Autoproteolysis of the Small Subunit of Calcium-dependent Protease II Activates and Regulates Protease Activity*

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Calcium-dependent protease II (CDP-II) from bovine heart is a heterodimer with subunit molecular weights of 80,000 and 26,000. Previous studies have demonstrated that the protease requires 350 μM Ca²⁺ for half-maximal activity and that the large subunit contains both the catalytic and Ca²⁺ binding functions of the enzyme. The function of the small subunit has been unclear. We have examined the effect of Ca²⁺ on structural and catalytic properties of CDP-II in the presence and absence of substrate proteins. When incubated with Ca²⁺ in the absence of substrate, CDP-II undergoes a series of autoproteolytic cleavages that sequentially reduce the small subunit's molecular weight from 26,000 to 24,000 to 22,000 to 17,000. During this time there is no detectable change in the 80-kDa subunit, which remains associated with the autolyzed small subunit. The rate of autoproteolysis is dependent on temperature and on the concentration of Ca²⁺ (half-maximal rate at approximately 600 μM Ca²⁺). The first cleavage appears to be unimolecular because its rate is unaffected by CDP-II concentration or by the presence of exogenous protein substrates. Subsequent cleavages result in the formation of the 80-kDa/17-kDa heterodimer and appear to occur by bimolecular reactions; rates of these reactions were slowed by decreasing CDP-II concentrations and by the presence of protein substrates.

Autoproteolysis of the small subunit has two distinct functional consequences, each of which is associated with different forms of the autolysed protease. Our results indicate that the 80-kDa/26-kDa form of CDP-II represents an inactive proenzyme and that the initial Ca²⁺-dependent cleavage of the 26-kDa subunit results in activation of the protease. The activated enzyme hydrolyzes protein substrates with a Ca²⁺ concentration requirement of 350 μM for half-maximal rates. The further autoproteolysis, which results in the formation of the 80-kDa/17-kDa heterodimer, serves to reduce the Ca²⁺ concentration requirement for protease activity by 25-fold. Thus, these results provide evidence for specific roles of the small subunit in the regulation of CDP-II activity.

Two calcium-dependent proteases (designated CDP-I and CDP-II) are present in most if not all tissues of vertebrates (1, 2). Although the physiological function(s) of these enzymes is not yet known many properties of the enzymes are becoming well-defined. For example, regardless of the source, the proteases are heterodimers with subunit molecular weights of approximately 80,000 and 30,000. It has been demonstrated that the large subunit contains both the active site and the calcium-binding sites of each enzyme (1–6). The role of the small subunit has remained obscure. Previous work has demonstrated that each subunit of CDP-II is autoproteolyzed during an incubation of the enzyme with Ca²⁺ in the absence of substrate (5, 7–9). In the present study we have examined the limited autoproteolysis of the small subunit of CDP-II from bovine heart. Based on our data, we propose a model in which sequential cleavages of this subunit serve first to activate the protease and subsequently to reduce the calcium concentration requirement of the enzyme.

MATERIALS AND METHODS

Preparation of Calcium-dependent Protease—CDP-II was prepared from bovine heart as described previously (10). The enzyme has been characterized as a heterodimer with subunit molecular weights of 80,000 and 26,000.

Assays of Protease Activity—Protease activity was measured by the hydrolysis of [methyl-¹⁴C]casein as described previously (4, 10). Casein was labeled by the method of Dottavio-Martin and Ravel (11) and had specific activity of 2500 dpm/μg casein.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed with 10% or 12% acrylamide slab gels as described previously (4, 10) and detailed in legends to figures. Gels were stained with Coomassie Blue R-250. Densitometry of the stained gels was performed with a Zeineh soft laser scanner; integration of the peaks was performed using a Zeineh I integrator program (Biomedical Instruments).

Casein-Sepharose Affinity Chromatography—Casein-Sepharose was prepared as described previously (4). Ca²⁺-dependent affinity chromatography of purified CDP-II was carried out as described previously (4) with several modifications. 1) The column buffers contained 150 mM NaCl instead of 400 mM KCl. 2) The column loading and elution was performed such that the time the enzyme was exposed to Ca²⁺ was reduced from approximately 90 to 15 min. 3) CDP-II was eluted from the column with 15 mM EGTA rather than 5 mM EGTA.

