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# Activation of the 20S Proteasome of *Xenopus* Oocytes by SDS: Evidence for the Substrate-Induced Conformational Change Characteristic of Trypsin-Like Peptidase

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**ABSTRACT**—The 20S proteasome of eukaryotic cells has at least three distinct peptidase activities (trypsin-like, chymotrypsin-like and peptidylglutamylpeptide (PGP) hydrolase activities). These peptidases are latent and require appropriate activators. SDS has been widely used as an activator of these peptidases, but the mechanism of its activation remains unresolved. In this study, we investigated the kinetics of the SDS-activated hydrolysis of the above three types of peptidase of the 20S proteasome purified from *Xenopus* oocytes. When the reaction was started by simultaneous adding both SDS and substrate, maximal rates of hydrolysis were reached after appreciable lag phases with the trypsin-type substrate [*t*-butyloxycarbonyl-Leu-Arg-Arg-4-methylcoumaryl-7-amide (Boc-LRR-MCA)], but no such lag phases were observed with the chymotrypsin-type and PGP hydrolase-type substrates [succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA), and benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide (Cbz-LLE-2NA), respectively]. Similarly, changes in the hydrolysis rate to a reduced level upon dilution of SDS occurred after an appreciable lag phase again in the trypsin-like peptidase, but not in the other types. The lag phase characteristic of the trypsin-like peptidase was dependent on the substrate concentration. Thus, the lag phase was less discernible at very low concentrations of the substrate (e.g. at concentrations in the order of 1/100 of the *K<sub>m</sub>* value), but became more conspicuous with the increases in the substrate concentration. This lag phase also vanished upon preincubation of the activator (SDS) for a short period of 5 sec. These results suggest that the formation of the enzyme-substrate complex in the trypsin-like reaction induces a conformational change in the enzyme which makes the SDS activator site(s) in an occluded form, reducing the rates of SDS binding and dissociation.

## INTRODUCTION

The proteasome is an intracellular multicatalytic proteinase that is distributed in all eukaryotic and prokaryotic cells. In eukaryotic cells, degradation of most intracellular proteins are catalyzed by the enzyme that accounts for up to 1% of the total cell proteins (Coux *et al.*, 1996; Baumeister *et al.*, 1997; Goldberg *et al.*, 1997). The enzyme (26S proteasome, 2000 kDa) which degrades ubiquitinated proteins in an ATP-dependent manner, is constructed by a 20S proteasome (700 kDa) that has several types of peptidase activity and two 19S regulatory complexes that perform several types of ATPase (Hilt *et al.*, 1996; Baumeister *et al.*, 1997). The peptidase activities are located in the 20S proteasome that takes the form of a cylindrical particle, composed of 28 subunits arranged in a

stack of four rings (Coux *et al.*, 1996; Baumeister *et al.*, 1997; Goldberg *et al.*, 1997). The 20S proteasome of eukaryotes has at least three distinct endopeptidase activities involved in cleaving bonds on the carboxyl side of basic (trypsin-like peptidase), hydrophobic (chymotrypsin-like peptidase) and acidic (PGP hydrolase) amino acid residues (Hershko and Ciechanover, 1992; Rivett, 1993). In addition to these three types, there appear to be several other types of protein-degrading and peptidase activities (Djaballah and Rivett, 1992; Orlowski *et al.*, 1993). The peptidase activities of the isolated 20S proteasome are extremely low without activators. It is postulated that the enzyme is regulated *in situ* by subunit-exchange, the 19S regulatory complex and PA28 activator, etc. (Coux *et al.*, 1996; Goldberg *et al.*, 1997). However, it is virtually impossible to perform kinetic studies of the enzyme activation by these putative regulators at the moment. Therefore, several exogenous activators, such as SDS, fatty acids, cardiolipin and polylysine have been used to obtain appre-

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ciable levels of peptidase activity. Among these, SDS has been widely used as the activator, but the reported results are contradictory: e.g., the activation by SDS is reversible (Tanaka *et al.*, 1989; Arribas and Castaño, 1990) or irreversible (Dahlmann *et al.*, 1993).

The aim of the present study is to elucidate the mechanisms by which the three types of peptidase activity of the 20S proteasome (trypsin-like, chymotrypsin-like, and PGP hydrolase) are activated by SDS. In this study, the activation of three peptidase activities by SDS were examined in detail by following the time-courses of hydrolysis. Here, we report that both acceleration and deceleration of the hydrolysis by SDS binding and dissociation occur after an appreciable lag phase in trypsin-like peptidase, but not in the chymotrypsin-like peptidase and PGP hydrolase. The lag phase increased with the increase of the substrate concentration. We propose that the trypsin-like peptidase reaction is mediated by the substrate-induced conformational change of the enzyme.

