Protein Substrates Activate the ATP-dependent Protease La by Promoting Nucleotide Binding and Release of Bound ADP*

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The interaction of protein substrates with protease La from Escherichia coli enhances its ability to hydrolyze ATP and peptide bonds. These studies were undertaken to clarify how unfolded proteins allosterically stimulate this ATPase activity. The tetrameric protease can bind four molecules of ATP, which activates proteolysis, or four molecules of ADP, which inhibits enzymatic activity. Protein substrates stimulate binding of the nonhydrolyzable ATP analog [3H]adenyl-5'-yl imidodiphosphate, although they do not increase the net binding of [3H]ATP or [3H]ADP. Once bound, ATP is quickly hydrolyzed to ADP, which remains noncovalently associated with protease La even through repeated gel filtrations. Exposure to protein substrates (e.g. denatured bovine serum albumin at 37 °C) induces the release of all the bound ADP from the enzyme. Nonhydrolyzable ATP analogs bound to the enzyme were not released by these substrates. Proteins that are not degraded (e.g. native bovine serum albumin) and oligopeptides that only bind to the catalytic site do not induce ADP release. Thus, polypeptide substrates have to interact with an allosteric site to induce this effect. The protein-induced ADP release is inhibited by high concentrations of Mg2+ and is highly temperature-dependent. Protein substrates promote [3H]ATP binding in the presence of ADP and Mg2+ (i.e. ATP-ADP exchange) and reduced the ability of ADP to inhibit the enzyme's peptidase and ATPase activities. These results indicate that: 1) ADP release is a rate-limiting step in protease La function; 2) bound ADP molecules inhibit protein and ATP hydrolysis in vivo; 3) denatured proteins interact with the enzyme's regulatory site and promote ADP release, ATP binding, and their own hydrolysis.

Protein breakdown in Escherichia coli, as in other cells, is a highly selective process that requires metabolic energy (1–3). Bacterial proteins with highly abnormal conformations, as may result from mutations, biosynthetic errors, or recombinant DNA manipulations, tend to be degraded much more rapidly than the bulk of cell proteins (1–3). Despite much progress recently in our knowledge about this process, the biochemical mechanisms that allow for the selective elimination of such abnormal proteins and that prevent excessive destruction of normal cell proteins are still not clear.

The energy requirement for protein degradation in E. coli results in large part from the involvement of protease La, the ion gene product. This enzyme requires ATP hydrolysis for proteolytic activity. During the degradation of various proteins, approximately two ATP molecules are hydrolyzed for each peptide bond cleaved (4). The precise function of ATP hydrolysis in the enzyme's catalytic mechanism is under intense study. Protease La preferentially degrades denatured proteins (1, 5–8) and certain short-lived normal proteins (9, 10). Transcription of this protease rises when cells produce large amounts of abnormal polypeptides, such as incomplete proteins or certain cloned foreign proteins (11, 12).

Studies with model peptides (13) indicate that this unusual protease interacts with protein substrates at two distinct sites: one is the active site, which preferentially hydrolyzes hydrophobic peptides (14), and the other is a regulatory site (13). Occupancy of this allosteric site by protein substrates increases both ATP hydrolysis (15) and proteolytic capacity (13). For example, protein substrates stimulate 2–4-fold the enzyme's ATPase activity (15) and its ability to hydrolyze model tetrapeptides. However, nondegraded proteins and small peptide substrates, which bind to the active site but not to the regulatory region, do not stimulate the enzyme's ATPase activity (13). This allosteric effect seems important in substrate recognition. Although many native proteins are not degraded and do not induce this activation, they do so after denaturation (13). This ability of denatured proteins to activate protease La, and the subsequent inactivation of this enzyme following ATP hydrolysis in vivo, may help prevent the inappropriate destruction of functional cell proteins (13). Thus, understanding the mechanism by which proteins activate ATP hydrolysis may help clarify the selectivity of intracellular proteolysis.

In the preceding paper (16), we showed that protease La binds four molecules of ATP, presumably one on each of its four identical subunits. However, these four sites were found to differ in their properties: two show low affinity, and two show high affinity for ATP (16). Nucleotide binding to one of the high affinity sites allows the hydrolysis of tetrapeptide substrates, while occupancy of the low affinity sites is necessary for maximal rates of protein hydrolysis (16). At each of these sites, ATP hydrolysis occurs rapidly after binding, and the resulting ADP remains tightly associated with the enzyme (16). In contrast to ATP, ADP is a potent inhibitor of proteolytic activity (13). Since the S0.5 of ADP binding to protease La (16) is far below its normal concentration in E. coli (0.1 mM), this inhibition by ADP probably occurs in vivo.

