Regulation of the Peptidylglutamyl-Peptide Hydrolyzing Activity of the Pituitary Multicatalytic Proteinase Complex†

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Received December 27, 1990

ABSTRACT: The finding that the activity of the multicatalytic proteinase complex (MPC) is greatly activated by low concentrations of sodium dodecyl sulfate (SDS) and fatty acids led to the proposal that the proteolytic activity of the complex is latent and that activation is needed for expression of full activity. Kinetic examination of the nature of the latency with Cbz-Leu-Leu-Glu-2-naphthylamide, a substrate cleaved by the peptidylglutamyl-peptide hydrolyzing activity (PGPH activity) of the complex, showed that plots of velocity versus substrate concentration yield sigmoidal curves, implying the presence of two or more substrate binding sites and the presence of cooperative interactions between the sites. Hill plots of log $[v/(V_{max} - v)]$ versus log [S] gave slopes with a Hill coefficient of 2.2–2.4, suggesting that more than two subunits are expressing the PGPH activity. At saturating substrate concentrations, SDS and lauric acid exposed a masked component of PGPH activity that was about equal in magnitude to the overt activity measured in the absence of these detergents, showing that under the latter conditions only about half of the enzyme activity is expressed. Activation by SDS and lauric acid was greater at low than at high substrate concentrations and was associated with a shift of the substrate concentration at half-V_{max} (apparent K_m) toward lower values. The decrease in the apparent K_m in the presence of SDS (but not in the presence of lauric acid) was associated with a decrease in cooperativity. The presence of at least two distinct PGPH activity components with different reactivities was also indicated by the finding of two distinct inactivation rate constants in reactions with 3,4-dichloroisocoumarin, an irreversible inhibitor of the enzyme. About 80% of the overt activity was inactivated with a rapid second-order rate constant, while the remainder was inactivated at a slower rate that was equal to the rate of inactivation of the masked activity exposed by SDS. The PGPH activity of the MPC was strongly inhibited by low concentrations (10^{-6}–10^{-7} M) of protein present in pituitary homogenates and also by β-casein, lysozyme, and bovine serum albumin. Binding of these proteins to the enzyme caused a concentration-dependent shift of the S-shaped substrate saturation curves with an increase in apparent K_m without change in maximal velocity, a finding that could indicate negative allosteric effects or competitive inhibition. It is concluded that cooperative interactions between more than two subunits of the complex determine the expression of the PGPH activity of the MPC and that full expression of this activity results from conformational changes induced by SDS or lauric acid binding at a site or sites (allosteric sites) different from the substrate binding site. These phenomena are in a major way responsible for the apparent "latency" of the MPC.

The "multicatalytic proteinase complex" (MPC), first described in bovine pituitaries (Orlowski & Wilk, 1981, 1988; Wilk & Orlowski 1980, 1983), is a high molecular mass (~700 kDa) proteinase present in all eukaryotic cells. The complex is composed of multiple low molecular mass (21–34 kDa) nonidentical subunits and exhibits three distinct endopeptidase activities, cleaving peptide bonds on the carboxyl side of acidic, basic, and hydrophobic amino acids [for reviews, see Rivett (1989) and Orlowski (1990)]. The three activities are referred to as trypsin-like (cleavage on the carboxyl side of basic residues), chymotrypsin-like (cleavage on the carboxyl side of hydrophobic residues), and peptidylglutamyl-peptide hydrolyzing (PGPH-hydrolyzing; cleavage on the carboxyl side of glutamyl residues). Evidence was presented that each of these activities is associated with a different component of the complex (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983). Specificity studies indicated that the component that catalyzes cleavage of bonds on the carboxyl side of glutamyl residues also exhibits chymotrypsin-like activity, though the chymotrypsin-like activity of the MPC does not attack bonds after acidic amino acid residues (Orlowski & Michaud, 1989). It is now clear that the complex constitutes a major extralysosomal proteolytic system apparently involved in both ubiquitin-dependent and ubiquitin-independent pathways of intracellular proteolysis (Rechsteiner, 1987; Eytan et al., 1989; Driscoll & Goldberg, 1990).

Intracellular proteolysis must be a highly regulated process since the rate of intracellular protein degradation can potentially affect the orderly functioning of cellular metabolism and cause considerable damage if not adequately controlled. The presence of relatively high concentrations of the MPC in the cytoplasm of cells (Hendil, 1988) therefore poses an important question regarding regulation of its proteolytic activity. Early studies have indicated that the PGPH activity, one of the main activities of the MPC, is only partially expressed and that it can be greatly activated by low concentrations of sodium dodecyl sulfate (SDS)[1] (0.01–0.04%) and fatty acids (Orlowski

† This work was supported by Grant DK 25377 (to M.O.), by NRSA Fellowship HL 08254 from the National Institutes of Health, and by the Stony Wold-Herbert Fund (to C.C.). M.C.H. is a postdoctoral fellow of the University of Granada, Granada, Spain.

