Components of the Bovine Pituitary Multicatalytic Proteinase Complex (Proteasome) Cleaving Bonds after Hydrophobic Residues†

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ABSTRACT: Two catalytic components of the multicatalytic proteinase complex (MPC, proteasome) designated as chymotrypsin-like (ChT-L) and branched chain amino acid preferring (BrAAP) cleave bonds after hydrophobic amino acids. The possible involvement of the ChT-L and peptidylglutamyl-peptide hydrolyzing (PGPH) activities in the cleavage of bonds attributed to the BrAAP component was examined. Several inhibitors of the ChT-L activity containing a phenylalaninal group did not affect the BrAAP activity at concentrations that were more than 150 times higher than their K_i values for the ChT-L activity. Concentrations of lactacystin that inactivated more than 90% of the ChT-L activity had no effect on the BrAAP activity. Concentrations of 3,4-dichloroisocoumarin (DCI) that inactivated the ChT-L activity activated by up to 10-fold the BrAAP activity toward synthetic substrates and by more than 2-fold the degradation of the insulin B chain in a reaction not inhibited by Z-LGF-CHO, a selective inhibitor of the ChT-L activity. These findings are incompatible with any significant involvement of the ChT-L activity in the cleavage of BrAAP substrates. Both the native and DCI-treated MPC cleaved the insulin B chain mainly after acidic residues in a reaction inhibited by Z-GPFL-CHO, an inhibitor of the BrAAP and PGPH activities. DCI exposure did not result in acylation of the N-terminal threonine in the active site of the Y subunit. These results suggest involvement of the PGPH activity in the cleavage of BrAAP substrates, but this conclusion is incompatible with DCI activation of the BrAAP activity and inactivation of the PGPH activity, and with the finding that proteins inhibiting the PGPH activity had no effect on the BrAAP activity. Rationalization of these contradictions is discussed.

The multicatalytic proteinase complex (MPC,[1] proteasome) is a multisubunit cytoplasmic and nuclear particle involved in the ubiquitin (Ub)-dependent and Ub-independent pathways of degradation of cytoplasmic, nuclear, and membrane-bound proteins. It is a cylindrical structure composed of four stacked rings each containing seven subunits (*1*−*3*). Each of the inner two rings contains seven different β-subunits, whereas the outer two rings contain seven α-subunits. Since each subunit is represented twice, the overall subunit structure can be presented as having the composition $\alpha_7\beta_7\beta_7\alpha_7$ (*4*, *5*). Work with lactacystin, a rather specific proteasome inhibitor, has established that an N-terminal threonine residue is involved in the catalytic mechanism of some of the β-subunits (*6*). Two other mechanism-based irreversible inhibitors, peptidyl-vinyl sulfones and 3,4-dichloroisocoumarin, have also been shown to react with the hydroxyl group of the N-terminal threonine in the catalytically active β-subunits (*7*, *8*). The catalytic function of the other four remaining β-subunits, if any, is not known. Similarly, the α-subunits were believed to have only structural functions, but a recent report credits one of the α-subunits with an RNAse activity (*9*). It seems therefore that knowledge of all the catalytic functions of the MPC is still incomplete.

Three catalytic activities, each associated with distinct components of the MPC, were identified in early studies. They were designated as chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl-peptide hydrolyzing (PGPH), on the basis of the nature of the amino acid residue providing the carbonyl group to the scissile bond (*3*, *10*−*13*). Work with irreversible inhibitors of the three activities has indicated that β-subunits X, Y, and Z of the mammalian proteasome are associated with expression of the ChT-L, PGPH, and T-L activities, respectively (*7*, *8*, *14*). The three activities can be irreversibly inactivated by exposure of the MPC to DCI (*15*), an agent that was shown to acylate the hydroxyl group of the N-terminal threonine in the X subunit (*8*, *16*).

X-ray diffraction studies of the proteasome from yeast *Saccharomyces cerevisiae* have identified the presence of three active sites associated with the Pre2, Pre3, and Pup1 subunits of the yeast homologues of the X, Y, and Z

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[1] Abbreviations: BrAAP, branched chain amino acid preferring; ChT-L, chymotrypsin-like; DCI, 3,4-dichloroisocoumarin; HPLC, high-pressure liquid chromatography; MCA, 7-amino-4-methylcoumaryl amide; MHC, major histocompatibility complex; MPC, multicatalytic proteinase complex; 2NA, 2-naphthylamide; pNA, *p*-nitroanilide; pAB, *p*-aminobenzoate; peptidyl-CHO, peptidyl aldehyde; PGPH, peptidyl-glutamyl-peptide hydrolyzing; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; Suc, succinyl; T-L, trypsin-like; Z, benzyloxycarbonyl.