The present studies were undertaken to clarify the mechanism by which protein substrates stimulate ATPase activity and promote the conversion of an inactive enzyme to a more active form. We have examined whether polypeptide substrates promote nucleotide binding to protease La, inhibit ADP binding, or induce the release of ADP bound to the enzyme.
Activation of Protease La by Substrates

**MATERIALS AND METHODS**

Protease La was purified as described earlier (13). The sources of all reagents were described in the preceding paper (16). To denature bovine serum albumin (BSA) (Miles, Detroit, Mich.), this protein was reduced and carboxymethylated according to Hirs (17). Alkalitreated BSA was prepared by heating a solution of BSA (10 mg/ml) in 10 mM NaOH for 60 min at 60 °C. Globin and methylated casein were prepared as described previously (14).

The binding of ATP or ADP to protease La was studied by centrifugation of a ligand-enzyme mixture through Sephadex G-50, as described in our preceding paper (16). The Sephadex was equilibrated with buffer containing native BSA (1 mg/ml); the binding assay mixtures contained the same amount of BSA (16). The protein substrates were present in the reaction mixture at a final concentration of 0.1 mg/ml along with BSA. The ATPase and protelytic activities of protease La were assayed as described previously (12-14).

To study the release of bound ATP from protease La the enzyme-[1H]ATP complex was isolated by centrifugation through Sephadex G-50, as described in our preceding paper (16). The Sephadex was equilibrated with buffer containing native BSA (1 mg/ml); the binding assay mixtures contained the same amount of BSA (16). The protein substrates were present in the reaction mixture at a final concentration of 0.1 mg/ml along with BSA. The ATPase and protelytic activities of protease La were assayed as described previously (12-14).

**RESULTS**

Effect on ATP and ADP Binding—When protease La is incubated with Mg2+ (10 mM) and ATP (10 μM), the enzyme binds two molecules of the nucleotide, but it binds four molecules of ATP at 100 μM or higher concentrations. Addition of protein substrates, such as α-casein or denatured BSA, to the reaction had no effect on the amount of ATP bound to either the low or high affinity sites after a 60- or 120-min incubation (Table I, A). Casein also did not increase the number of ATP molecules bound under conditions (0.05 μM ATP) where only one site on the enzyme was occupied by ATP (data not shown (16)). In addition, protein substrates did not seem to increase the rate of ATP binding to either class of sites when measured with 10 or 100 μM ATP after 15 or 30 min (data not shown) when binding is still incomplete (16). Therefore, the stimulation of ATPase activity by protein substrates does not appear to be due simply to increased affinity of the enzyme for ATP, although the rapid hydrolysis of the bound ATP (16) may mask such effects.

In similar experiments, we also tested whether protein substrates might alter the binding of [1H]ADP, which normally has a higher affinity for this enzyme than ATP (16) (Table I). Neither casein nor denatured BSA had any effect on the binding of [1H]ADP whether this ligand was added at high or low concentrations. Also, at ADP concentrations (0.05 μM) where only one high affinity site was occupied, no inhibitory effect of these proteins was observed. Several other protein substrates had no effect on ADP binding to the enzyme either at 0 or at 37 °C (data not shown).

The Effect of Substrates on AMP-PNP Binding—Because the ATP bound to the protease is rapidly hydrolyzed to ADP, even at 0 °C, these experiments might not have detected an increase in the affinity of the enzyme for ATP. Since the ATP analog AMP-PNP is not hydrolyzed (5, 6, 15) and binds with high affinity to only one site on the enzyme (16), we tested whether protein substrates promoted [1H]AMP-PNP binding (Table II). In the presence of casein, 2 mol of AMP-PNP were bound at 10 μM, and 3 mol of AMP-PNP were bound at 100 μM. Thus, even at 0 °C, protein substrates increase the affinity of protease La for this nucleotide analog.