Protein Determinations—Protein was determined by the method of Bradford (12) using premixed reagents purchased from Bie-Rad. Bovine serum albumin was the standard.

RESULTS

When calcium-dependent protease II (CDP-II) was incubated at 25 °C in the presence of 2 mM Ca²⁺, but in the absence of a protein substrate, the 26-kDa subunit was rapidly (i.e. within 30 s) cleaved to a form which migrated on SDS-PAGE (12) with an apparent molecular weight of 24,000. 1 The abbreviations used are: CDP-II and -I, calcium-dependent protease II and -I, respectively; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethyl)iminotri]acetic acid.
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PAGE with $M_r = 17,000$ (Fig. 1A). This cleavage appears to result from autoproteolysis because it is dependent on Ca$^{2+}$ and is completely inhibited by inhibitors of CDP-II such as leupeptin. The proteolyzed subunit remained associated with the 80-kDa catalytic subunit as determined by their co-migration on gel filtration chromatography (data not shown). The rate of appearance of the 17-kDa peptide was dependent on temperature and on the concentration of calcium. At 4°C and/or at limiting Ca$^{2+}$ concentrations (<1 mM) the rate of formation of the 17-kDa peptide was slowed and two transient intermediates of molecular mass = 24,000 and 22,000 were observed (Fig. 1B). Each of these intermediates, as well as the 17-kDa peptide, was derived from the 26-kDa subunit because each reacted with an antibody raised against the 26-kDa subunit but not with an antibody raised against the 80-kDa subunit (data not shown). The rates of appearance of these three autolytic fragments were affected differently by enzyme concentration. For example, over a 60-fold CDP-II concentration range (1.7-0.03 mg/ml) the rate of appearance of the 24-kDa peptide varied less than 2-fold. On the other hand, the rate of appearance of the 17-kDa peptide was approximately 25-fold faster at the highest enzyme concentration than at the lowest over this same concentration range (data not shown, see below).

During the time that the small subunit was undergoing these various proteolytic cleavages no detectable changes were observed in the 80-kDa subunit. These included molecular weight (judged by SDS-PAGE), quantity of the peptide (judged by densitometric scanning of the stained gel), and charge (judged by O'Farrell-type two-dimensional gel electrophoresis (13)). In addition, there was no significant change in total protease activity against protein substrates such as casein. After longer incubation times, the quantities of both the 80- and 17-kDa peptides were diminished. Peptides in the molecular weight range of 65,000-40,000 were produced and this was associated with loss of proteolytic activity (data not shown).

CDP-II requires approximately 350 μM Ca$^{2+}$ for half-maximal activity against casein and is fully active at Ca$^{2+}$ concentrations above 1 mM (10). As indicated above, protease activity measured at an optimal Ca$^{2+}$ concentration (e.g. 3.5 mM) did not change appreciably during autoproteolysis of the 26-kDa subunit. During that time, however, protease activity measured at a low Ca$^{2+}$ concentration, e.g. 71 μM, increased from an undetectable level to a value equal to that measured at 3.5 mM Ca$^{2+}$ (Fig. 2). The kinetics of the appearance of this lower Ca$^{2+}$ requiring activity coincided with the kinetics of the formation of the 17-kDa peptide. This relationship was demonstrated clearly in a series of autoproteolytic incubations in which temperature, Ca$^{2+}$ concentration, and enzyme concentration were systematically varied (Figs. 3, 4, and 5). As described above, these factors controlled the rate of formation of the 17-kDa peptide. Under conditions where this rate varied widely, it was the only process observable by SDS-PAGE which correlated with the alteration of the Ca$^{2+}$ concentration requirement of the protease. Thus, although a series of autoproteolytic cleavages are made on the small subunit, only one of these, the formation of the 17-kDa peptide, correlates with the lowering of the Ca$^{2+}$ concentration requirement of the enzyme. The autolyzed enzyme could be isolated in the 80-kDa/17-kDa heterodimer form by terminating autoproteolysis with EGTA. This enzyme was half-maximally activated by 15 μM Ca$^{2+}$ and thus was approximately 25-fold more sensitive to Ca$^{2+}$ than the original protease (Fig. 6).

