Activation of the 20S Proteasome of Xenopus Oocytes by Cardiolipin: 
Blockage of the Activation of Trypsin-like Activity by the Substrate

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The effects of an activator, cardiolipin, on the three peptidase activities of the 20S proteasome of Xenopus oocytes were examined. The trypsin-like activity was activated when the enzyme was treated with cardiolipin before the addition of the substrate, but there was no appreciable activation when cardiolipin was added concomitantly with the substrate. On the other hand, the chymotrypsin-like peptidase and peptidylglutamylpeptide hydrolase (PGPH) were activated regardless of the sequence of addition. When very low concentrations of the substrate (e.g. 0.1-0.5 μM; about 1/100 of the K_m) were used, cardiolipin strongly activated trypsin-like peptidase by the simultaneous addition but not after substrate addition. These results suggest that the trypsin-type substrate produces a conformational change in the enzyme in a concentration-dependent manner which makes the activator sites inaccessible to cardiolipin.

Key words: 20S proteasome; trypsin-like activity; activator cardiolipin; Xenopus oocytes; conformational change

In eukaryotic cells, degradation of most intracellular disused proteins is catalyzed by the proteasome which accounts for up to 1% of the total cell proteins. The 20S proteasome (2,000 kDa), which degrades ubiquitinated proteins in an ATP-dependent manner, is constructed from the 20S proteasome (700 kDa), which has several types of peptidase activity, and two 19S regulatory complexes that include six ATPase subunits. The three distinct types of peptidase activities of the 20S proteasome have been reported to catalyze by individual catalytic sites: viz. the endopeptidase activities involved in cleaving bonds on the carboxyl side of basic (trypsin-like activity), hydrophobic (chymotrypsin-like activity), and acidic (PGPH activity) amino acid residues. In addition to these three types, there appear to be several other types of peptidase activity. The peptidase activities of the purified 20S proteasome are essentially latent. That is, appropriate activators are required for its peptidase activities, and mostly SDS and sometimes fatty acids have been used as the activators in previous studies. According to recent reports, cardiolipin (phospholipid that was first isolated from the cardiac muscle, and is now known as a physiological substance present in various tissues) is a potent activator of the major peptidase activities of the 20S proteasome described above. Thus, it is important to characterize the mode of activation of these peptidases by cardiolipin, because it has physiological relevance. However, very few such studies have been done. Moreover, there is a strong controversy about the reported effects of cardiolipin on the trypsin-like activity: activation or no effect.

The aim of this study is to discover the mechanisms by which the three types of peptidase activities of the 20S proteasome (trypsin-like, chymotrypsin-like, and PGPH activities) are activated by cardiolipin. We preliminarily report here that the activation of the trypsin-like peptidase occurs when the enzyme has reacted with cardiolipin before the addition of the substrate peptide, but there is no activation if cardiolipin is added at or after the peptide reaction with the substrate. This suggests that the substrate binding to the enzyme produces a conformational change characteristic of the trypsin-like activity, which makes cardiolipin inaccessible to its activator site(s). These results thus provide a reasonable explanation for the controversial reports described above.

We investigated the three types of peptidase activity of the purified 20S proteasome using fluorogenic substrates specific to each of these peptidases: Succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA; chymotrypsin-like peptidase), benzoyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide (Cbz-LLE-2NA; PGPH), and tert-butyloxycarbonyl-Leu-Arg-Arg-4-methylcoumaryl-7-amide (Boc-LRR-MCA; trypsin-like peptidase). The 20S proteasome was prepared from oocyte cytosol of Xenopus laevis ovaries as described previously, with minor modifications. Reactions of peptidyl substrate hydrolysis were started by adding a peptidyl substrate dissolved in dimethylsulfoxide (DMSO). After incubation for various times, the reaction was stopped by adding 1% SDS. The activity of peptidyl substrate hydrolysis was measured as described previously.

During the course of this study, we found that the activation of trypsin-like (but not the other types) peptidase by cardiolipin is considerably different depending upon whether cardiolipin is added before or at the same time.
Fig. 1. Cardiolipin-dependent Activation Profiles of Three Peptidase Activities When the Activator is Added before (A) and at the Same Time with (B) the Addition of Substrate.

Reactions were started by adding various substrates, Suc-LLVY-MCA (LLVY) (○), Cbz-LLE-2NA (LLE) (▲) or Boc-LRR-MCA (LRR) (▽) at 2 min after adding various concentrations of cardiolipin to the enzyme (A) or at the same time as adding various concentrations of cardiolipin (B). The reaction solution contained 50 mM Tris-HCl, pH 8.5, 10% DMSO, 1 mM EDTA, 1.9 μg/ml 20 S proteasome, 200 μM Suc-LLVY-MCA or Cbz-LLE-2NA or 10 μM Boc-LRR-MCA and 0–2 μg/ml cardiolipin. After 5 min, the reaction was stopped by adding an excess of SDS. The peptidase activity was expressed as relative values of amount of substrate hydrolyzed during the reaction period. The K_m values for hydrolysis reaction of Suc-LLVY-MCA, Cbz-LLE-2NA, and Boc-LRR-MCA determined under these conditions were 380, 530, and 20 μM, respectively.

Fig. 2. Activation of Trypsin-like Activity by Concomitant Addition of Cardiolipin When the Concentration of Substrate Was Extremely Low.

