. Lys-362 is supposed to interact directly with the β- or γ-phosphate of the bound ATP [8]. We investigated the structural and functional properties of this K362A mutant and asked whether this conserved lysine is responsible for the binding of ATP in protease La and to what extent the proteolytic activity of La is affected.

2. Materials and methods

2.1. Molecular cloning procedures

Site-directed mutagenesis was carried out by the method of Kunkel [9] using the helper phage M13K07 [10] to isolate uridinylated, single-stranded DNA of the La expression plasmid pLON [4]. The K362A mutation was introduced by using the following oligonucleotide: 5′ GGACTGACCAAGAGACGTCGCACCTACCCCCGGCGG 3′. The mutant was identified by dideoxy sequencing [11] using T7 DNA polymerase (Pharmacia) according to the manufacturer's instructions.

2.2. Protein expression and purification

Protease La and the K362A mutant were expressed in the *lon*-deficient *E. coli* strain BL21 [12] and purified by sequential chromatography on heparin agarose and Sephadex S300 as described previously [4]. Since ATP hydrolysis may stimulate the dissociaton of peptides bound to La, the fractions obtained after the heparin column step were incubated in 5 mM ATP and 7.5 mM $MgCl_2$ in standard buffer (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM β-mercaptoethanol, 10% (v/v) glycerol) at 4°C for 1 h directly before the application of the protein onto the Sephadex S300 column.

The protein concentration was determined by measuring the absorbance at 280 nm (ε_{280} = 47,000 $cm^{-1} \cdot M^{-1}$).

2.3. Enzyme assays

The ATPase activity of protease La was measured at 37°C in standard buffer as described elsewhere [4,13] by detecting the inorganic phosphate as a complex with Malachite green and ammonium molybdate at 660 nm. The stimulation of the ATPase activity by dephosphorylated α-casein (Sigma) was investigated at casein concentrations of 1 mg/ml.

Peptide hydrolysis was assayed at 37°C in standard buffer containing 10% (v/v) DMSO with the fluorogenic peptide Glt-Ala-Ala-Phe-MNA (1.2 mM). Formation of the reaction product MNA was measured 'on

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Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; Glt, glutaryl; MNA, 4-Methoxy-2-naphtylamine; Tris, Tris(hyrdoxymethyl)aminomethane.

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line' by the increase in fluorescence at 415 nm (excitation at 335 nm) as described [4] using a Hitachi F-4500 fluorescence spectrophotometer.

3. Results and discussion

3.1. The Walker A motif is fully conserved in known protease La (lon) sequences

The Walker A motif consists of the consensus sequence GXXGXGKT that can be found in several nucleotide-binding protein families [8,7]. These residues form a loop that interacts with the nucleotides (Table 1). The *lon* gene products of *E. coli*, *B. brevis*, and *M. xanthus* exhibit about 50% sequence identity [14] and about 30% of the residues in the human protease La are identical to those in eubacterial La proteases [14,15]. In comparison to this extent of amino acid conservation, the Walker A motif is conserved to 100% within the family of La proteases (Table 1). Based on this fact and on described examples of lysine replacements in the GKT sequences [16,17], the lysine at position 362 of protease La from *E. coli* was exchanged with an alanine to inactivate its ATPase function.

3.2. The exchange of the conserved lysine results in an ATPase-deficient mutant

Protease La wild-type and its mutant K362A were purified from the *lon*-deficient *E. coli* BL21 harboring the corresponding expression plasmids. The K362A mutant showed no differences to La wild-type or the previously described proteolytically inactive S679A mutant [4] in terms of expression yield, solubility, and spectroscopic (fluorescence, far-UV circular dichroism) characteristics (data not shown). Thus, the mutation did not alter the overall three-dimensional structure of the protein significantly. We then compared the ATPase activities of protease La wild-type and the K362A mutant. The K362A mutant possesses less than 10% of the ATPase activity of the wild-type in the presence of 5 mM ATP, where the wild-type is fully saturated with ATP (Fig. 1A). Dephosphorylated α-casein, which is a protein substrate of protease La and stimulates its ATPase activity, did not markedly increase the ATPase activity of the K362A mutant. Lineweaver–Burk analysis of the two variants of protease La revealed not only a 30-fold lowered catalytic activity (V_{max}) but also a severalfold increased apparent K_M value of K362A compared to the wild-type (Fig. 1B, Table 2). The K_M and the V_{max} value of K362A could not be determined very accurately because of its very low specific activity (Table 2) and probably because of an increased cooperativity of ATP