The data presented above suggest that the initial autoproteolytic cleavage (i.e. 26-24 kDa) occurred principally, but perhaps not exclusively, by a unimolecular event while the further cleavage to the 80-kDa/17-kDa form of the enzyme was a bimolecular event. If so, the rate of the initial cleavage should be relatively unaffected by the presence of an exogenous substrate while further autoproteolysis should be markedly slowed by such a substrate. In order to test this, CDP-II (100 μg/ml) was incubated in the presence or absence of lysozyme (2.2 mg/ml) at 1.6 mM Ca$^{2+}$. At various incubation times, samples were taken for analysis by SDS-PAGE.

![Fig. 1. Effect of autoproteolysis on calcium-dependent protease II. Panel A, CDP-II (800 μg/ml in 50 mM Tris-HCl, pH 7.6, 5 mM β-mercaptoethanol) was incubated at 25°C in the presence of either 5 mM EGTA (lane 1) or 2.2 mM Ca$^{2+}$ (lane 2) for 30 s. Panel B, CDP-II (200 μg/ml in 50 mM Tris-HCl, pH 7.6, 5 mM β-mercaptoethanol) was incubated at 4°C in the presence of 1.7 mM Ca$^{2+}$ for the indicated time.](image1)

![Fig. 2. Effect of autoproteolysis on the activity of CDP-II. CDP-II (225 μg/ml in 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol) was preincubated at 4°C in the presence of 2 mM Ca$^{2+}$. At various times, samples of the preincubation were diluted 500-fold in 5 mM Tris-HCl, pH 7.5, and then assayed for protease activity at a final Ca$^{2+}$ concentration of either 3570 or 71 μM.](image2)
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FIG. 3. Correlation between formation of the 17-kDa subunit and appearance of protease activity at 71 μM Ca\(^{2+}\). CDP-II (250 μg/ml) was subjected to autoproteolysis in the presence of 2 mM Ca\(^{2+}\) (○, ●) or 714 μM Ca\(^{2+}\) (□, ■). Preincubations were carried out at either 4 °C (○, □) or 10 °C (●, ■). At various times (0–45 min), samples for each of these conditions were either: 1) processed for SDS-PAGE or 2) diluted 500-fold for subsequent assay of protease activity at 71 μM Ca\(^{2+}\). The amount of 17-kDa subunit was quantitated by densitometry of the SDS gel as described under "Materials and Methods." The maximum quantity of 17-kDa peptide was set at 100% and other values calculated as a percent of that value. Similarly, the maximum value for protease activity at 71 μM Ca\(^{2+}\) was set at 100% and other values were calculated as a percent of that value.

17 K (% Max)

FIG. 4. Ca\(^{2+}\) concentration requirement for autolysis-dependent appearance of protease activity at 71 μM Ca\(^{2+}\) and formation of 17-kDa subunit. CDP-II (1.6 mg/ml in 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol) was preincubated at 4 °C with various concentrations of Ca\(^{2+}\). After 10 min, samples were either processed for SDS-PAGE or diluted to 500-fold for subsequent protease activity assay at a final Ca\(^{2+}\) concentration of 71 μM. Protease activities are expressed as a percentage of the maximum observed activity. The SDS gel was stained and the amount of the 17-kDa peptide formed under each condition was quantitated and expressed as a percentage of the maximum value.