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β-subunits of the mammalian enzyme. Like their counterparts in the yeast MPC, the X, Y, and Z subunits each contain an N-terminal Thr residue (*17*−*19*). Mutagenesis studies indicate that the Pre2, Pre3, and Pup1 subunits and their X, Y, and Z homologues in the mammalian MPC are involved in expression of the ChT-L, PGPH, and T-L activities, respectively (*8*, *20*, *21*), but the crystallographic studies do not exclude the possibility of the presence of additional active sites of yet undetermined structure and mechanism. The catalytic functions of the remaining four β-subunits, one of them the N3 subunit having an N-terminal Thr residue, are not known.

Cleavage of bonds after hydrophobic amino acid residues is catalyzed by at least two distinct catalytic components (*22*). One, designated as ChT-L, is capable of cleaving peptidyl−arylamide bonds after aromatic or branched chain amino acid residues in such model synthetic substrates as Z-Gly-Gly-Phe-*pAB (pAB being *p*-aminobenzoate; the asterisk denotes the site of cleavage), Z-Gly-Gly-Leu-*pNA (pNA being *p*-nitroanilide) (*3*, *10*−*12*), or suc-Leu-Leu-Val-Tyr-*MCA (MCA being methylcoumarylamide). The second, designated as BrAAP, cleaves preferentially bonds after the branched chain amino acids Leu, Ile, and Val, in synthetic substrates and natural peptides, including neurotensin and the connecting peptide (C-peptide) of proinsulin (*13*, *22*). This component is resistant to inactivation by DCI (*22*), an irreversible inhibitor of the ChT-L, PGPH, and T-L activities of the MPC. Indeed, exposure of the proteasome to DCI, rather than inhibiting activity, greatly accelerates the degradation of a series of natural peptides, including neurotensin, the B chain of insulin, and casein (*22*, *23*). Experiments with [^{14}C]DCI have shown that the inactivation of the ChT-L activity is associated with acylation of the hydroxyl group of the N-terminal threonine in the active site of the X subunits of the pituitary MPC (*8*). Examination of products formed during degradation of neurotensin and proinsulin by the MPC treated with DCI has shown that the predominant cleavage site in neurotensin was the carboxyl-terminal Ile^{12}−Leu^{13} bond, whereas in proinsulin, cleavage occurred at the Leu^{44}−Ala^{45} and Val^{39}−Gly^{40} bonds of the C-peptide (*22*). In two of the three cleavage sites, a Pro residue was found in the P_3 position, as shown in the sequence of the C-terminal portion of neurotensin, Arg-Pro-Tyr-Ile*-Leu, and the sequence around the cleavage site in the C-peptide of proinsulin, Gly-Pro-Gln-Val-*Gly.

On the basis of these findings, we synthesized two model substrates, Z-Gly-Pro-Ala-Leu-*Ala-pAB and Z-Gly-Pro-Ala-Leu-*Gly-pAB. These substrates were cleaved almost exclusively at the Leu−Ala and Leu−Gly bonds, respectively, by an activity of a component that was resistant to inactivation by DCI. Indeed, activity toward these substrates was activated by up to 10-fold by pretreatment of the MPC with DCI. An enzyme assay in the presence of excess aminopeptidase N that led to the release of the aromatic amine (pAB) was therefore used to measure this activity (*13*). Other experiments indicated that the presence of a Pro residue in the P_3 position was unfavorable for substrate binding to the active site of the ChT-L activity and that peptidyl aldehyde inhibitors, such as Z-Gly-Pro-Phe-Leu-CHO and related compounds, are efficient inhibitors of the BrAAP activity but are poor inhibitors of the ChT-L activity (*24*). These findings suggested that the ChT-L activity as measured by the extent of cleavage of amino acid−arylamide bonds is catalyzed by a component that is distinct from that hydrolyzing the Leu−Gly and Leu−Ala bonds in Z-GPALG-pAB and Z-GPALA-pAB, respectively.

An open question is whether the BrAAP activity is a manifestation of an additional active site with a different mechanism of action, or instead is an expression of one or more of the three MPC activities catalyzed by the known active sites of the X, Y, and Z subunits. The strongest argument supporting the existence of a novel active site has been that at concentrations of DCI causing complete inactivation of the ChT-L, T-L, and PGPH activities of the bovine pituitary MPC, the BrAAP component is activated by up to 10-fold. Because experiments indicate that the BrAAP activity is an important factor in protein degradation by the MPC (*8*, *22*, *24*) and suggest an important role for this activity in cleaving bonds after branched chain amino acids [potentially important during processing of major histocompatibility complex (MHC) class I-restricted antigenic peptides], this question takes on functional importance. Here we report the results of experiments with peptidyl−aldehyde inhibitors, lactacystin, DCI, synthetic peptides, and the B chain of insulin aimed at examining the relationship between the ChT-L, PGPH, and BrAAP activities.