## MATERIALS AND METHODS

### Materials

Cbz-LLE-2NA and 2-naphthylamide (2NA) were purchased from Sigma (St. Louis, MO, USA). Suc-LLVY-MCA, Boc-LRR-MCA, *t*-butyloxycarbonyl-Gln-Ala-Arg-4-methylcoumaryl-7-amide (Boc-QAR-MCA), *t*-butyloxycarbonyl-Phe-Ser-Arg-4-methylcoumaryl-7-amide (Boc-FSR-MCA), and 7-amino-4-methylcoumarin (AMC) were purchased from Peptide Institute (Osaka, Japan). These fluorogenic peptides were dissolved in dimethylsulfoxide (DMSO) and used. Sepharose CL-6B, phenyl-Sepharose CL-4B and Mono Q were from Pharmacia (Uppsala, Sweden), DEAE-cellulose (DE52-cellulose) was from Whatman (Maidstone, UK) and hydroxylapatite was from Wako Pure Chemical (Osaka, Japan). All other reagents used were of analytical grade.

### Purification of 20S proteasome

The 20S proteasome was prepared from *Xenopus* oocytes as described previously with slight modifications (Yamada *et al.*, 1998). The ovaries of adult *Xenopus* (10-15) were washed three times with 200 ml of 20 mM Tris-HCl (pH 7.5), 20% glycerol, 10 mM 2-mercaptoethanol and 1 mM EDTA (TGME buffer), and then centrifuged in minimum volumes of the TGME buffer (100,000 × g, 50 min) (Tokumoto and Ishikawa, 1993). The supernatant was centrifuged again at 20,000 × g for 30 min to remove lipid materials and the resultant clear supernatant (~5 ml) was applied to a Sepharose CL-6B column (2.7 × 98 cm) in TGME buffer. The fractions with Suc-LLVY-MCA-hydrolyzing activity (active fractions) were pooled and applied again to a DE52-cellulose column (1.6 × 10 cm) in TGME buffer containing 100 mM KCl. The column was washed with the same buffer and then eluted with a linear gradient of 100-300 mM KCl in TGME buffer. The active fractions were collected, diluted to 100 mM KCl by adding TGME buffer and applied to a second DE52-cellulose column (1.2 × 4 cm). This column was washed with TGME buffer containing 100 mM KCl, and eluted with a linear gradient of 100-300 mM KCl in TGME buffer. The active fractions were pooled, diluted and concentrated through a Mono Q column (HR 5/5) equilibrated with TGME buffer. The Mono Q column was washed with TGME buffer containing 100 mM KCl, and eluted with a linear gradient of 100-300 mM KCl in TGME buffer. The active fractions were dialyzed against 5 mM potassium phosphate (pH 7.6) containing 20% glycerol, 10 mM 2-mercaptoethanol and 1 mM EDTA. The dialysate was applied to a column (1.6 × 10 cm) of hydroxylapatite equilibrated with the same buffer, and elution was conducted with a linear gradient of potassium

phosphate (pH 7.6) (5-350 mM, 300 ml). The active fractions were dialyzed against TGME buffer, and the dialysate was applied again to a Mono Q column (HR 5/5) equilibrated with TGME buffer and eluted with a linear gradient of KCl in TGME buffer (0-350 mM KCl, 40 ml). Several mg of 20S proteasome were purified in these procedures. To examine the purity and the subunit composition of the 20S proteasome, the active fraction was subjected to non-denaturing- and SDS-PAGEs (Laemmli, 1970; Yamada *et al.*, 1995). The sample was stored at -85°C and used within 6 months.