FIG. 5. Effect of CDP concentration on appearance of protease activity at 71 μM Ca\(^{2+}\) and formation of 17-kDa subunit. Panel A, CDP-II was preincubated in 50 mM Tris-HCl, pH 7.5, at 4 °C at the indicated concentrations in the presence of 2.2 mM Ca\(^{2+}\). At various times, samples of the preincubation were diluted 750-fold with 50 mM Tris-HCl, pH 7.5, and assayed for protease activity at 71 μM Ca\(^{2+}\). Panel B, preincubation samples of CDP-II as described above were subjected to SDS-PAGE. The 17-kDa subunit was quantitated by densitometry as described under "Materials and Methods." The maximum amount of 17-kDa subunit was set at 100% and other values were expressed as a percent of that value.

FIG. 6. Ca\(^{2+}\) concentration requirement for calcium-dependent proteases. CDP-II (80-kDa/26-kDa), autolyzed CDP-II (80-kDa/17-kDa), and CDP-II isolated after affinity chromatography on casein-Sepharose (80-kDa/24-22-kDa) were assayed for activity with [methyl-\(^{14}\)C]casein under standard conditions.

Assessed the effect of substrate on the formation of this peptide. As shown in Fig. 7, the presence of substrate, in this instance, casein, greatly reduced the rate of appearance of low Ca\(^{2+}\)-requiring activity and thus, presumably, formation of...
pyrocarbonate. The concentrations of enzymes were those very slowly and approximately 60% remained after 60 min. These results provide further evidence that the initial cleavage susceptible to proteolysis (see "Discussion").

Although this experiment indicates that there is not significant bimolecular autoproteolysis of the 26-kDa/26-kDa heterodimer. The proteins were mixed at a low molar ratio (3:1; 0.89 mg/ml of 80-kDa/26-kDa to 0.29 mg/ml of 80-kDa/26-kDa) and incubated at 4 °C for periods up to 60 min. The incubation contained 100 μM Ca²⁺, a concentration sufficient to completely activate the 80-kDa/17-kDa form of the enzyme but not the native enzyme. After various incubation times at 4 °C samples were prepared for SDS-PAGE. No detectable cleavage of the 26-kDa peptide was observed even after 60 min (data not shown). Although this experiment indicates that there is not significant bimolecular autoproteolysis of the 26-kDa subunit, the incubation conditions differ from those in other experiments because of the lower Ca²⁺ concentration. In order to examine autoproteolysis at high Ca²⁺ concentrations, but eliminate unimolecular autoproteolysis of the 80-kDa/26-kDa heterodimer, we performed the experiment with 80-kDa/26-kDa CDP-II which had been irreversibly inactivated with diethylpyrocarbonate. The concentrations of enzymes were those described above and the incubations were carried out at 100 μM Ca²⁺ and at 2.2 mM Ca²⁺. No detectable cleavage of the 26-kDa peptide was observed after 60 min of incubation at 100 μM Ca²⁺. At 2.2 mM Ca²⁺, the 26-kDa subunit was cleaved very slowly and approximately 60% remained after 60 min. These results provide further evidence that the initial cleavage of the 80-kDa subunit does not occur rapidly via a bimolecular mechanism even at high calcium concentrations. These results also indicate that high Ca²⁺ concentrations may be required by the CDP-II to assume a conformation which is susceptible to proteolysis (see "Discussion").

Because the initial cleavage of the 26-kDa subunit was distinct from the formation of the more Ca²⁺-sensitive enzyme, we questioned whether there was a separate functional event associated with this autoproteolysis. One possibility is that cleavage of the 26-kDa peptide results in activation of the protease and that the 80-kDa/26-kDa enzyme, regardless of the presence or absence of Ca²⁺, is an inactive proenzyme.

Several lines of evidence suggest that this possibility may be correct.