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& Wilk, 1981; Wilk & Orlowski, 1983). This activation has been later found to be a constant property of virtually all preparations of the MPC isolated from a variety mammalian and nonmammalian sources (Dahlmann et al., 1985; Ray & Harris, 1986; Tanaka et al., 1988). The extent of activation by SDS reported by different authors varied within a wide range, reaching values as high as 30-fold. Activation of some preparations of the MPC was also observed after short heating at 55–65 °C (Mykles, 1989) and by dialysis against distilled water. On the basis of these observations, it was proposed that the proteolytic activity of the complex is normally latent and that activation is needed for expression of its full intracellular activity (McGuire et al., 1989). The factors, however, affecting the expression of the proteolytic activity of the MPC remain to be identified.

We report here studies on the kinetics of hydrolysis of the synthetic substrate Cbz-Leu-Leu-Glu-2NA[1] and the kinetics of inactivation of the enzyme by 3,4-dichloroiscoumarin indicating that more than two subunits express the PGPH activity of the complex, and that cooperative phenomena between the subunits regulate the expression of this activity. Furthermore, SDS and lauric acid, two activators of the MPC binding at a site or sites different from the substrate binding site (allosteric site/s), were shown to expose a masked component of the PGPH activity that was not evident in the absence of these detergents. Evidence is also presented that the expression of the PGPH activity of the MPC can be greatly repressed by the presence of even small amounts of contaminating proteins. These findings can contribute in an important manner to the apparent latency of the MPC observed under different conditions.

Materials and Methods

Materials

Frozen bovine pituitaries were obtained from Pel Freeze Inc. (Rogers, AR). 3,4-Dichloroisocoumarin (Harper et al., 1985), bovine serum albumin, lysozyme, β-casein, and Z-Leu-Leu-Glu-2NA were obtained from Sigma Chemical Co. (St. Louis, MO). Z-Gly-Gly-Leu-pNA was synthesized as described previously (Wilk et al., 1979). Ultrogel-AcA-22 was obtained from Biotechnics Inc. (Savage, MD).

Methods

Determination of Enzyme Activity. The chymotrypsin-like and PGPH activities were determined with Z-Gly-Gly-Leu-pNA and Z-Leu-Leu-Glu-2NA, respectively, as described previously (Wilk & Orlowski, 1980, 1983). Measurements are based on determination of the amounts of aromatic amines released from the synthetic substrates using a diazotization procedure. Incubations were at 37 °C. Activity is expressed in units, 1 unit being defined as the amount of enzyme that liberates 1 μmol of product/h. Specific activity is expressed in units per milligram of protein.

Purification of the Enzyme. The enzyme was purified from bovine pituitaries as previously described (Orlowski & Michaud, 1989). The progress of the purification was followed by determining the activity with both Z-Leu-Leu-Glu-2NA and Z-Gly-Gly-Leu-pNA. A summary of the specific activities, recoveries, and extent of purification with each of the two substrates is given in Table I.

Inhibition Kinetics. Two microliters of a 1.0 mM solution of 3,4-dichloroisocoumarin in dimethyl sulfoxide (Me_2SO) was added to 198 μL of an enzyme solution (after step 7 of the purification procedure) containing 0.5–0.6 mg of protein/mL in 0.01 M Tris–EDTA buffer, pH 7.5. The mixtures were incubated at 25 °C in the absence and presence of 0.02% SDS. Aliquots of the incubation mixture (2–3 μg of enzyme) were then transferred at various time intervals to incubation mixtures (final volume 0.25 mL) at 37 °C containing 0.48 mM Z-Leu-Leu-Glu-2NA in 0.05 M Tris-HCl buffer, pH 8.0, for determination of residual enzyme activity as described above. Activity measurements were carried out both in the presence and in the absence of 0.02% SDS. The final concentration of Me_2SO did not exceed 4%. The pseudo-first-order inactivation constants were obtained from plots of $\ln(v_i/v_0)$ versus time. Correlation coefficients were generally better than 0.98.

[1] Abbreviations: 2NA, 2-naphthylamide; pNA, *p*-nitroanilide; SDS, sodium dodecyl sulfate; Z, benzyloxycarbonyl; Me_2SO, dimethyl sulfoxide.

Table I: Summary of Purification of the Multicatalytic Proteinase Complex from Bovine Pituitaries[a]

purification step	peptidylglutamyl-peptide hydrolyzing		chymotrypsin-like[b]	
	sp act.	recovery	sp act.	recovery
(1) homogenate	0.008	100 (0)	0.0069	100 (0)
(2) supernatant	0.023	105 (2.7)	0.019	80 (2.8)
(3) ammonium sulfate	0.099	76 (12)	0.067	37 (9.7)
(4) DEAE-Sephacel chromatography (pH 7.34)	0.87	62 (109)	1.13	56 (164)
(5) Ultrogel chromatography (pH 8.3)	13.2	183 (1650)	4.08	52 (591)
(6) DEAE-Sephacel chromatography (pH 8.3)	26.2	286 (3275)	4.34	33 (629)
(7) Ultrogel chromatography (pH 7.5)	25.1	114 (3140)	16.5	35 (2390)

[a] The PGPH activity and the chymotrypsin-like activities of the MPC were determined with 0.8 mM Cbz-Leu-Leu-Glu-2NA and 0.4 mM Cbz-Gly-Gly-Leu-pNA as substrates, respectively. [b] Data from Orlowski and Michaud (1989). Specific activity is defined as described under Materials and Methods. Values in parentheses indicate extent of purification.