The Release of ADP from the Enzyme—When ATP bound to protease La is hydrolyzed, the ADP generated remains tightly bound to the enzyme. One possible mechanism for the protein-stimulated ATPase activity would be if ADP release were the rate-limiting step and if polypeptides enhanced release of ADP from the enzyme. To test this possibility, the enzyme was incubated with [1H]ADP and the enzyme-nucleotide complex was isolated by centrifugation through Sephadex G-50 columns and then incubated with a substrate (e.g. α-casein) or a nondegraded protein (native BSA) at 37 °C. Upon exposure to the casein, the bound ADP molecules were released (Table III, A). By contrast, in the presence of native BSA, the ADP remained bound to the enzyme even through repeated centrifugations through the Sephadex column. Thus, protein substrates must reduce dramatically the affinity of the enzyme for bound ADP.

Similar results were also obtained when the protease was incubated initially with [1H]ADP instead of [1H]ATP. Upon incubation of the enzyme-ADP complexes with α-casein at 37 °C, the bound ADP was rapidly released from the enzyme. Most of this ADP was released within 10 min of exposure to the substrate at 37 °C. By contrast, there was no dissociation of ADP upon incubation with native BSA (Fig. 1). These results also indicate that protein substrates can induce ADP release without being hydrolyzed themselves, since under these conditions (with only ADP present) breakdown of peptide bonds does not occur.

Protein substrates differ in their ability to cause the release of ADP from the protease (Table III). α-Casein, methylated

| Table I  |
| --- |
| Effects of protein substrates on binding of [1H]ATP to protease La |
| Protease La (4-5 μg) was incubated in the presence of [1H]ATP (10 μM) for 60 min or with 100 μM [1H]ATP for 120 min with Mg2+ (10 mM) or EDTA (10 mM). The enzyme was incubated with [1H]ADP (10 or 100 μM) for 120 min. The protein substrates were present at 0.1 mg/ml. Native BSA (1 mg/ml) was present in all assay mixtures, and the number of nucleotides bound was determined as described under "Materials and Methods." |
| Incubation with | ATP | ADP |
| --- | --- | --- |
| 10 μM | 100 μM | 10 μM | 100 μM |
| molecules/tetramer La |
| A. Binding in the presence of Mg2+ (10 mM) |
| Native BSA | 2.5 | 4.3 | 4.6 | 4.4 |
| α-Casein | 2.2 | 4.2 | 4.0 | 4.3 |
| Alkylated BSA | 2.1 | 4.3 | 3.6 | 4.6 |
| B. Binding in the presence of EDTA (10 mM) |
| Native BSA | 1.2 | 2.6 | 4.0 | 4.0 |
| α-Casein | 0.5 | 1.0 | 4.2 | 4.2 |
| Alkylated BSA | 0.3 | 1.2 | 4.6 | 4.2 |

| Table II  |
| --- |
| Casein promotes [1H]AMP-PNP binding to protease La at 0 °C |
| Protease La (3-4 μg) was incubated with 10 μM [1H]AMP-PNP for 60 min or with 100 μM [1H]AMP-PNP for 120 min at 0 °C with 10 mM Mg2+ in the presence and absence of α-casein, as described in Table I. The number of [1H]AMP-PNP molecules associated with the enzyme was then determined. Native BSA is not a substrate for the enzyme. |
| Incubation with | 10 μM | 100 μM |
| --- | --- | --- |
| AMP-PNP/tetramer La |
| Native BSA | 1.1 | 1.4 |
| α-Casein | 2.0 | 3.0 |

1 The abbreviations used are: BSA, bovine serum albumin; AMP-PNP, adenyl-5'-yl imidodiphosphate; Glt, glutaryl; MNA, methoxy-naphthylamine; MesSO, dimethyl sulfoxide.
Protein substrates induce the release of \([^3]H\)ADP but not \([^3]H\)AMP-PNP bound to protease La

Protease La (4-5 \(\mu\)g) was incubated with Mg\(^{2+}\) at 0 \(^\circ\)C for 60 min with 10 \(\mu\)M \([^3]H\)ATP or \([^3]H\)AMP-PNP and for 120 min with 100 \(\mu\)M \([^3]H\)ATP. At the end of the incubation period, the enzyme-nucleotide complex was isolated (16), and to this complex either Tris-HCl, pH 7.9 (0.05 M), or the protein substrates (0.1 mg/ml) were added. These mixtures were then incubated for 10 min at 37 \(^\circ\)C and centrifuged through Sephadex G-50 columns again. At each step, identical experiments were carried out using nonradioactive ATP to estimate the recovery of protease La. The relative sensitivities of different proteins to digestion is based on published data (13, 14).

