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Role of ATP Hydrolysis in the Degradation of Proteins by Protease La From *Escherichia coli*

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Protein degradation in animal and bacterial cells is dependent upon ATP [1,2]. In bacteria [3–6] and mammalian mitochondria [7], this energy dependence arises in large part from the involvement of a novel type of proteinase whose function is directly coupled to ATP hydrolysis [2,3]. The best-characterized of these ATP-dependent enzymes is protease La from *Escherichia coli* [3–7]. This unusual enzyme is both a serine proteinase and an ATPase [4,5]. In fact the rate of protein degradation is directly proportional to the rate of ATP consumption [3–5]. Protein substrates stimulate ATP cleavage by protease La, but nonhydrolyzable proteins or small peptide substrates have no effect on this ATPase activity [4,5]. Previous studies [5] have shown that ATP hydrolysis is required for the degradation of proteins to acid-soluble products, but the hydrolysis of small peptides requires only the binding of a nucleotide to the enzyme. For example, nonhydrolyzable ATP analogues can support hydrolysis of oligopeptides but not protein degradation [5]. On this basis, a multistep model of protein degradation has been proposed in which ATP binding activates the enzyme for each round of proteolysis (Fig. 1) [5].

Since peptide bond cleavage in small peptides does not require ATP hydrolysis [5], the latter process must serve another important function in the degradation of large polypeptides. For example, ATP consumption may promote the release of the polypeptide products from the enzyme; alternatively, ATP hydrolysis may permit the translocation of the enzyme along the substrate to the next cleavage site [5]. One prediction of such mechanisms is that in the presence of a nonhydrolyzable ATP analogue, a single cleavage or only a limited number of cleavages should occur. Previous studies of the function of protease La have measured the generation of acid-soluble peptides and therefore would not have detected limited proteolysis that resulted in large acid-precipitable fragments.

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To test these possible roles of ATP hydrolysis in protein breakdown, we studied the degradation of [^{14}C]-β-casein in the presence of ATP and nonhydrolyzable ATP analogues, using SDS polyacrylamide electrophoresis to analyze reaction products.

MATERIALS AND METHODS

Protease La from *E coli* was purified to homogeneity as determined by SDS gel electrophoresis (see Fig. 2) as described previously [6]. Degradation of [^{14}C]-β-casein (1,000 cpm/μg) was carried out at 37°C in a medium containing 50 mM TRIS HCl pH 8.0, 10 mM Mg acetate, 1 mM nucleotide, 100 μg/ml β-casein, and 20 μg/ml protease La. At the times indicated, 50-μl aliquots were removed, and the reaction was terminated either by addition of SDS sample buffer [8] and boiling or by the addition of 10% TCA and 10% BSA (bovine serum albumin) [4]. SDS-PAGE was carried out in 15–20% gradient gels according to Laemmli [8] and stained with Coomassie blue. Loss of intact casein molecule was monitored using a Hoefer GS 3000 densitometer in conjunction with a Waters 740 Data Module integrator.

The following nonhydrolyzable analogues of ATP were used: α,β-methyleneadenosine 5'-triphosphate (AMPCPP), β,γ-methyleneadenosine 5'-triphosphate (AMPPCP), and adenyl-5'-yl imidophosphate (AMPPNP).

RESULTS

In the presence of ATP, the purified protease rapidly degrades casein to acid-soluble peptides without the appearance of large (> 5,000-D) protein fragments

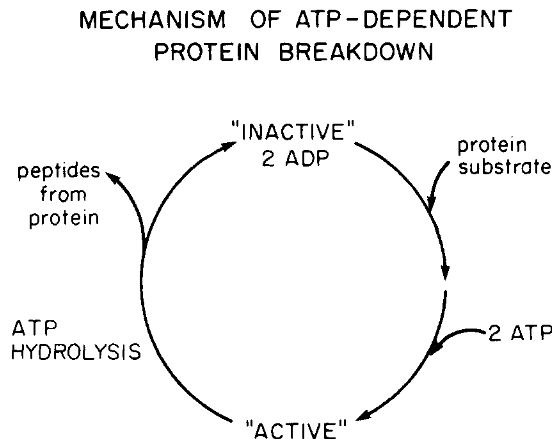

Fig. 1. This scheme is based on kinetic studies with fluorogenic peptide substrates of the ATP-dependent proteases from *Escherichia coli* by Waxman and Goldberg [4,5,12]. Its main features include 1) activation of peptide cleavage by ATP binding, which allows active site formation and peptide cleavage; 2) ATP hydrolysis, which is necessary for cleavage of large proteins (this ATPase step must inactivate the enzyme temporarily until additional ATP residues are bound); and 3) an ability of protein substrates to bind to a regulatory site, distinct from the proteolytic site, and thus to activate the enzyme further. Protein binding leads to greater ATP binding, peptide cleavage, and ATP hydrolysis. The degradation of proteins by this mechanism should involve repeated cycles until oligopeptides are generated.