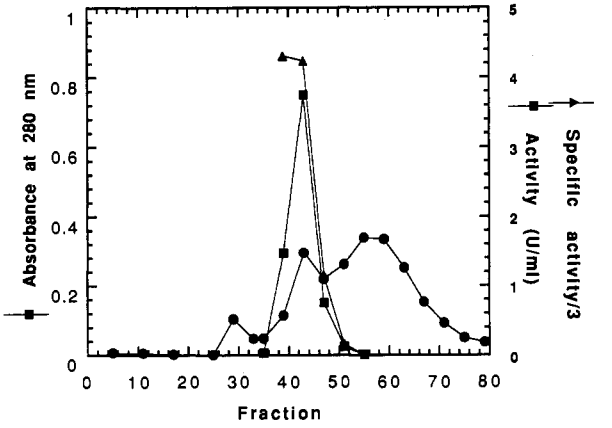

FIGURE 1: Elution profile of protein and of the peptidylglutamyl-peptide hydrolyzing activity of the MPC from an Ultrogel-Ac22 column (step 5 of the purification; Table I).

Results

The purification and recovery data for the PGPH activity and the chymotrypsin-like activity of the complex are shown in Table I. It is notable that while only about 35% of the initial chymotrypsin-like activity was recovered after step 7 of the purification, the apparent recovery of the PGPH activity amounted to more than 100%. Indeed, after step 6, the total amount of this activity exceeded by almost 3 times the amount measured in the initial homogenate. This suggested that the activity in the homogenate and after the initial purification steps was underestimated because of repression by inhibitory proteins. That this is indeed the case became evident from examination of the specific activity of the fractions emerging from the Ultrogel-AcA 22 column in the first molecular sieving

Table II: Inhibition of the Peptidylglutamyl-Peptide Hydrolyzing Activity of the Complex by Proteins[a]

protein	amount (μg)	concn (μM)	inhibn (%)
fraction 53	2	0.1	79
fraction 57	2	0.1	68
fraction 61	2	0.1	79
lysozyme	3	0.9	70
casein	1.5	0.25	74
bovine serum albumin	60	3.6	70

[a] Incubation mixtures contained Cbz-Leu-Leu-Glu-2NA (0.64 mM) as the substrate, enzyme (1.7 μg), Tris-HCl buffer (0.05 M; pH 8.0), and proteins in the amounts indicated in a final volume of 0.25 mL. Controls contained only the same additions except for the proteins.

chromatography purification step (step 5 Table I and Figure 1). While the initial fractions within the activity peak showed a uniform specific activity, a precipitous decrease occurred in the trailing fractions, indicating possible contamination with inhibitory proteins. This was tested by determining the effect of proteins present in fractions emerging from the Ultrogel column (step 5, Table I) immediately after the main activity peak on the activity of the isolated MPC (after step 7 of the purification procedure). The data summarized in Table II show that addition of as little as 2 μg of protein from these fractions to incubation mixtures containing the pure enzyme caused a 70–80% inhibition of the PGPH activity. Assuming that the approximate molecular weight of the inhibitory proteins (based on their elution volume from the Ultrogel column) is about 100 000, it can be calculated that concentrations as low as 10^{-7}–10^{-8} M of these proteins caused a pronounced inhibition of activity. It remains to be established whether this inhibition is a property specific for only a single protein present in pituitary homogenates or whether it is the result of a high-affinity enzyme–substrate interaction between the PGPH activity of the complex and proteins in general. The finding that several other proteins such as lysozyme, β-casein, and bovine serum albumin (BSA) were also inhibitory argues for the latter possibility. Of the proteins examined, β-casein, a good substrate of the MPC, inhibited at the lowest concentrations, whereas much higher concentrations of BSA were required for inhibition. Inhibition of PGPH activity of the complex even by very low concentrations of proteins derived from pituitary homogenates stresses the importance of using highly purified preparations of the MPC for the evaluation of its activity. Furthermore, the data indicate that some of the "latency" of the MPC reported in the literature could have resulted from the use of insufficiently purified enzyme preparations and inhibition of activity by contaminating proteins.

Examination of the kinetics of the PGPH activity of the MPC with Cbz-Leu-Leu-Glu-2NA as the substrate showed that plots of reaction velocity (v) as a function of substrate concentration ([S]) (Figure 2) yielded distinct sigmoidal curves. Double-reciprocal plots (not shown) yielded curves concave upward. Both types of plots are generally interpreted as indicating the presence of positive cooperative effects. The apparent K_m, defined as substrate concentration at half-V_{max}, was 290 μM, and the V_{max} was about 29 units/mg. Addition of 1.5 μg each of either albumin, lysozyme, or casein caused a shift in the apparent K_m toward higher substrate concentrations with preservation of the sigmoidal shape of the plots and with no apparent change in maximal velocity. This pattern of inhibition suggested competition between the proteins and the synthetic substrate for the active site of the PGPH activity, but could also have resulted from negative allosteric effects. It is of interest that concentrations of the same proteins as high as 13–100-fold greater than those used here had no effect on the chymotrypsin-like and trypsin-like activities of the complex