After incubation at 37 \(^\circ\)C with

- Native BSA
- Alkali-denatured BSA
- Globin
- \(\alpha\)-Casein
- Carboxyanidemethylated BSA
- Methylated \(\alpha\)-S-casein

were then incubated for 10 min at 37 \(^\circ\)C and centrifuged through Sephadex G-50 columns again. At each step, identical experiments were carried out using nonradioactive ATP to estimate the recovery of protease La. The relative sensitivities of different proteins to digestion is based on published data (13, 14).

| Conditions | Susceptibility to protease La |
|------------|-----------------------------|
|            | 10 \(\mu\)M \* | 100 \(\mu\)M \* |
| A. Preincubated with \([^3]H\)ATP | 2.4 | 4.0 |
| When isolated | | |
| After incubation at 37 \(^\circ\)C with | | |
| Native BSA | None | 2.4 |
| Alkali-denatured BSA | + | 3.2 |
| Globin | + | 2.6 |
| \(\alpha\)-Casein | ++ | 0.5 |
| Carboxyanidemethylated BSA | ++ | 0.4 |
| Methylated \(\alpha\)-S-casein | ++ | 0.8 |
| B. Preincubated with \([^3]H\)AMP-PNP | 1.3 |
| When isolated | | |
| After incubation at 37 \(^\circ\)C with | | |
| Native BSA | None | 1.2 |
| Casein | ++ | 1.0 |

* Values represent molecules bound/tetramer La.

Fig. 1. \(\alpha\)-casein, at low concentrations, can induce the release of bound ADP from protease La at 37 \(^\circ\)C. The experiment was carried out as described under Table III using \([^3]H\)ADP (10 \(\mu\)M) as the ligand. After separation, the enzyme-nucleotide complex was incubated at 37 \(^\circ\)C for varying periods of time with or without \(\alpha\)-casein (5 \(\mu\)g/ml), and the amount of nucleotide remaining bound to the enzyme at each time point was determined.

Casein, and alkylated BSA are rapidly degraded by protease La (6, 13), and all three proteins induced the release of most of the bound ADP from the enzyme within 10 min. On the other hand, globin and alkali-denatured BSA, which are poorer substrates for protease La (6, 13), induced the release of only one (or at most two) of the bound ADP molecules in this period (Table III). Thus, the ability of protein substrates to cause ADP release correlated with their relative susceptibilities to the enzyme, as had been found for the protein-induced stimulation of ATPase and peptidase activities (13, 15). Moreover, casein and BSA caused ADP release at the same low concentrations (5 \(\mu\)g/ml) at which they allosterically activated peptide and ATP hydrolysis (13).

In addition to the catalytic site where peptide bonds are hydrolyzed, protease La has an allosteric site whose occupancy by protein substrates (13) leads to an increased capacity for peptide bond and ATP hydrolysis. We tested whether substrate-induced release of ADP involves an interaction with this allosteric site or with the active site. Unlike casein, tetrapeptide substrates, like Glt-Ala-Ala-Phe-MNA, bind only to the catalytic site and not to the allosteric site (13). When Glt-Ala-Ala-Phe-MNA was added to the enzyme-[^3]H ADP complex, this peptide did not cause ADP release (Table IV). Thus, those factors that induce dissociation of ADP are identical to those that allosterically activate peptide and ATP hydrolysis (13, 15).

In this experiment, the tetrapeptide substrate had to be dissolved in MeSO before being added to the reaction mixture (13). This solvent by itself can stimulate the ATPase activity of protease La by as much as 50% (15). Since MeSO did not cause the release of any of the bound ADP (Table IV), the stimulation of ATPase activity by this solvent involves a different mechanism from the stimulation by protein substrates.

Although the binding of ATP, ADP, and AMP-PNP and even the ATP hydrolysis can occur rapidly at 0 \(^\circ\)C (16), the protein-induced ADP release was highly temperature-dependent (Table IV). This effect was clearly observed at 37 \(^\circ\)C. By contrast, at 0 \(^\circ\)C, the addition of protein substrates caused almost no release of ADP (Table IV), even though these polypeptides could interact with the enzyme and could enhance AMP-PNP binding to this low temperature (0 \(^\circ\)C) (Table II). The sharp temperature dependence of the protein-induced ADP release reaction (Table IV, Fig. 1) indicates that this process has a high activation energy and that the ATPase reaction must be limited by the rate of dissociation of the ADP produced and not by ATP binding or hydrolysis.