### Enzyme assays

The activity of various peptidases was determined as described previously with slight modifications (Yamada *et al.*, 1998). Reactions were carried out at 37°C in 50 mM Tris-HCl buffer (pH 8.5) containing 1.5 or 2.9 µg/ml 20S proteasome, with or without 0.06% SDS, 0.2-500 µM peptidyl substrate, 10% DMSO and 1 mM EDTA. The reaction of peptidyl substrate hydrolysis was started by adding peptidyl substrate which was dissolved in DMSO. The SDS resolved in reaction solution was added to the enzyme solution before, concomitant with or after the starting of the peptidase reaction which was carried out in reaction solution containing Tris-HCl buffer (pH 8.5), 20S proteasome, peptidyl substrate, DMSO and EDTA (total volumes of 0.2-5.0 ml). At appropriate times, 0.1 or 0.2 ml of the reaction solution was mixed with an equal volume of 1% SDS solution to stop the reaction and the mixture was diluted to 2.2 ml with 100 mM Tris-HCl buffer (pH 9.0). Then, the AMC or 2NA liberated during the reaction was measured fluorometrically (380 nm excitation/460 nm emission for AMC and 335 nm excitation/410 nm emission for 2NA) with a fluorescence spectrophotometer (F-3000, Hitachi, Japan). The activity of peptidyl substrate hydrolysis was presented as nmol of substrate hydrolyzed per µg protein of 20S proteasome (Yamada *et al.*, 1995).

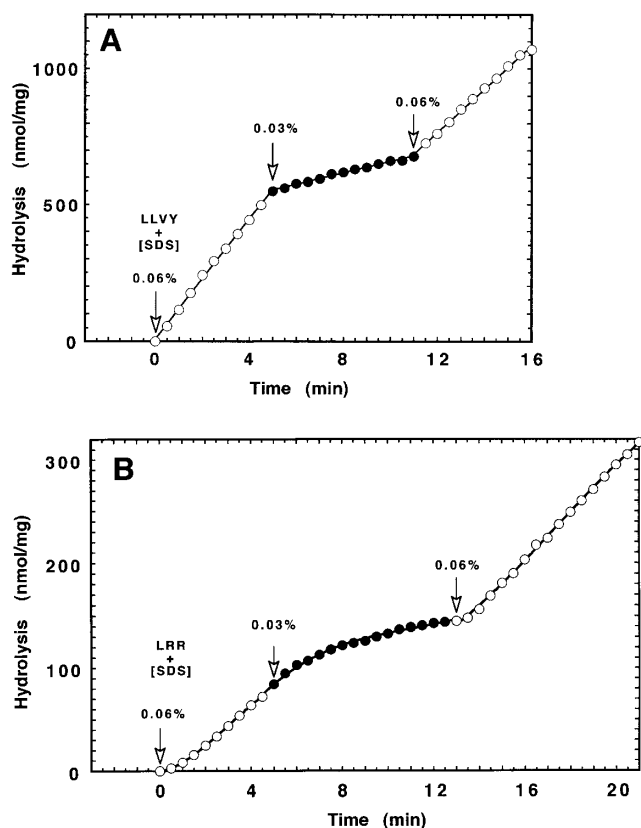
### Protein concentration

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The concentration of the 20S proteasome of *Xenopus* oocytes was calculated from the absorbance at 280 nm, assuming  $E_{1\text{cm}}^{1\%}$  value of 12.3 (Tanaka *et al.*, 1988).

## RESULTS

SDS has been widely used as a common activator of various peptidases of the 20S proteasome (Tanaka *et al.*, 1989; Kinoshita *et al.*, 1990; Pereira *et al.*, 1992; Ozaki *et al.*, 1992), but little is known about the mechanism of its action. In the present study, we used SDS as the activator of three types of peptidase (trypsin-like, chymotrypsin-like, and PGP hydrolase) using the fluorogenic substrate peptides specific to each of these peptidases. The concentration of SDS used in this study, 0.06%, was chosen since it produced about maximal activation of all of these peptidases under the conditions used (Yamada *et al.*, 1995; Watanabe and Yamada, 1996).

In the experiment shown in Fig. 1 peptidase reactions were started by adding Suc-LLVY-MCA (Fig. 1A) and Boc-LRR-MCA (Fig. 1B) and SDS at the same time to the enzyme solution, and the peptide hydrolysis was followed for 5 min (1st phase). Then, the SDS concentration was reduced to 0.03% to examine the effects of dilution on the hydrolysis rate (2nd phase), and the SDS concentration was increased back to 0.06% to follow the subsequent reaction (3rd phase). As shown in Fig. 1A, chymotrypsin-like hydrolysis proceeded at a constant rate immediately after the start of the reaction. Upon