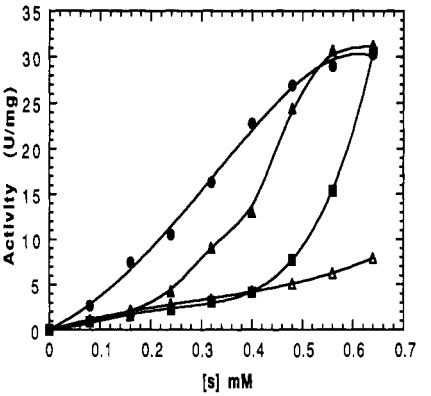

FIGURE 2: Effect of proteins on the peptidylglutamyl-peptide hydrolyzing activity of the MPC. All reaction mixtures contained Cbz-Leu-Leu-Glu-2NA at the indicated concentrations, Tris-HCl buffer (0.05 M; pH 8.0), and the MPC (1.65 μg of protein) after step 7 of purification (Table I) in a final volume of 0.25 mL. Tubes contained in addition either 1.5 μg of casein (△), 1.5 μg of lysozyme (■), 1.5 μg of bovine serum albumin (▲), or no added protein (●) (control). Activity was determined as described under Materials and Methods and expressed in units per milligram of protein. One unit is defined as the amount of enzyme that liberates 1 μmol of product per hour.

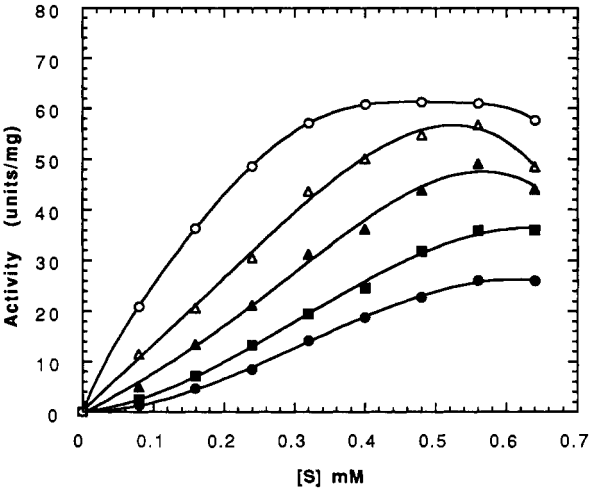

FIGURE 3: Effect of SDS on the rate of hydrolysis of Cbz-Leu-Leu-Glu-2NA as a function of substrate concentration. Reaction mixtures (final volume 0.25 mL) contained enzyme (1.65 μg of protein after step 7 of the purification procedure), substrate, and Tris-HCl buffer (0.05 M; pH 8.0). The concentrations of SDS were (○) 0.02%, (△) 0.016%, (▲) 0.012%, (■) 0.004%, and (●) 0% (control). Definition of units is the same as given in Figure 2.

(data not shown). Of the three proteins, casein was most inhibitory, while albumin was a relatively weak inhibitor; 1.5 μg (4×10^{-7} M) of lysozyme caused doubling of the apparent K_m, indicating high affinity of this protein for the enzyme. In separate experiments (data not shown), it was shown that concentrations of casein as low as 10^{-8} M caused a 50% inhibition of the enzyme at a substrate concentration of 0.64 mM.

The effect of SDS on the rate of reaction is shown in Figure 3. With an increase in SDS concentration, there was a progressive increase in the reaction rate and V_{max} with a shift of the substrate concentration at half-maximal velocity toward lower substrate concentrations. The sigmoidicity of the curves was generally preserved at low SDS concentrations but seemed to decrease at optimal SDS concentrations (0.02%), indicating loss of cooperativity. At these concentrations, SDS caused a pronounced activation, with the degree of activation being higher at low substrate concentrations and decreasing with approach to saturation. Thus, for example, the activation was

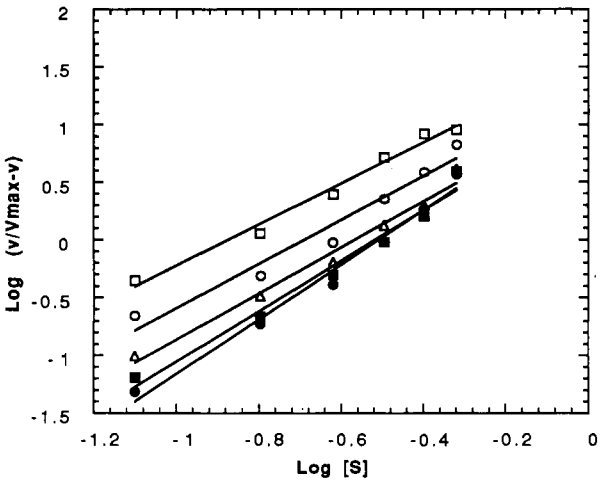

FIGURE 4: Hill plots of the data from Figure 3. The substrate concentrations for the calculation of log [S] were from 0.08 to 0.48 mM, a range below values for which substrate inhibition becomes evident.