A; Hydrolysis reactions were done at 0.2 μM Boc-LRR-MCA (LRR(1/100 of K_m value, see legends for Fig. 1A). 0.6 μg/ml cardiolipin was added 2 min before (○), concomitantly with (▲), and 5 min after (▽) the start of reaction. Reaction without cardiolipin is shown (△). The reaction conditions were the same as for Fig. 1B except that concentrations of 205 proteasomes, Boc-LRR-MCA, and cardiolipin were 0.95 μg/ml, 0.2 μM, and 0.6 μg/ml, respectively. B; 0.6 μg/ml cardiolipin was added before and concomitantly with the start of reaction, which was done for 5 min in various concentrations of Boc-LRR-MCA as indicated in the abscissa. The ratio of [(the hydrolysis rate when cardiolipin was added concomitantly with the start of reaction)−(the rate without cardiolipin)]/[(the rate when cardiolipin was added 2 min before starting the reaction)−(the rate without cardiolipin)] were plotted against the substrate concentration in the reaction solution.

as the addition of substrate (Figs. 1A-B).

Figure 1A depicts the dose-dependence of cardiolipin activation of the three types of peptidases when various concentrations of cardiolipin (as indicated on the abscissa) were added and incubated for 2 min before the addition of the substrate. As seen, cardiolipin activated all of the three peptidases. Maximum activation was obtained at the concentration of 0.6 μg/ml, and at 0.2 μg/ml about half maximum activation was observed. The general pattern of activation is essentially identical for all of the three types of peptidase. Figure 1B shows the dose-dependence of cardiolipin activation when cardiolipin was added simultaneously with the substrates. Cardiolipin had no effect on the trypsin-like activity regardless of the concentrations, while with the chymotrypsin-like peptidase and PGPH, cardiolipin produced the same dose-dependent activation pattern as that produced by the cardiolipin prior incubation. There is a sharp contrast in the requirement for prior incubation with cardiolipin for its activation between the two groups of peptidase activity. In one group, namely in hydrolysis of Boc-LRR-MCA (trypsin-like activity), an appreciable activation by cardiolipin was observed only when the enzyme reaction was started after cardiolipin incubation (Figs. 1A-B). In another group, namely in the chymotrypsin-like peptidase and PGPH, however, the same extent of activation was obtained regardless of the sequence of the additions (Figs. 1A-B). The above results suggest that the binding of the substrate Boc-LRR-MCA to the enzyme produces a conformational change characteristic of the trypsin-like hydrolytic reaction, which excludes the binding of cardiolipin to its activator site(s). In order to investigate this hypothesis, the courses of hydrolysis of Boc-LRR-MCA were measured at much lower substrate concentrations (0.1–0.5 μM;
$K_a = 20 \mu M$, see legend for Fig. 1). Figure 2A compares the rates of hydrolysis of 0.2 $\mu M$ Boc-LRR-MCA (1/100 of $K_a$) obtained by (a) prior incubation with, (b) simultaneous addition of, or (c) after substrate addition of 0.6 $\mu g/\text{ml}$ cardiolipin. In contrast to the observation in Fig. 1B, the simultaneous addition of cardiolipin with the low concentration of the substrate produced a significant level of activation. However, the addition of cardiolipin 5 min after the substrate addition was incapable of producing an appreciable activation. Figure 2B shows the extent of enzyme activation by 0.6 $\mu g/\text{ml}$ cardiolipin as a function of the concentration of Boc-LRR-MCA that was added simultaneously with cardiolipin. It is clearly shown here that the simultaneous addition of cardiolipin with the substrate Boc-LRR-MCA can produce an appreciable activation if the substrate concentration is significantly low, the conditions under which the substrate-induced enzyme conformational change described above would proceed at a much slower rate. These results are consistent with the concept that the binding of Boc-LRR-MCA produces a new conformational state in which cardiolipin becomes inaccessible to its activator site(s). Furthermore, these results can be analyzed using the simple model: $E+S \rightarrow E:S \rightarrow E\cdot Pr \rightarrow E + Pr$, where $E$, unreacted enzyme; $E:S$, enzyme-substrate complex; $Pr$, hydrolytic product. Calculating from several parameters used in the experiment in Fig. 2A (0.2 $\mu M$ Boc-LRR-MCA, which is the 1/100 of $K_a$ (20 $\mu M$); 2.7 nM proteasome; the rate of hydrolysis (0.56 mol substrate/mol enzyme/min)), $E$ would have decayed at $t_{1/2} \sim$ several sec under the conditions of Fig. 2A. Under the conditions in Fig. 1B (10 $\mu M$ Boc-LRR-MCA), on the other hand, $E$ would have decayed almost instantly after the substrate addition. From the above consideration, we propose that cardiolipin can bind to the activator site(s) for trypsin-like activity if the enzyme is in the E state, but the activator site(s) become inaccessible to cardiolipin in the E:S and E:Pr states. Therefore, the site(s) on the enzyme to which cardiolipin binds for activation appears to be different for the two groups of peptidase (a, trypsin-like; b, chymotrypsin-like and PGPH). The intriguing feature characteristic of the trypsin-like peptidase is that upon initiating the hydrolytic reaction with the substrate the activator site(s) become inaccessible to the cardiolipin binding, suggesting of a new type of enzyme conformational change involved in the enzyme reaction of this particular peptidase.

The other important aspect of this paper is the finding that the modes of cardiolipin activation are clearly distinguishable between the two groups of peptidase activities. Namely, both chymotrypsin-like peptidase and PGPH were activated regardless whether cardiolipin was added before or after the addition of the substrates for these peptidases. In contrast, the trypsin-like peptidase was activated only when the enzyme was treated with cardiolipin before starting the hydrolytic reaction with the substrate Boc-LRR-MCA, but there was no activation either when cardiolipin was added concomitantly with the substrate or after the addition of the substrate. This discrete difference in the activation pattern depending upon the sequence of additions provides a reasonable explanation for the contradictory reports about the cardiolipin effect on the trypsin-like activity described above.10-12

References