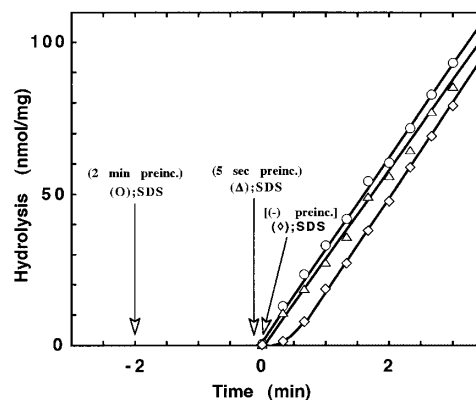
**Fig. 1.** Characteristic appearance of the lag phases in the SDS-activated hydrolysis of the trypsin-type substrate. Reactions were carried out initially in a total volume of 5.0 ml containing 50 mM Tris-HCl, pH 8.5, 2.9  $\mu\text{g/ml}$  20S proteasome, 0.06% SDS, 100  $\mu\text{M}$  Suc-LLVY-MCA (LLVY; **A**), or 10  $\mu\text{M}$  Boc-LRR-MCA (LRR; **B**), 1 mM EDTA and 10% DMSO. Reactions were started by adding Suc-LLVY-MCA (**A**) or Boc-LRR-MCA (**B**) and SDS solutions which were containing other components of the reaction solution to the enzyme solution. At 5 min after the start of the reaction, an equal volume of the same reaction solution without SDS and the enzyme was added. Furthermore, at 11 min (**A**) or 13 min (**B**) after starting the reaction, a small volume of the same reaction solution but with a higher concentration of SDS and no enzyme was added to adjust the SDS concentration back to 0.06%. Reactions were stopped by adding 100  $\mu\text{l}$  of the reaction solutions to 100  $\mu\text{l}$  of 1% SDS solution at various times. Similar results were obtained in individual assays in 10 or more separate experiments.

dilution of SDS in the 2nd phase, the hydrolysis rate was reduced instantly to a new rate, and the increase of SDS to the original concentration (0.06%) in the 3rd phase resulted in an instant increase to a new rate, which was approximately identical to the rate in the 1st phase. Essentially the same result was obtained with PGP hydrolase-type substrate Cbz-LLE-2NA (data are not shown to avoid redundancy).

In contrast to the two types of peptidases, if Boc-LRR-MCA was used as the substrate there was an appreciable lag period at each phase of the SDS modulated reactions described above (Fig. 1B). Interestingly, the enzyme activation by 0.06% SDS that was added together with the substrate took place after a lag period of about 30 to 40 sec as determined by extrapolation of the linear portion of the time-course to the abscissa. Alternatively, dilution of SDS (2nd phase) pro-

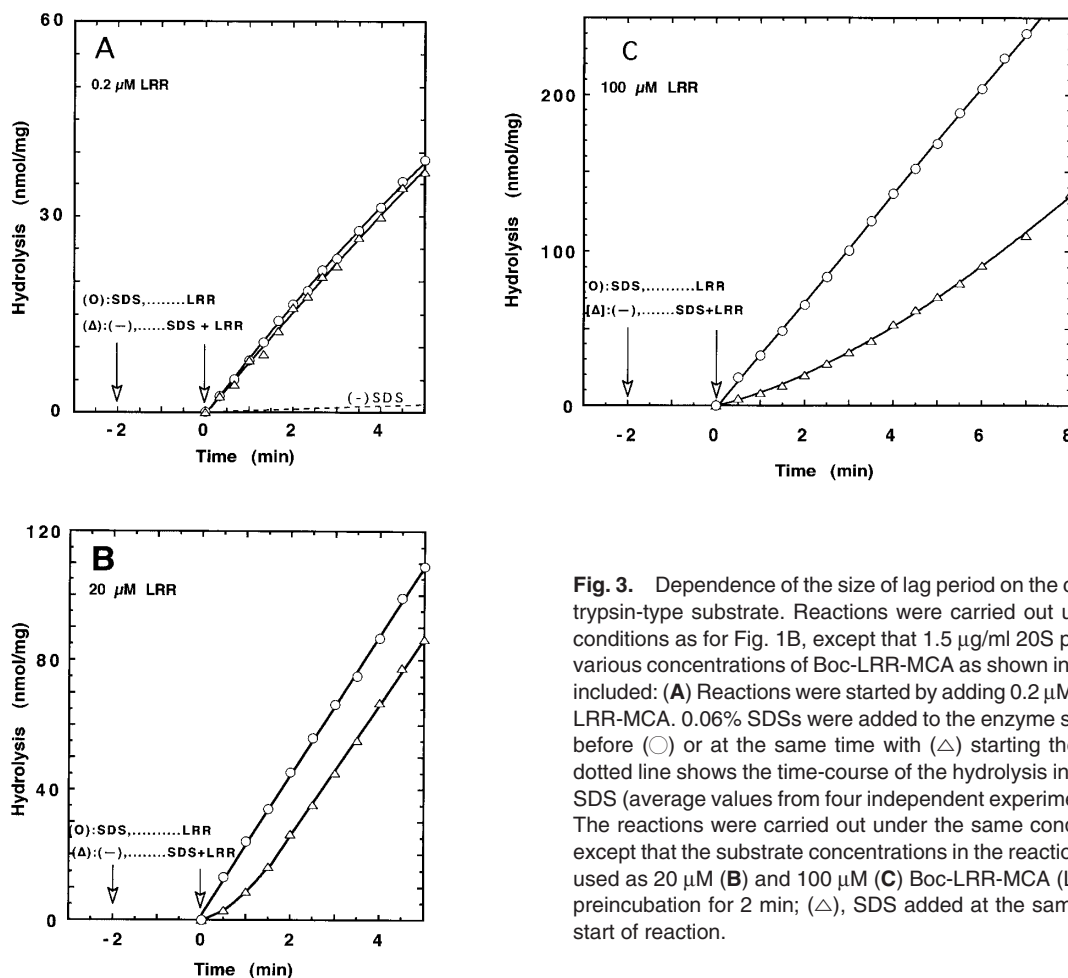
duced a reduction of the hydrolysis rate after a delay period, during which time the higher rate of hydrolysis that had been obtained with 0.06% SDS continued for a while. The increase of the SDS concentration (3rd phase) produced an acceleration in hydrolysis again after an appreciable lag period. Quite similar time courses with lag periods were obtained in the reactions of other trypsin-like substrates, Boc-FSR-MCA and Boc-QAR-MCA (data are not shown). These results suggest that the mechanism by which SDS modulates the peptidase activities of the proteasome is distinctly different between the two groups of peptidase: (a) chymotrypsin-like and PGP hydrolase activities, and (b) trypsin-like activity. The results also suggest a hypothesis that the putative modulator site(s) become less accessible to the binding and dissociation of SDS upon the binding of the trypsin-type substrate to the enzyme.