(Figs. 2, 3A). In the absence of any nucleotide or in the presence of the nonhydrolyzable ATP analogue AMPPCP, very little degradation of casein occurs (Fig. 3A,B). Surprisingly, in the presence of a different nonhydrolyzable ATP analogue, AMPCPP, the disappearance of the intact casein molecules is as rapid as in the presence of ATP (Figs. 2, 3B). However, in this case, proteolysis results in the appearance of two large protein fragments (21 and 19 kD) and in much slower production of acid-soluble peptides than occurs with ATP (Fig. 3A). Previous studies (L. Waxman, unpublished observations) have confirmed that AMPCPP is not hydrolyzed by protease La. Therefore, this analogue supports limited cleavages of casein by protease La through some allosteric effect on the protease, which does not require high-energy phosphates. The 19- and 21-kD polypeptides were generated in much larger amounts than protease La; thus they are being produced catalytically by the enzyme in the presence of AMPCPP.

Another nonhydrolyzed analogue, AMPPNP, also supports the degradation of the casein molecule by protease La although not as well as either ATP or AMPCPP (Figs. 2, 3B). AMPPNP also allowed some production of the 21- and 19-kD protein fragments, although production of acid-soluble peptides was even slower than with AMPCPP.

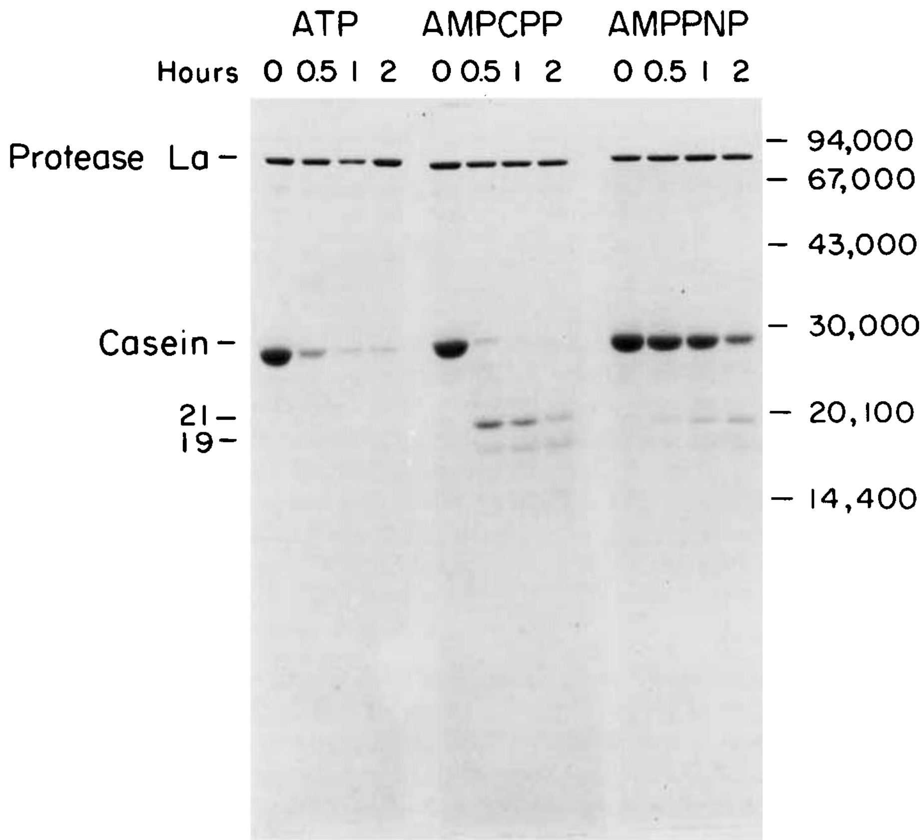

Fig. 2. SDS gels of β-casein degradation by protease La. One hundred microliters of a sample containing 2 μg of protease La and 5 μg of digested casein was loaded onto each lane.