about 8.5-fold at a substrate concentration of 80 μM, whereas at saturation the activation was only about 2-fold. It is notable that in the presence of lysozyme, β-casein, or BSA at concentrations similar to those given in Figure 2, maximal velocities obtained in the presence of optimal SDS concentrations were reached at substrate concentrations similar to those obtained in the absence of these proteins (see Figure 3), suggesting decreased protein binding. As a result, the degree of activation under such conditions was more than 30-fold because the basal activity in the absence of the detergent was markedly repressed by the presence of protein. This finding suggests that the degree of activation by SDS increases in the presence of some contaminating proteins and that consequently the high activations (30-fold) reported in the literature could have resulted from the use of insufficiently purified enzyme preparations, augmenting thereby the impression of enzyme latency.

Activation by SDS was associated with a shift in the velocity–substrate saturation curves with a decrease in the apparent K_m to 145 μM. The simultaneous increase, however, in the maximal velocity of the reaction in the presence of SDS also indicated that the detergent does not act as a simple allosteric activator but also exposes a component of the MPC not evident in its absence. It is notable that in the presence of optimal concentrations of SDS and of saturating substrate concentrations the increase in activity was about equal to that present in the absence of the detergent, causing as a consequence doubling of the activity. This indicated that the component exposed by SDS is about equal in activity to that measured in its absence. This component could not be detected by increasing substrate concentrations in the absence of detergent, and indeed an increase in the concentration of Cbz-Leu-Leu-Glu-2NA beyond 0.64 mM caused substrate inhibition, complicating kinetic evaluation of the reaction at higher substrate concentrations.

The presence of sigmoidal kinetics and the pattern of activation by SDS suggested that the PGPH activity of the MPC behaves as an allosteric enzyme and that at least two components of activity must be assumed to be present: one overt activity evident in the absence of the detergent and the other masked and becoming exposed in its presence. Plots of log [S] versus log $[v/(V_{max} - v)]$ (Hill plots) were linear at substrate concentrations between 0.08 and 0.48 mM with correlation coefficients of 0.98–0.99 and a Hill coefficient for the reaction in the absence of the detergent of 2.4 (Figure 4).

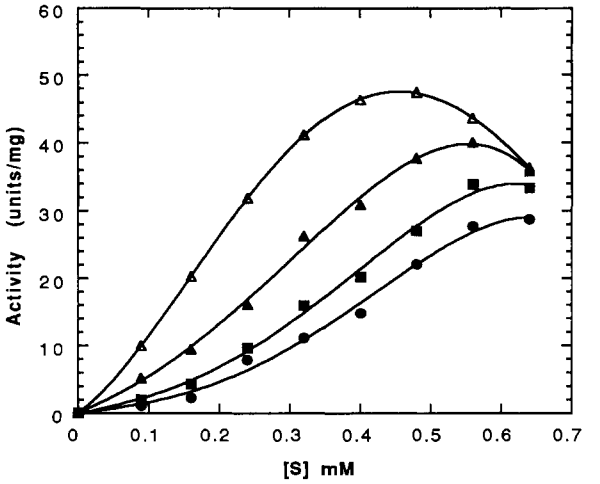

FIGURE 5: Effect of lauric acid on the rate of hydrolysis of Cbz-Leu-Leu-Glu-2NA as a function of substrate concentration. The concentrations of lauric acid were (△) 2.4, (▲) 1.6, and (■) 0.8 mM; (●) control (no lauric acid). Other conditions were the same as given in the legend to Figure 2.

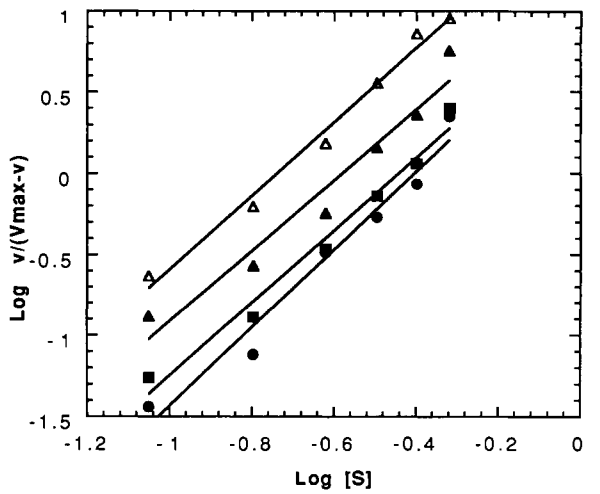

FIGURE 6: Hill plots of the data from Figure 5. Other conditions were the same as those described in legends to Figures 2 and 4.

With increasing SDS concentration, the Hill coefficient progressively decreased to a value of 1.79 at 0.02% SDS, suggesting a progressive loss of cooperativity.