We have examined the kinetics of casein hydrolysis by CDP-II under a number of incubation conditions (Fig. 8). At optimal Ca²⁺ concentrations and 25 °C, the initial rates of casein hydrolysis are constant and acid-soluble peptides are produced at linear rates. Under these same conditions, complete conversion of the 26- to the 17-kDa subunit had occurred by the first assay time points. However, we have also examined rates of casein hydrolysis at various limiting Ca²⁺ concentrations and/or at temperatures below 25 °C (e.g. 4 and 10 °C), conditions which decrease the rate of hydrolysis of the 26-kDa subunit. If proteolysis of the 26-kDa subunit is obligatory for protease activity, the rate of casein hydrolysis should increase with time of incubation as more of the 26-kDa subunit is cleaved. In fact, under such conditions, the rate of casein hydrolysis increased progressively during the course of the assay. Such kinetics could be explained by the formation of increasing amounts of active enzyme during assay. Conversely, these kinetics would not be expected for a form of the enzyme which is already "activated." We have used the 80-kDa/17-kDa heterodimer produced by limited proteolysis as an example of such an enzyme. Indeed, under all conditions tested, the rates of casein hydrolysis by this form of the enzyme were constant.

This mechanism of protease activation may account for previous results by us and others demonstrating that CDPs isolated by substrate affinity chromatography lack the native small (i.e. unautoyzed) subunit (4, 10, 14, 15). This result would be expected if the Ca²⁺-dependent interaction between

![Fig. 7. Effect of casein substrate on the autolysis-dependent formation of protease activity at 71 μM Ca²⁺. CDP-II (220 μg/ml in 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptopropanol) was preincubated at 4 °C in the presence of 2 mM Ca²⁺ and in either the presence (●, ●) or absence (○, □) of 5 mg/ml α-casein. At various times, samples of this preincubation were diluted 500-fold in 50 mM Tris-HCl, pH 7.5, and assayed for protease activity at final Ca²⁺ concentrations of either 357 (●, ●) or 71 μM (○, □).](image-url)

![Fig. 8. Kinetics of casein hydrolysis by calcium-dependent proteases. Calcium-dependent protease activity was determined using [methyl-¹⁴C]casein substrate as described previously (4, 10). In addition to the indicated concentrations of Ca²⁺, each assay contained 50 mM Tris-HCl, pH 7.6 (at the indicated temperature), 1 mM dithiothreitol, 0.07 μg of protease, and 16 μg of casein. Data for Panels A and B were obtained using the 80-kDa/26-kDa heterodimer. Data for Panels C and D were obtained using the 80-kDa/17-kDa heterodimer isolated after autoproteolysis. Each data point represents the average of three determinations. For each panel, similar results were obtained in four separate experiments using three different enzyme preparations. Panel A, incubations at 25 °C; ●, 3570 μM Ca²⁺; ○, 714 μM Ca²⁺; ▲, 357 μM Ca²⁺. Panel B, incubations at 4 °C; ●, 3570 μM Ca²⁺; ○, 714 μM Ca²⁺; ▲, 357 μM Ca²⁺. Panel C, incubations at 25 °C; ●, 3570 μM Ca²⁺; ○, 357 μM Ca²⁺; ▲, 18 μM Ca²⁺; △, 4 μM Ca²⁺. Panel D, incubations at 4 °C; ●, 357 μM Ca²⁺; ○, 18 μM Ca²⁺; ▲, 4 μM Ca²⁺.](image-url)
the protease and substrate requires prior cleavage of the small subunit. Although we and others have reported the isolation of an 80-kDa monomer by this technique, certain conditions of the chromatography such as salt concentration and the extended time of exposure to Ca\(^{2+}\) appear to contribute to the complete loss of the small peptide. To re-examine this issue, we subjected purified CDP-II to affinity chromatography on casein-Sepharose using conditions designed to reduce salt concentration and significantly decrease the time of the chromatography (see "Materials and Methods"). The isolated enzyme consisted of the 80-kDa subunit and a peptide with M\(_r\) = 22,000. In some preparations small amounts of the 17-kDa peptide were also present. The affinity-isolated enzyme required approximately 300 \(\mu\)M Ca\(^{2+}\) for half-maximal activity against \(\alpha\)-casein, a value similar to that for the 80-kDa/26-kDa enzyme (Fig. 6). This result provides direct evidence that the initial cleavage of the 26-kDa subunit is not responsible for the decrease in the Ca\(^{2+}\) concentration requirement of the protease. Furthermore, preincubation of the affinity-purified enzyme with Ca\(^{2+}\) also resulted in its conversion to a form which required only 15 \(\mu\)M Ca\(^{2+}\) for half-maximal activity. SDS-PAGE demonstrated that this enzyme was composed of 80-kDa/17-kDa subunits (data not shown).