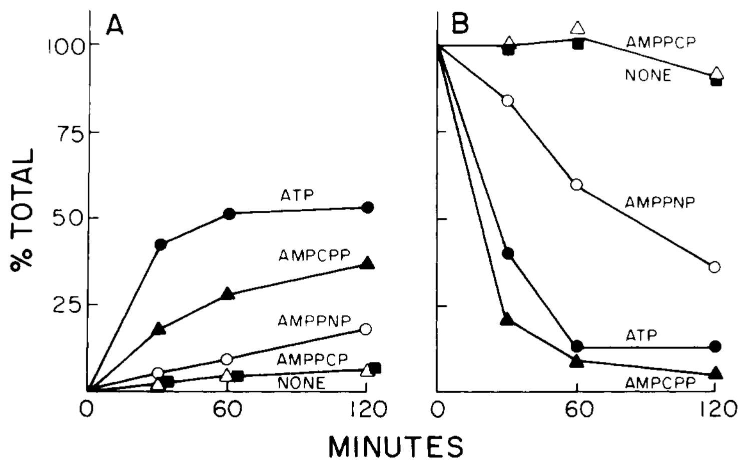

Fig. 3. Analysis of casein degradation products generated in the presence of ATP and nonmetabolizable ATP analogues. A) Appearance of acid-soluble peptides. B) Loss of intact casein from SDS gels. Results are presented as percent of casein initially present; 100% represents 5 μg of casein degraded.

TABLE I. Products of Casein Degradation With ATP and AMPPNP at Different Temperatures

	Casein degraded (μg/h)	TCA-soluble material produced (μg/h)	Polypeptides generated	
			21 kD	19 kD
ATP 37°C	4.24	3.00	–[a]	–
ATP 10°C	0.19	0.13	–	–
AMPPNP 37°C	2.42	0.19	+	+
AMPPNP 10°C	0.00	0.01	–	–

[a]None detected.

This lack of intermediate fragments with ATP was not due to the rapidity of proteolysis under these conditions. In additional experiments, the reaction temperature was lowered to 10°C in the presence of ATP in order that the production of acid-soluble peptides from the casein by protease La was as slow with ATP as with AMPPNP at 37°C (Table I). Nevertheless, with ATP, no large intermediates accumulated, and the amount of acid-soluble products accounted for the casein degraded. In other words, the mechanism of casein degradation was qualitatively different when ATP could be split (at 10° or 37°) from when AMPPNP was present.

DISCUSSION

These experiments demonstrate that ATP binding without nucleotide hydrolysis can support limited cleavage of peptide bonds in proteins by protease La. These findings thus extend recent observations with small peptides as substrates [9] and are consistent with the multistep mechanism (Fig. 1) proposed earlier [5,12]. The earlier conclusion [3,4] that ATP hydrolysis was required for degradation of large proteins was due to our assaying proteolytic activity by measuring the production of acid-soluble peptides (which would not detect the production of large proteolytic fragments) and our use of ATP analogues (AMPPCP and AMPPNP) that support protein degradation much more poorly than AMPCPP.

Although protein breakdown occurs with AMPCPP, the mechanism of protease La function appears quite different from that with ATP. Thus ATP hydrolysis is not required for peptide bond hydrolysis or for the initial cleavage of casein but is important for some other function of the enzyme. The lack of large polypeptide fragments during proteolysis with ATP is consistent with a processive mechanism in which each protein molecule is completely degraded before another substrate molecule can be attacked. Since large protein fragments are produced catalytically with the nonhydrolyzable analogues and are released from the enzyme, the energy obtained from ATP hydrolysis appears to be important for maintaining a processive mechanism. These observations can thus account for our prior failure and that of other laboratories to isolate polypeptide intermediates during protein degradation in vivo. Analogous mechanisms are found in the type I endonucleases from *E coli* [11]. However, this is the first time a processive mechanism has been reported for a protease, and this mechanism may explain the energy requirement for its function.

It is interesting that only two large polypeptides are produced in the presence of the nonhydrolyzable ATP analogues. Therefore the binding of protease La to the substrate must occur at very few sites (1 or 2, at most). We therefore propose that the enzyme initially recognizes only limited regions for attack. It then acts processively, provided the nucleotide can be hydrolyzed (Figs. 2, 3). Somehow the energy consumption allows rapid translocation of the protein along the substrate or simply prevents substrate release from the enzyme. In hydrolyzing proteins, protease La consumes about two ATP molecules for each peptide bond cleaved [10]. Although such a mechanism may appear to waste a great deal of metabolic energy unnecessarily [12], it could be highly advantageous to the cell and would allow the degradation of proteins in vivo without an accumulation of partially hydrolyzed proteins, which could be highly toxic to the cell.

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