In view of the finding that fatty acids activate the PGPH activity of the complex in a manner similar to that observed for SDS, it was of interest to examine the effect of some fatty acids on the kinetics of this reaction. Figure 5 shows the effect of increasing concentrations of lauric acid on the kinetics of the reaction. As in the experiments with SDS, increasing concentrations of lauric acid caused a shift in the apparent K_m toward lower substrate concentrations with a concomitant increase in V_{max}. At optimal concentrations, the maximal velocity measured in the presence of lauric acid was again almost twice as high as that in its absence. Unlike the decrease in cooperativity found with SDS, increases in velocity with increasing concentrations of lauric acid occurred with preservation of the sigmoidal shape of the velocity curve, suggesting preservation of cooperative effects. Hill plots (Figure 6) were linear in the concentration range of 0.08–0.48 mM with a Hill coefficient in the range of 2.2–2.4 and a correlation coefficient of 0.98–99.

Activation by SDS indicated the presence in the complex of two PGPH activity components with approximately equal activity; one overt and reactive in the absence of detergent and the other masked and becoming exposed in the presence of the

Table III: Kinetic Parameters of Inactivation of Components of the Peptidylglutamyl-Peptide Hydrolyzing Activity by 3,4-Dichloroisocoumarin[a]

condn of measurement	$t_{1/2}$ (min)	k_{obs}/I (s^{-1} M^{-1})
(1) preincubn in absence of SDS (plot A, Figure 7)	14.9	78
(2) condn as in (1) (plot B, Figure 7)	59	20
(3) preincubn in presence of 0.02% SDS; assay in absence of SDS (plot C, Figure 7)	58	20
(4) preincubn as in (3); assay in presence of 0.02% SDS (plot D, Figure 7)	61	19

[a] The concentration of 3,4-dichloroisocoumarin was 10 μM in all experiments. Data for (1) and (2) are mean values obtained from four separate determinations. Data for (3) and (4) were obtained from single experiments. The details of experimental conditions are given in the legend to Figure 7.

detergent. Furthermore, the detergent caused a shift in the apparent K_m of the overt component toward lower values (lowering of the apparent K_m) and produced a substrate–velocity plot with a single V_{max} and apparent K_m. It was therefore of interest to determine whether the two components would also show differences in reactivity with 3,4-dichloroisocoumarin, an effective irreversible inhibitor of the MPC. Figure 7 shows data obtained when the enzyme was incubated with 3,4-dichloroisocoumarin (10 μM) in the absence and presence of 0.02% SDS. Plots of log remaining activity versus time in the absence of detergent showed the presence of two components with distinct inactivation rate constants. The initial rate of inactivation was rather fast, having a $t_{1/2}$ of about 15 min and a second-order inactivation rate constant, k_{obs}/I, of about 80 s^{-1} M^{-1} (Table III; Figure 7, plot A). After inactivation of about 80% of the initial activity the reaction rate slowed by almost 4-fold (Figure 7, plot B; Table III). When the enzyme was preincubated with 3,4-dichloroisocoumarin in the presence of 0.02% SDS, and aliquots of the preincubation mixture were subsequently assayed for activity either in the presence or in the absence of SDS, the $t_{1/2}$ and the inactivation rate constants (Figure 7, plots C and D; and Table III) were almost identical with those observed for the slow component (Figure 7, plot B) seen in the absence of the detergent. These data are consistent with the presence of two PGPH activity components, one with a low substrate affinity and a fast inactivation rate constant and the other with a high substrate affinity and a slow inactivation rate constant. Furthermore, the data also indicate that the kinetic parameters with both substrate and inhibitor of the overt component (low affinity and fast inactivation) change when exposed to SDS and become identical with those measured in the presence of SDS. This also suggests that the two activities are probably expressed by identical subunits and are not the expression of subunits with overlapping specificities positioned on different nonidentical subunits of the complex.

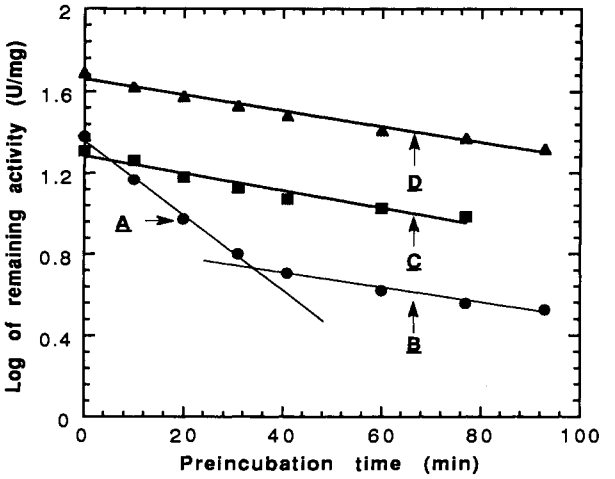

FIGURE 7: Inactivation of the peptidylglutamyl-peptide hydrolyzing activity by 3,4-dichloroisocoumarin. Preincubation mixtures in the absence of SDS (plots A and B) contained 0.198 mL of enzyme (after step 7 of the purification; about 0.5 mg of protein/mL) and 2 μL of a 1 mM solution of 3,4-dichloroisocoumarin in dimethyl sulfoxide (final inhibitor concentration 10 μM). Preincubation mixtures in the presence of SDS contained 0.194 mL of enzyme, 4 μL of a 1% SDS solution (final concentration 0.02%), and 2 μL of 3,4-dichloroisocoumarin in dimethyl sulfoxide. Aliquots of the mixtures (4–5 μL) were transferred at indicated time intervals into reaction mixtures at 37 °C (final volume 0.25 mL) containing 0.48 mM Cbz-Leu-Leu-Glu-2NA in Tris-HCl buffer (0.05 M; pH 8.0) for determination of enzyme activity either in the presence (plot D) or in the absence of 0.02% SDS (plots A, B, and C). Preincubations were at 25 °C. Controls in which the enzyme was preincubated in the absence of inhibitor were also included in the experiments. Definition of units is the same as described in Figure 2.