Protein substrates thus seem to activate protease La in part by promoting the release of ADP, which is both a product of the enzyme and a strong inhibitor of its function (15). However, this effect can only be functionally important if proteins do not also induce release of undegraded nucleotides. To test this possibility, we studied the effects of casein on AMP-PNP release (Table III, B). In contrast to the findings with ADP, protein substrates did not cause any dissociation of the bound AMP-PNP. Thus, substrate-induced release of the bound nucleotide requires that it be hydrolyzed and perhaps does not occur if the enzyme is in the activated (nucleotide-bound) state (14, 15).

**Table IV**

The influence of temperature on the ability of proteins and peptide substrates to induce[^3]HADP release

| Conditions | ADP/tetramer La |
|------------|----------------|
| Preincubated at 0 \(^\circ\)C with 50 \(\mu\)M \([^3]H\)ADP | 4.3 |
| Then incubated for 10 min with | |
| Native BSA | 4.4 |
| Alkali-denatured BSA | 4.1 |
| Globin | 4.0 |
| \(\alpha\)-Casein | 3.6 |
| Methylated \(\alpha\)-S-casein | 4.0 |
| Glt-Ala-Ala-Phe-MNA (500 \(\mu\)M) | 4.8 |
| MeSO | 4.2 |

* Values represent molecules bound/tetramer La.
Influence of Mg$^{2+}$ and EDTA on Substrate-induced Effects—Maximal ATP binding to protease La requires only 20–30 $\mu$M Mg$^{2+}$ (16), but maximal ATPase and proteolytic activity require 5–10 mM of this ion (14). Therefore, in addition to facilitating nucleotide binding, Mg$^{2+}$ at some low affinity site must play a role in catalysis. We, therefore, compared the release of bound ADP (Table V) either at low (10 or 100 $\mu$M) or at a high (1 mM) Mg$^{2+}$ concentration, which allows near maximal proteolysis (15). The preceding experiments on ADP release (Tables III and IV, Fig. 1) were all performed in the absence of added Mg$^{2+}$, and a similar substrate-induced release was observed when casein was added in the presence of EDTA (Table V). However, when Mg$^{2+}$ was present at 1 mM, it blocked protein-stimulated ADP release. At lower concentrations of Mg$^{2+}$ (<50 $\mu$M), there was no such inhibitory effect (Table V). Thus, although some Mg$^{2+}$ appears necessary for catalysis (14), the continued presence of this ion may retard or even prevent substrate-induced ADP release. Since proteins activate the ATPase in the presence of Mg$^{2+}$, substrate-induced ADP release may be linked to another event, such as the binding of new ATP molecules (see below).

In the absence of EDTA, protease La binds half as many ATP molecules as with Mg$^{2+}$ present (16). With EDTA present, addition of $\alpha$-casein decreased net $[^{3}H]$ATP binding by 70–80% at both 10 and 100 $\mu$M (Table I, B), and alkylated BSA, another substrate of protease La, decreased $[^{3}H]$ATP binding by 50–60%. Proteins that were not substrates did not have any effect under these same conditions (Table I). Clearly, protein substrates can interact with the allosteric site and induce alterations in structure of protease La in the absence of Mg$^{2+}$. However, these findings differ sharply from those obtained in the presence of Mg$^{2+}$, where protein substrates either had no effect on $[^{3}H]$ATP binding (Table I) or enhanced the binding of nonmetabolized analogs (Table II). It is unclear why protein substrates have different allosteric effects in the presence of EDTA or Mg$^{2+}$.

Protein Substrates and the Competition between ATP and ADP—ADP has a higher affinity for protease La than ATP (16) and saturates all four sites even at 10 $\mu$M concentration. Additional experiments tested whether protein substrates, by reducing its affinity for ADP, enhance ATP binding and promote enzyme activation. When present with 100 $\mu$M [H]$ATP$ and 10 mM Mg$^{2+}$, 10 $\mu$M ADP prevents ATP binding to at least two sites on the enzyme at 0°C (Table VI). Addition of $\alpha$-casein reduced the ability of ADP to inhibit ATP binding. For example, at 0°C, protein substrates increased the number of $[^{3}H]$ATP bound from 2.00 to 3.00. This ability of proteins to block the inhibitory effect of ADP could be overcome if the ADP concentration was increased 5-fold.