**DISCUSSION**

Despite the strong evidence that calcium-dependent proteases can be purified as heterodimers with subunit molecular weights of approximately 80,000 and 30,000, to our knowledge there is no direct evidence that enzymes in this form are catalytically active. In fact, affinity purification techniques which require active protease have consistently isolated CDPs as monomers of approximately 80 kDa (4, 10, 14, 15). Because the monomers retain calcium-dependent proteolytic activity, such work has raised questions about the function of the small peptide and the basis for its loss during affinity chromatography. On the one hand, it has been argued that the small subunit is a contaminant, unrelated to the protease and therefore is removed by affinity chromatography. On the other, it has been suggested that the small subunit is highly susceptible to autoproteolysis and consequently is removed during any affinity chromatography which requires enzyme activation. We have considered an alternative possibility that affinity chromatography isolates only activated forms of the protease and that an autolytic cleavage of the small subunit is obligatory for subsequent Ca\(^{2+}\)-dependent protease activity against protein substrates.

Our data indicate that Ca\(^{2+}\) promotes sequential autoproteolytic cleavages of the small subunit of CDP-II and this effect has two distinct consequences. First, autoproteolysis activates the protease and second it reduces the Ca\(^{2+}\) concentration requirement for protease activity against protein substrates. We propose the following model for the autoproteolytic activation and conversion of the CDP-II. 1) Ca\(^{2+}\) binds to the 80-kDa subunit of the 80-kDa/26-kDa protease. Ca\(^{2+}\) binding is necessary but not sufficient for protease activity. Ca\(^{2+}\) promotes a unimolecular cleavage of the 26-kDa subunit, an event obligatory for subsequent protease activity. 2) The resulting 80-kDa/24-kDa heterodimer is an active protease characterized by a high Ca\(^{2+}\) concentration requirement (350 \(\mu\)M Ca\(^{2+}\) required for half-maximal activity against casein). Although this enzyme may also act to cleave the 26-kDa subunit of other protease molecules, the bimolecular reaction appears to be less favored than the unimolecular reaction. 3) The activated 80-kDa/24-kDa protease autolyzes further to form an 80-kDa/22-kDa form and finally an 80-kDa/17-kDa form of CDP-II. In vitro these reactions are slow in the presence of substrate proteins. The 80-kDa/17-kDa heterodimer is approximately 25-fold more sensitive to Ca\(^{2+}\) than the original enzyme.

Although this model is consistent with our data we cannot exclude completely the possibility that the various functional changes associated with autoproteolysis are attributable to alterations of the large subunit. For example, it is possible that proteolytic cleavages very near a terminus of this subunit remain undetected by either one- or two-dimensional SDS-PAGE. We have attempted to perform NH\(_2\)-terminal amino acid analysis of the 80-kDa subunit before and after autoproteolysis but find that it appears to be blocked in each case. Such a result is consistent with an unproteolyzed large subunit but also does not exclude the possibility that a cleavage occurs at the carboxyl terminus.