In order to investigate the above hypothesis, the effects of SDS preincubation which is carried out in the absence of substrate on the lag period were examined in Fig. 2. When SDS was added together with the substrate under the same reaction conditions as for Fig. 1B, a similar lag period of about 30 sec was observed. Therefore, the lag phase completely disappeared due to SDS preincubation for 2 min, and preincubation for as short as 5 sec was found to be sufficient to eliminate the lag period (Fig. 2). Furthermore, in order to examine the above hypothesis, we carried out trypsin-like peptidase reactions using various concentrations of the substrate Boc-LRR-MCA in the presence of 0.06% SDS (Fig. 3A-C). In these experiments, we compared two different modes of addition of SDS at each concentration of the substrate: (1) the addition of SDS together with the substrate, and (2) the addition of SDS at 2 min before the substrate. At low concentrations of the substrate (0.2  $\mu\text{M}$  Boc-LRR-MCA), there was virtually no difference in the kinetics of hydrolysis regardless of the mode of addition (Fig. 3A). At higher concentrations of the substrate (20 and 100  $\mu\text{M}$ ), however, the kinetics became dis-



**Fig. 2.** Disappearance of the lag phase by preincubation of SDS for a short period. Reaction conditions were the same as for Fig. 1B except that the time for SDS addition is different. 0.06% SDS was added at 2 min before (O), 5 sec before (Δ) or the same time with (◇) the start of the reaction which is initiated by adding a small volume (1/100 of reaction solution) of the same reaction solution but with a higher concentration of Boc-LRR-MCA (LRR; final concentration of 10  $\mu\text{M}$ ).