Since these effects of proteins were clear but small at 0°C (Table IV), a similar experiment was carried out at higher temperatures. At both 23 and 37°C, more dramatic effects of

| Table V |
|-----------------|-----------------|
| **Mg$^{2+}$ inhibits the casein-induced release of $[^{3}H]$ADP from protease La** |
| The experiment was carried out as described under Table III. The final concentration of EDTA was 10 mM and of $\alpha$-casein was 0.1 mg/ml. |
| **Treatment** | **ADP/trimer La** |
| Preincubation at 0°C with 50 $\mu$M $[^{3}H]$ADP | 3.8 |
| Then incubated at 37°C with EDTA | 4.2 |
| $\alpha$-Casein + EDTA | 0.6 |
| $\alpha$-Casein + 10 mM Mg$^{2+}$ | 0.9 |
| $\alpha$-Casein + 100 mM Mg$^{2+}$ | 1.4 |
| $\alpha$-Casein + 1000 $\mu$M Mg$^{2+}$ | 3.5 |

Protease La (7–8 $\mu$g) was incubated at 0°C with 100 $\mu$M [H]$ATP$ at 23 and 37°C with 50 $\mu$M [H]$ATP$ and Mg$^{2+}$ (10 mM). The reaction mixtures were incubated for 120 min at 0 and 23°C and for 10 min at 37°C. Casein (0.1 mg/ml) and ADP (10 $\mu$M at 0°C and 50 $\mu$M at 23 and 37°C) were added as indicated.

| Table VI |
| **Protein substrates can block the inhibition by ADP of $[^{3}H]$ATP binding** |
| Protease La (2–3 $\mu$g) was incubated with 10 mM ADP for 60 min on ice. Then ATP was added to the tubes at 1 mM (final concentration) for the protease assay or 0.5 mM for the ATPase assay. Casein was added at a final concentration of 0.1 mg/ml. The protease and ATPase assays were carried out as described by Waxman and Goldberg (6). |
| **Temperature** | **Addition to assay** |
| **C** | **[^{3}H]ATP/trimer La** |
| 0 | 0.7 | 2.1 | 3.0 |
| 23 | 4.5 | 4.3 | 1.5 |
| 37 | 4.2 | 0.9 | 4.2 |

Protease La (5–6 $\mu$g) was incubated with Mg$^{2+}$ (10 mM) and either ADP (0.05 or 0.5 mM), ATP (1 mM), or both, together with the fluorogenic substrate Gly-Ala-Ala-Phe-MNA (500 $\mu$M) for 60 min at 37°C in the presence and absence of casein (0.1 mg/ml). The fluorescence generated was estimated as described previously (13).

| Table VII |
| **The effect of ADP on the proteolytic and ATPase activities of protease La** |
| Protease La (5–6 $\mu$g) was incubated with Mg$^{2+}$ (10 mM) and either ADP (0.05 or 0.5 mM), ATP (1 mM), or both, together with the fluorogenic substrate Gly-Ala-Ala-Phe-MNA (500 $\mu$M) for 60 min at 37°C. Casein was added in the presence of 0.1 mg/ml. The protease and ATPase activities were assayed as described by Waxman and Goldberg (6). |
| **Addition** | **Protein activity** | **Inhibition by ADP** | **ATPase activity** | **Inhibition by ADP** |
| **$[^{3}H]$casein degraded/h** | **%** | **nmol P$_{i}$ released/h** | **%** |
| ATP | 3.00 | 0 | 9 |
| ATP + ADP | 2.40 | 20 | 4 | 57 |
| ATP + casein | 25 |
| ATP + casein + ADP | 21 | 16 |

**Table VIII**

Casein reduces the inhibition by ADP of ATP-dependent peptide hydrolysis by protease La

Protease La (5–6 $\mu$g) was incubated with Mg$^{2+}$ (10 mM) and either ADP (0.05 or 0.5 mM), ATP (1 mM), or both, together with the fluorogenic substrate Gly-Ala-Ala-Phe-MNA (500 $\mu$M) for 60 min at 37°C in the presence and absence of casein (0.1 mg/ml). The fluorescence generated was estimated as described previously (13).