Discussion

Evidence based on specificity studies and the effect of inhibitors and activators indicates that the MPC exhibits at least three distinct proteolytic activities designated as chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing, and that each of these activities is associated with a different component of the complex. The complex is composed of 12–13 nonidentical subunits with molecular masses ranging from 21 to 32 kDa. The association, however, of the three activities with defined subunits of the complex has not yet been determined nor is it even clear which of the subunits are proteolytically active. The possibility must also be considered that formation of a proteolytically active site, and thereby expression of proteolytic activity, requires the participation and interaction of sequences located on two subunits, since attempts to isolate proteolytically active subunits have not been successful, and dissociation of the complex uniformly results in loss of all proteolytic activity. Furthermore, determination of amino acid sequences of some of the subunits showed the presence of homologies between subunits, but no homology with the amino acid sequences of any of the known proteases, nor any homology with the sequences around the active serine residues in the class of serine proteases [Lee et al., 1990; Lilley et al., 1990; and review by Orlowski (1990)].

Assuming an overall molecular mass of the complex of 700–750 kDa, and an average molecular mass of a subunit of 26–27 kDa, the total number of subunits in the complex amounts to about 28 ± 2 subunits. This number of subunits is also consistent with the electron microscopic picture of the complexes isolated from different sources, which shows a consistent pattern composed of four stacked rings each containing six to eight subunits (Kopp et al., 1986; Arrigo et al., 1988). Consequently, some of the subunits must be represented 2 or more times. Indeed, the banding pattern of the complex after separation by polyacrylamide gel electrophoresis under dissociating conditions and the intensity of staining of different bands by Coomassie brilliant blue indicates that some of the subunits may be represented as many as 4 times. The possibility must therefore be considered that each of the proteolytic activities is associated with more than a single subunit or with more than a pair of subunits if formation of a proteolytically active site would require the interaction of two subunits.

Of the three active components of the complex, the PGPH activity, which also attacks bonds on the carboxyl side of hydrophobic residues and therefore exhibits chymotrypsin-like activity, is of particular interest. It is one of the most active components of the complex; it is greatly activated by low

concentrations of SDS and fatty acids and interacts with proteins as shown by inhibition of cleavage of synthetic substrates even by low concentration of various proteins. The great activation of this component of the complex by fatty acids and low concentrations of SDS led to the proposal that the in vivo proteolytic activity of the complex is latent and that activation is required for expression of its full activity. These considerations induced us to examine in greater detail factors that determine the expression of this activity.

Plots of the rate of degradation of Cbz-Leu-Leu-Glu-2NA versus substrate concentration consistently produced sigmoidal curves, and double-reciprocal plots of the same data gave curves concave upward, both generally associated with allosteric enzymes and indicating the presence of positive cooperativity (Levitzki & Koshland, 1976). The presence of positive cooperativity would necessitate the assumption of at least two substrate binding sites and two subunits participating in the catalytic process. Indeed, Hill coefficients obtained from plots of log $[v/(V_{max} - v)]$ versus log [S] gave values of 2.2–2.4, suggesting the participation in catalysis of more than two and possibly as many as four subunits.

That the PGPH activity is expressed by at least two components of the complex is also indicated by two independent lines of experimental evidence. The first relates to the activating effect of low concentrations of SDS and fatty acids, and the second to the kinetics of inactivation by 3,4-dichloroisocoumarin. At saturating substrate concentrations and in the presence of optimal concentrations of SDS or lauric acid, the PGPH activity was about twice as high as in the absence of the detergents. This indicated the presence of a masked activity expressed only in the presence of the detergents that was equal to the overt activity expressed in the absence of the detergents. The exposure of the masked activity was associated with an apparent increase in affinity (decrease in the apparent K_m) toward the synthetic substrate, indicating that the detergents do not exert their effect by binding to the active site but rather by inducing changes in activity through conformational changes as a result of binding to allosteric sites of the complex.