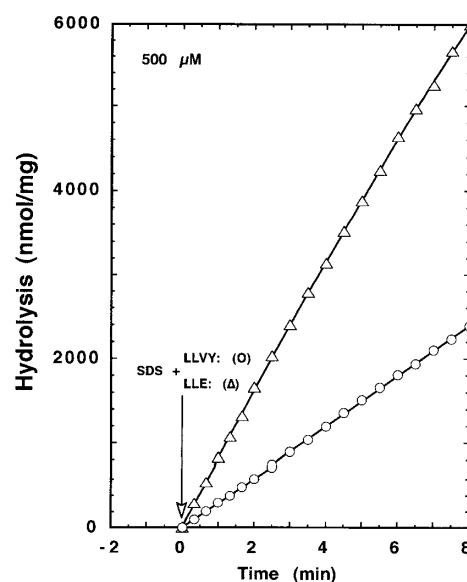


**Fig. 3.** Dependence of the size of lag period on the concentration of trypsin-type substrate. Reactions were carried out under the same conditions as for Fig. 1B, except that 1.5  $\mu\text{g/ml}$  20S proteasome and various concentrations of Boc-LRR-MCA as shown in the figure were included: **(A)** Reactions were started by adding 0.2  $\mu\text{M}$  substrate Boc-LRR-MCA. 0.06% SDSs were added to the enzyme solution at 2 min before (○) or at the same time with (Δ) starting the reaction. The dotted line shows the time-course of the hydrolysis in the absence of SDS (average values from four independent experiments). **(B and C)** The reactions were carried out under the same condition as for **(A)** except that the substrate concentrations in the reaction solution were used as 20  $\mu\text{M}$  **(B)** and 100  $\mu\text{M}$  **(C)** Boc-LRR-MCA (LRR): (○), SDS preincubation for 2 min; (Δ), SDS added at the same time with the start of reaction.

tinctly different depending upon the mode of addition. Namely, the lag period became clearly discernible when SDS was added together with the substrate. As clearly seen here, the extent of delay (i.e. the size of lag period) increased with the increase of substrate concentration (Figs. 1B, 2 and 3A-C). On chymotrypsin-like and PGP hydrolase activities at the high concentrations of substrate (e.g. 500  $\mu\text{M}$  Suc-LLVY-MCA and 500  $\mu\text{M}$  Cbz-LLE-2NA; about  $K_m$  values, data are not shown) the lag periods in the reaction were examined in Fig. 4. As seen in the figure, no appreciable lag phases were detected.

## DISCUSSION

The 20S proteasome is a multi-catalytic proteinase, and the three major peptidase activities (trypsin-like, chymotrypsin-like and PGP hydrolase) have been investigated using the peptidase-specific fluorogenic substrate peptides whose hydrolysis results in high fluorescent products. However, all of these peptidase activities are latent, and require appropriate activators such as SDS, fatty acids, cardiolipin and polylysine. Among these activators, SDS was most frequently used in the literature. Although there is a general consensus that SDS serves as an activator of several peptidases, many important



**Fig. 4.** Undetectable lag phase in the reaction with high concentration of substrates Suc-LLVY-MCA and Cbz-LLE-2NA. Reaction conditions were the same as for Fig. 1 except that the substrate and the concentration were different. Reactions were started by simultaneous addition of SDS and substrate solutions: (○), 500  $\mu\text{M}$  Suc-LLVY-MCA (LLVY); (Δ), 500  $\mu\text{M}$  Cbz-LLE-2NA (LLE).

questions remain unresolved especially as to whether common or differentiated mechanisms are operating for the activation of the different types of peptidase described above.

One of the most important aspects of the present study is the finding that both acceleration and deceleration of the trypsin-like hydrolysis by SDS binding and dissociation occur after appreciable delays. There are no such delays in the chymotrypsin-like peptidase and PGP hydrolase reactions. These results suggest that the substrate-induced conformational change of the enzyme characteristic of the trypsin-like peptidase is involved in the peculiar mode of SDS activation of this particular peptidase. This notion is supported by the following pieces of evidence. First, the extent of delay in the activation by SDS (viz. the size of the lag period) increased considerably with the increase of the substrate concentration. This indicates that the formation of the enzyme-substrate complex induces a conformational change in the enzyme which makes the activator site(s) in an occluded form, reducing the rate of SDS binding to the site(s). This mechanism is also supported by the present observation that the deceleration of the hydrolysis by dilution of the enzyme-bound SDS occurred after the appreciable delay (Fig. 1B). As shown in the preincubation experiments, treatment of the enzyme with SDS for a short period before the substrate addition eliminates the delay of activation, indicating that the activator site(s) is readily accessible to SDS before the substrate addition, viz. prior to the substrate-induced enzyme conformational change. Since SDS instantly activates the chymotrypsin-like and PGP hydrolase activities regardless of the sequence of addition, we propose that the trypsin-like peptidase reaction is mediated by the conformational change mechanism specifically involved in this particular type of peptidase.

In conclusion, the modes of action of the widely used activator of the 20S proteasome, SDS, were investigated using the fluorogenic substrate peptides specific to the three major types of peptidase. The present studies have revealed new conformational change in the enzyme involved in the SDS-activated peptidase reactions. The trypsin-like peptidase reaction is mediated by the substrate-induced conformational change characteristic of this particular peptidase which can be characterized by an occlusion phenomenon of the activator site(s).

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