Table 1
Occurrence of the Walker A motif in different nucleotide binding proteins and in the known La-type proteases from different organisms

Protein	Residues	Walker A sequence	References
ATPase α (*E. coli*)	169–176	**G** D R **G** T **G K T**	[7]
ras p21 (human)	10–17	**G** A G **G** V **G K S**	[19]
Myosin (rabbit)	178–185	**G** E S **G** A **G K T**	[7]
Ef-Tu (*E. coli*)	18–25	**G** H V D H **G K T**	[19]
Protease La (*E. coli*)	356–363	**G** P P **G** V **G K T**	[14]
Protease La (*M. xanthus*, IonD)	379–386	**G** P P **G** V **G K T**	[14]
Protease La (*M. xanthus*, IonV)	368–375	**G** P P **G** V **G K T**	[14]
Protease La (*B. brevis*)	355–362	**G** P P **G** V **G K T**	[14]
Protease La (human)	409–416	**G** P P **G** V **G K T**	[15]

Letters in bold represent the conserved residues of the Walker A sequences.

Table 2
Kinetic constants of the ATPase activities of protease La wild-type and the Walker A point mutant K362A

Protein	V_{max} [a]	K_m(μM)	V_{max}/K_m
La wild-type	44 ± 5	200 ± 20	220 ± 30
La K362A	1.3 ± 0.3	1700 ± 300	0.7 ± 0.2

Data were obtained as described in Fig. 1B.
[a] V_{max} = ATP molecules hydrolyzed per min per subunit of protease La.

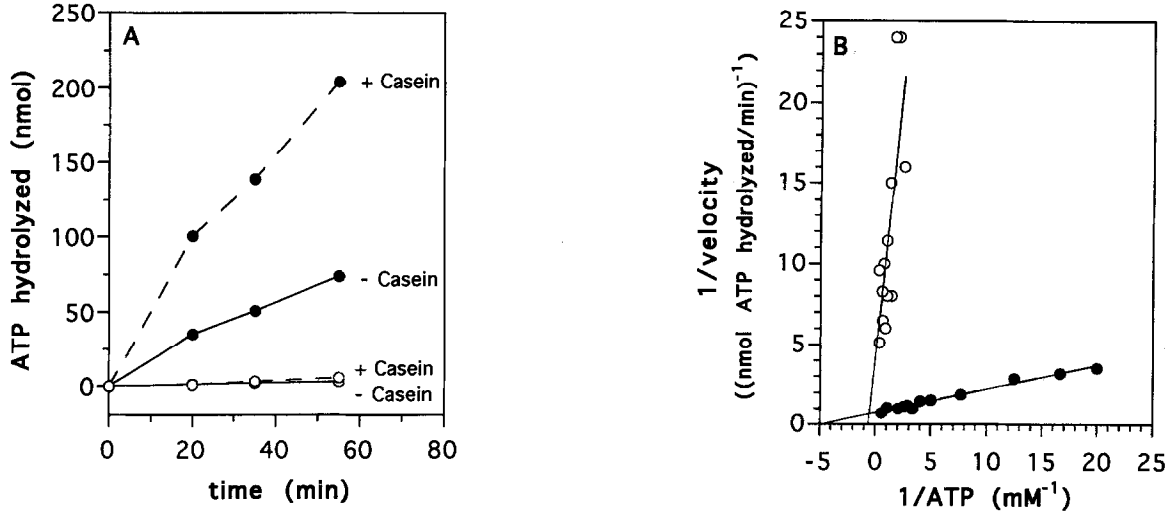

Fig. 1. (A) Hydrolysis of ATP by protease La wild-type (●) and protease La K362A (○) at saturation with ATP and stimulation by the protease substrate α-casein. The tests were performed as described in section 2 at 37°C. The assays contained 400 nM protease La (monomer), 5 nM ATP, and 7.5 mM $MgCl_2$ in standard buffer. Dephosphorylated α-casein was added to a final concentration of 1 mg/ml (45 μM). (B) Lineweaver–Burk analysis of ATP hydrolysis by protease La wild-type (●) and by protease La K362A (○) mutant at 37°C. The assays contained 100 nM La (monomer) wild-type or 800 nM (monomer) La K362A and 7.5 mM $MgCl_2$ in 300 μl standard buffer. The ATP concentrations were varied netween 0.05 and 5 mM.