| **Addition** | **Fluorescence** | **Inhibition** | **Fluorescence** |
| **%** | **units/h** |
| None | 3.0 |
| ADP (0.05 mM) | 0.1 | 80 | 0.2 |
| ADP (0.5 mM) | 0.0 | 100 | 0.1 |
| ATP | 5.1 | 0 | 7.0 |
| ATP + ADP (0.05 mM) | 4.6 | 15 | 7.0 |
| ATP + ADP (0.5 mM) | 2.3 | 55 | 7.0 |

**Table IX**

*Casein reduces the inhibition by ADP of ATP-dependent peptide hydrolysis by protease La*

Protease La (5–6 $\mu$g) was incubated with Mg$^{2+}$ (10 mM) and either ADP (0.05 or 0.5 mM), ATP (1 mM), or both, together with the fluorogenic substrate Gly-Ala-Ala-Phe-MNA (500 $\mu$M) for 60 min at 37°C. Casein was added in the presence of 0.1 mg/ml. The protease and ATPase activities were assayed as described by Waxman and Goldberg (6).
release (Table V). Apparently, the reaction involving exchange of bound ADP with a free ATP molecule differs from the simple dissociation of bound ADPs in its sensitivity to Mg²⁺.

**Influence of Protein Substrates and ADP on Enzyme Activity**—ADP is a potent inhibitor of the proteolytic, peptidase, and ATPase activities of protease La (14, 15). Because of its high affinity for protease La (18, 19) and because it is present in all cells at about 10⁻⁵ M (18, 19), ADP probably serves as an inhibitor of protease La in vivo (16). We, therefore, tested if protein substrates reduce the sensitivity of the enzyme to ADP. In the presence of 10 μM ADP, the basal ATPase activity of protease La is inhibited by about 60%, but with casein present, ATPase activity increases and is much less sensitive to this level of ADP (Table VII), presumably because the protein substrate promotes the dissociation of bound ADP (Fig. 1). In the presence of 1 mM ATP, 0.5 mM ADP inhibits by 55% the peptidase activity (Table VIII). The addition of casein to the reaction mixture stimulates the peptidase activity and prevents almost completely its inhibition by ADP.

Because enzyme activities were assayed in these experiments (Table VIII), they were carried out in the presence of 10 mM Mg²⁺. It is interesting that even the low ATP-independent activity (13, 14) Table VIII) was prevented by low concentrations (50 μM) of ADP ((14) Table VIII) and was stimulated severalfold by protein substrates ((13) Table VIII). The inhibition of this ATP-independent activity by ADP was not reversed by protein substrates (Table VIII), perhaps due to the high Mg²⁺ levels which block protein-induced ADP release (Table V). However, when ATP was also present, the protein substrate blocked the inhibition by ADP of peptidase (Table VIII) and ATPase activities (Table VIII), as expected from its ability to promote ADP-ATP exchange (Table VI) in the presence of Mg²⁺.

**DISCUSSION**

Although protease La by itself has a higher affinity for ADP than for ATP (16), upon interaction with a denatured protein, it releases the ADP readily. In effect, the protein substrate induces an exchange of bound ADP for ATP (Table VII). These findings demonstrate a new mechanism regulating intracellular proteolysis. Because the intracellular concentrations of ADP (about 100 μM) (18, 19) are severalfold greater than the S₀₅ for ADP (16), the enzyme probably exists in vivo associated with four ADP moieties such that its ATPase and proteolytic activities are markedly inhibited (Tables VI and VIII (13, 14)). Thus, ADP seems to be a physiological regulator of protease La, which helps prevent excessive ATP consumption and proteolysis. By such a mechanism abnormal cell proteins can activate protease La and promote their own degradation.

In large part, this ATP-ADP exchange is due to a reduction in the affinity for bound ADP, but other allosteric changes occur as well. Protein substrates increase 3-fold the number of AMP-PNP molecules associated with the protease (Table III, B). It is, therefore, surprising that these substrates were not found to enhance significantly ATP binding to the enzyme (Table I). This negative result is consistent with the observation that proteins stimulate the enzyme’s ATPase activity by increasing its Vₘₐₓ (14) and not by reducing its Kₘ for ATP.