Our model has several implications regarding the function and regulation of CDP-II in vivo but also raises a number of questions. First is the fact that of the CDP-II isolated from tissue, none (as detected by SDS-PAGE) is in an autolyzed form. This result indicates either that tissues do not contain significant amounts of activated protease or that activated protease is not isolated by our purification scheme. In either case, CDP-II may be similar to many other calcium-dependent enzymes which are synthesized and stored as inactive proenzymes and subsequently activated by limited proteolysis; in many of these instances this limited proteolysis serves as a major point of regulation of protease activity (16). It is unclear, however, to what extent the proteolytic activation of CDP-II is important as a regulatory process in vivo because both autoproteolytic activation and the resulting active enzyme are dependent on Ca\(^{2+}\) to the same extent. This may suggest that in addition to Ca\(^{2+}\), other factors such as the specific inhibitor (1, 2, 17, 18) and stimulator proteins (19) are important in the regulation of CDP-II activity in intact cells. Information about the relative localization and interaction of these components in vivo may provide insight to this issue. The second functional consequence of autoproteolysis, i.e. the conversion of CDP-II to an enzyme active at more physiologic Ca\(^{2+}\) concentrations, also provides an attractive mechanism for the control of CDP-II activity. The significance of this mechanism is unclear, however, because it also requires high concentrations of Ca\(^{2+}\). Our results indicate that at low Ca\(^{2+}\) concentrations, other Ca\(^{2+}\)-dependent proteases cannot promote this conversion and there is no evidence that any other protease or peptidase can mimic this effect. Nevertheless, this latter possibility cannot be excluded. In addition, other factors may influence autoproteolysis. For example, Coolican and Hathaway (9) have recently demonstrated that phosphatidylinositol can decrease the Ca\(^{2+}\) concentration requirement for autoproteolysis of chicken gizzard CDP-II by 25-fold. Interestingly, this compound had no effect on the Ca\(^{2+}\) concentration requirement for protease activity against exogenous substrates. This or related mechanisms may be of considerable physiologic significance.

Several groups have previously documented a reduction in the calcium-concentration requirement of calcium-dependent proteases as a result of autoproteolysis (7-9, 20-22). These results, however, appear to contain inconsistencies with regard to the relationship between this functional alteration and the exact form of the proteolyzed CDP-II. For example, this change has been ascribed alternately to cleavage of the large subunit, cleavage of the small subunit, or both. Although the basis for these discrepancies is unclear, we believe that the model described here may be common to CDP-II from

\[2 \text{A. F. Clark, D. E. Croell, and G. N. DeMartino, unpublished observations.}\]
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other sources because similar results have been obtained with the enzyme from rat liver and rat heart.²

The suggestion that autoproteolysis may be related to CDP activation also has been made previously, but as with the alteration of the Ca²⁺ concentration requirement, the physical basis for this has been unclear. For example, Pontremoli et al. (22), using CDP-I from erythrocytes, have claimed that the enzyme is activated as a result of autoproteolytic cleavage of the large subunit. Although these workers reported that the small subunit was concomitantly degraded they did not attribute any functional significance to its proteolysis. However, in experiments with CDP-I from bovine heart we have demonstrated that the small subunit of this enzyme undergoes a series of autoproteolytic cleavages which are indistinguishable from those observed for CDP-II. Furthermore cleavage of the 26-kDa subunit of CDP-I also appears to result in protease activation while formation of the 17-kDa peptide correlates with a reduction in the Ca²⁺-concentration requirement of the protease.³ Because the small subunits of CDP-I and CDP-II previously have been shown to be identical peptides (10), it seems reasonable to expect that they play the same role in the function of each enzyme. Interestingly, in agreement with the results of Pontremoli et al. (22) we also have observed that unlike CDP-II, the large subunit of CDP-I is autoproteolyzed during short preincubations with Ca²⁺. This event, however, occurs only after proteolysis of the 26-kDa subunit and its significance is unclear. Finally, several reports have suggested novel aspects of the mechanism of autoproteolytic activation of calcium-dependent proteases involving interaction of the proteases with substrates or red cell membranes; these interactions have been reported to lower the Ca²⁺ concentration requirement for protease activation (21–23). We are uncertain of the generality of such a mechanism because in related experiments with both CDP-I and CDP-II we consistently have failed to detect any effect of substrate on the Ca²⁺ concentration requirement for autolysis.³

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³ G. N. DeMartino, C. A. Huff, and D. E. Croall, unpublished observations.

⁴ CDP-I from bovine heart requires less Ca²⁺ for activity than CDP-II (30 μM Ca²⁺ versus 350 μM Ca²⁺ for half-maximal activity, Ref. 10). Autoproteolysis of CDP-I reduced its Ca²⁺ concentration requirement to 2 μM for half-maximal activity. G. N. DeMartino, C. A. Huff, and D. E. Croall, unpublished observations.