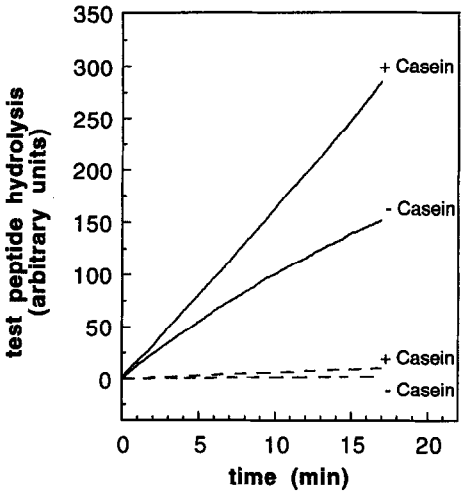

Fig. 2. Hydrolysis of Glt-Ala-Ala-Phe-MNA by protease La wild-type (—) and the K362A mutant (- - -) at 37°C. Measurements were performed as described in section 2. The assays contained 100 nM (monomer) protease La or K362A, 1.2 mM Glt-Ala-Ala-Phe-MNA, 5 mM ATP, and 7.5 mM $MgCl_2$. Dephosphorylated α-casein was added to a final concentration of 1 mg/ml (45 μM).

binding to the mutant. The measured V_{max} value for ATP hydrolysis by La wild-type (44 ± 5 min^{-1}) differs by a factor of 2 from our previous results (about 22 min^{-1}). This may be due to the fact that peptides bound to La can inhibit the ATPase activity of the enzyme [4,18]. As a result of our altered purification procedure, inhibitory peptides may have been released from La by the additional treatment of La with Mg-ATP prior to the gel-filtration step (see section 2) thus leading to a higher V_{max} value.

Taken together, the exchange of the conserved lysine in the P-loop sequence of protease La results in a drastic change of the Michaelis–Menten parameters. The catalytic efficiency of this point mutant is decreased by at least two orders of magnitude.

3.3. *Loss of proteolytic activity in the ATPase-deficient mutant*

The proteolytic activity of protease La has been believed to be essentially linked to its ATPase activity, using assays lacking ATP or containing non-hydrolyzable ATP analogues. Nevertheless, at least one example of ATP-independent substrate degradation has been reported [20]. We compared the proteolytic activities of protease La wild-type and La K362A in the presence of 5 mM ATP. The K362A mutant showed very weak proteolytic activity (about 2% of wild-type activity) toward the test peptide Glt-Ala-Ala-Phe-MNA. Dephosphorylated α-casein, which not only increases the ATPase activity but also the peptidase activity of wild-type La by binding to a regulatory site [3], stimulated the proteolytic activity of K362A only slightly.

In principle, the strongly reduced peptidase activity of the K362A mutant might also result from a failure to release bound degradation products that might have been co-purified along with the enzyme. However, it appears likely that the ability of La to release peptides is directly coupled to its ATPase activity. Since the K362A mutant still possesses a residual ATPase activity, it should also be able to release bound peptides, albeit with a decreased turnover. Therefore, the reduced peptidase acivity of La K362A is presumably only a consequence of its low specific ATPase activity, and the mechanistic linkage between ATPase and peptidase activity is still present in the mutant.

In summary, the reduction of the catalytic efficiency of the ATPase of La concomitantly results in a drastic drop in its proteolytic activity. The results provide further evidence for the tight mechanistic coupling of ATPase and peptidase activity in protease La. However, this linkage is only uni-directional. The ATPase activity is essential for the simultaneous cleavage of peptide bonds, whereas the loss of the proteolytic activity does not affect the ATPase activity.

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