The stimulation of ADP release seems to be the critical factor underlying the enhancement by proteins of ATPase activity. Unlike ATP binding and the hydrolysis of the enzyme-bound nucleotide (which occur even at 0 °C), the ADP release induced by proteins is sharply temperature-dependent (Tables IV and VI), as is the protein-stimulated ATPase activity. Therefore, there must be a large activation energy for ADP release, and this step must be the rate-limiting one in the ATPase reaction. Another observation supporting this conclusion is that at low temperatures protein substrates promote AMP-PNP binding, but not ADP release. Thus, substrates can interact with the enzyme at low temperatures and induce some conformational changes; however, only at higher temperatures do additional allosteric effects occur that lead to ADP release and enhanced ATPase activity. The activation of the ATPase by proteins thus results from two effects: 1) the release of ADP is stimulated, and consequently, 2) the binding of ATP is facilitated. Although we have studied these two processes as distinct reactions (except in Table IV), they appear coupled. For example, substrates promote ATP-ADP exchange even in the presence of high concentrations of Mg²⁺ (10 mM) which prevents protein-induced release of ADP, when measured as an isolated process (Table IV).

In inducing the release of ADP from the enzyme (Table III, Fig. 1), protein substrates must be interacting with an inactive form of the protease. With the nonhydrolyzed analog AMP-PNP bound, protease La remains continually active against peptides, and under these conditions, protein substrates do not release the bound AMP-PNP molecules. Somehow ADP release is induced only by proteins that are substrates and not by proteins that are not degraded (Table III). However, hydrolysis of the polypeptide is not required for this effect; for example, it occurs in the presence of ADP when the enzyme is inactive (Fig. 1). Also, tetrapeptide substrates which bind only to the active site do not induce ADP dissociation (Table IV). This inability of peptide substrates to release ADP from the enzyme may explain why their hydrolysis is particularly sensitive to inhibition by ADP (Table VIII).

Together, these observations indicate that the unfolded proteins induce ADP release by interacting with the allosteric site. The resulting conformational changes that cause ATP-ADP exchange presumably are the same ones that lead to the enhancement of peptidase activity (13). Both effects contribute to enzyme activation, and though coupled, have been studied as separate effects (13). The release of ADP seems to be an early step that follows binding of an appropriate substrate and must precede maximal peptide bond hydrolysis.

**Proposed Mechanism of Protease La**—These observations

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2 L. Waxman and A. L. Goldberg, unpublished observations.
and related ones (4, 13–16) provide a more complete understanding of the activation-inactivation cycle underlying ATP-dependent proteolysis (Fig. 2). 1) The initial interaction of the inactive protease with an appropriate substrate leads to the release of the four bound ADP molecules. Concomitantly, four molecules of ATP-Mg become associated with it, and the enzyme is maximally activated (as judged with oligopeptide substrates (13)). 2) For each peptide bond cleaved, two ATP molecules are utilized (4). The P, groups produced are released (16), as are the ADP moieties, provided the allosteric regions remain occupied by part of the polypeptide substrate. 3) With the binding of new ATPs this cycle would be repeated. 4) Once the protein is completely hydrolyzed to oligopeptides, the enzyme should return to an inactive state with ADP tightly bound to it and remain in that state until a new substrate molecule interacts with the protease. Such a mechanism would help ensure that this protease is activated only in the presence of a substrate and thus should help prevent the accidental degradation of essential cellular proteins. Even if the enzyme binds ATP occasionally in the absence of a substrate, the nucleotide would be immediately hydrolyzed to ADP (16) and the enzyme would again become inactive.

In certain respects, this substrate-induced activation mechanism appears analogous to that of mammalian tissue plasminogen activator, which exists in circulation but becomes maintained in an inactive form by receptor occupancy (31). Several important nonproteolytic enzymes contain substrate-stimulated ATPase activity, including rec A (26), rho (27), the clathrin-uncoating ATPase (28), and certain restriction endonucleases (29). Similar mechanisms to that in Fig. 2 may account for their activation. If these ATPases also bind ADP very strongly, they may thus be maintained in an inactive form in vivo, and their substrates may activate them by inducing allosterically the release of the bound ADP.

Acknowledgments—We are grateful to Drs. Lloyd Waxman and David Chin for helpful discussions, to Renee Solomon for help in purifying the enzyme, and to Aurora Scott and Nancy Fischbein for assistance in preparing this manuscript.

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