The presence of two components expressing the PGPH activity is also supported by the demonstration of a biphasic inactivation rate with 3,4-dichloroisocoumarin. About 80% of the activity measured in the absence of SDS was inactivated with a short $t_{1/2}$ and a high second-order inactivation rate constant (k_{obs}/I). The remainder was inactivated with a longer half-life and a lower rate constants. In the presence of SDS, only a monophasic inactivation rate was observed, with a slow $t_{1/2}$ and a low k_{obs}/I, both almost identical with those measured in the second inactivation phase observed in the absence of the detergent. This behavior would be consistent with the interpretation that the PGPH activity of the MPC is expressed by two components, one of which is evident in the absence of the detergent and the other being expressed only in the presence of the detergent. The component active in the absence of detergent (overt component) shows a low substrate affinity but is rapidly inactivated by 3,4-dichloroisocoumarin, whereas the component exposed after addition of SDS (masked component) shows a high substrate affinity but a slow inactivation rate with 3,4-dichloroisocoumarin. The finding that the biphasic inactivation kinetics and the high apparent K_m of the overt activity are changed into monophasic inactivation kinetics and a single, lower apparent K_m in the presence of the detergent favors the conclusion that both components of the PGPH activity are associated with identical subunits. Exposure of the full activity in the presence of the detergent is probably due to conformational changes that make all the active sites equally accessible toward the substrate and inhibitor.

The structure of the complex with its multiple nonidentical subunits makes an attempt to determine the number of subunits expressing the PGPH activity on the basis of kinetic experiments alone difficult if not impossible. The sigmoidal relationship between the reaction rate and substrate concentration manifested by the overt activity alone would imply participation of at least two subunits. Hill coefficients greater than 2 and the presence of a masked activity equal in magnitude to the overt activity would argue for the participation of as many as four subunits. While these studies were in progress, Arribas and Castano (1990) reported on kinetics of hydrolysis of Z-Leu-Leu-Glu-2NA by the MPC isolated from rat liver. They find a Hill coefficient of 1.6 and assume the presence of two components, a high-affinity component with a low V_{max} and a noncooperative component with a lower affinity and higher V_{max}. SDS caused a decrease in cooperativity but only a slight increase in V_{max}. Thus, these results differ from those found for the pituitary MPC in the magnitude of the Hill coefficient and the effect of SDS and lauric acid, both acting as allosteric activators and causing the appearance of a masked component equal in magnitude to the overt component. It remains to be established whether these differences express differences in the subunit organization of the MPC derived from different tissues and species. Determination, therefore, of the exact number of subunits expressing the PGPH activity must await the synthesis of specific ligands capable of covalently modifying the amino acids participating in this reaction.

Examination of the specific activity of the PGPH activity at the different purification steps (Table I) points to the importance of using highly purified preparations of the complex for appropriate evaluation of its kinetic behavior. Indeed, the repression of activity by even minute protein impurities is the main cause of the initial observations that cleavage of Z-Leu-Leu-Glu-2NA shows first-order kinetics (Wilk & Orlowski, 1980, 1983). The PGPH activity of the MPC is greatly repressed in crude homogenates by the presence of various proteins, and the extent of this repression is evident from the fact that the number of activity units recovered after several purification steps was apparently greater than that measured in the crude homogenates. That this repression of activity is caused by proteins is also shown by the finding that the PGPH activity measured with Cbz-Leu-Leu-Glu-2NA as the substrate is inhibited 70–80% after addition of proteins derived from pituitary homogenates and also several other proteins in amounts that were on a weight basis similar or smaller than those of the enzyme protein present in the incubation mixtures (Table II).

The activation of the MPC by SDS is the main basis for the proposal that the MPC is latent and requires activation for expression of full activity. The cooperativity phenomena described here can account for a major part of the reported "latency" of the enzyme. The degree of activation by both SDS and fatty acids was higher at low substrate concentrations than at saturating concentrations. This is related to the shift of the apparent K_m in the presence of SDS toward lower substrate concentrations and also partially to the decrease of cooperativity induced by the detergent. Since the maximal velocity obtained in the presence of SDS and attributed to the MPC was virtually the same for the isolated enzyme as for the enzyme suppressed by the presence of contaminating proteins, the degree of activation is higher in impure than in

pure enzyme preparations. This finding could account for the different degrees of activation (from 2- to 30-fold) described for enzyme preparations from different sources and accordingly for the different degrees of "latency" reported in the literature.

Current evidence indicates that the MPC participates both in ubiquitin-dependent and in ubiquitin-independent pathways of cellular proteolysis (Rechsteiner, 1987; Hough et al., 1987, 1988; Eytan et al., 1989; Driscoll & Goldberg, 1990). Functioning of cellular metabolism and even the integrity of the cell depends on an orderly intracellular protein turnover which in turn depends on regulation of intracellular proteolysis. Factors, therefore, that affect the proteolytic function of the complex merit careful exploration. The presence of several proteolytic activities in a multisubunit particle of high molecular weight having an orderly structure suggests the need for regulatory mechanisms in the expression of its function. Some indication of the functioning of such mechanisms was provided by the observation that inhibition of the chymotrypsin-like activity by Cbz-Gly-Gly-leucinal, a reversible transition-state analogue inhibitor, and by 3,4-dichloroisocoumarin, an irreversible inhibitor (at concentrations of 2–4 μM) of the same activity, causes a marked activation of the trypsin-like activity (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983; Orlowski & Michaud, 1989). The demonstration that the PGPH activity is composed of at least two components, that only part of this activity is manifested in the absence of detergent, that the expression of this activity is under positive cooperative control, and that it is affected by the presence of proteins points toward the presence of such regulatory mechanisms.

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