EXPERIMENTAL PROCEDURES

Isolation of the MPCs from Bovine Pituitaries. The pituitary MPC was isolated as previously described (*15*) from 200 g of bovine pituitaries (Pel Freeze, Rogers, AR) with minor modifications. The DEAE-Sephacel chromatography step (step 4) was replaced by chromatography on a DEAE-Toyopearl column (100 mL; TOSOHAAS, Montgomeryville, PA) at pH 7.34, and the second DEAE-Sephacel chromatography step at pH 8.3 (step 6) was eliminated. Elution was carried out with a linear gradient established between 500 mL of 0.01 M Tris-EDTA buffer (pH 7.34) and 500 mL of a 0.5 M Tris-EDTA buffer at the same pH. Fractions of 10 mL were collected and assayed for MPC activity. The active fractions were collected, concentrated by ultrafiltration, and subjected to chromatography on an Ultragel ACA22 column (5 cm × 50 cm) equilibrated with a 0.05 M Tris-EDTA buffer (pH 8.3). Active fractions (10 mL) were collected, concentrated, and rechromatographed on a Bio-Gel A-1.5m column of the same size, equilibrated with a 0.05 M Tris-EDTA buffer (pH 7.5). Active fractions were concentrated, and the buffer was exchanged with a 0.01 M Tris-EDTA buffer at the same pH by ultrafiltration. Aliquots of the enzyme (0.1 mL) containing 100 μg of enzyme protein were stored frozen at −70 °C and thawed before being used for experiments.

Isolation of highly purified preparations of the MPC required the use in the two molecular sieving steps of columns with a resin packing that provided sharp protein bands with minimum band broadening. Before chromatography, each column was therefore tested for performance by applying a solution of myoglobin and observing the pattern of the migrating protein bands. The final specific activity of the PGPH activity was particularly dependent on the purity of the preparation, probably because of the inhibitory effect of even small amounts of contaminating proteins on the activity of this component (see the Results). Thus, for

example, the specific activity of the PGPH activity in pure MPC preparations reached values of 20−30 μmol mg of MPC^{-1} h^{-1} at a substrate concentration (Z-LLE-2NA) of 0.64 mM, whereas the activity obtained from improperly packed columns could be lower by as much as 60−70%.

Synthesis of Peptidyl−Aldehyde Inhibitors. Peptidyl−aldehyde inhibitors were synthesized by oxidation of the corresponding peptidyl alcohols by a modification (*12*, *24*, *25*) of the dimethyl sulfoxide−carbodiimide reaction described by Pfitzner and Moffatt (*26*). The progress of the oxidation reaction was followed by assessing the formation of dinitrophenylhydrazones in a reaction with 2,4-dinitrophenylhydrazine (*27*).

Determination of Enzyme Activities. The ChT-L, PGPH, and BrAAP activities were determined as previously described using the substrates Z-GGF*-pAB, Z-LLE*-2NA, and Z-GPAL*G-pAB or Z-GPAL*A-pAB, respectively. The sites of substrate cleavage are marked by asterisks. Syntheses of substrates and methods of activity measurements were previously described (*10*−*13*, *28*). The level of cleavage of the BrAAP substrates was determined in a coupled enzyme assay in the presence of excess aminopeptidase N which permits the determination of pAB by diazotization after its release from the reaction product Gly-pAB or Ala-pAB.

Reactions with 3,4-Dichloroisocoumarin (DCI). Pituitary MPC (49 μL, 49 μg of protein) was treated with 1 μL of 2.5 mM DCI in dimethyl sulfoxide (final concentration of 50 μM) and incubated at 26 °C for 120 min. While the ChT-L activity is completely inactivated under these conditions after 30 min, the rather long preincubation period of 120 min was necessary to achieve a more than 90% inactivation of the PGPH and T-L activities. Exposure of the proteasome to DCI for more than 120 min was not practical because of hydrolytic modification of DCI. Aliquots of the enzyme, usually 1−3 μL, were withdrawn at different time intervals for determination of the ChT-L, PGPH, and BrAAP activities at substrate concentrations of 1.0, 0.64, and 1.0 mM, respectively. Incubation mixtures at 37 °C contained the appropriate substrate, Tris-HCl buffer (pH 8.0), enzyme, and inhibitor where indicated. The rate of release of the aromatic amine was determined as described above. Controls containing the same amount of DMSO but not DCI were also carried through the procedure. Under the conditions described above, the ChT-L and PGPH activities were virtually completely inactivated (see below), whereas the rate of cleavage of the BrAAP substrate increased up to 10-fold.

Reactions with Lactacystin. A 10 mM solution of lactacystin (*6*), a specific inhibitor of the MPC, was prepared in anhydrous dimethyl sulfoxide and then appropriately diluted for the required concentrations in experiments on inhibition of MPC components. Typically, to 25 μL of an MPC solution (25 μg of protein) and 24 μL of 0.05 M Tris-HCl buffer (pH 8.0) was added 1 μL of an appropriate solution of lactacystin to obtain the desired concentration. Aliquots of the enzyme were withdrawn at time zero and at different time intervals for determination of residual activity as described above. The pseudo-first-order inactivation rate constants (k_{obs}) were obtained from plots of $\ln(v_t/v_o)$ versus time.

Degradation of the B Chain of Insulin. The oxidized B chain of insulin (3.5 mg) (Sigma) was dissolved in 0.5 mL of 0.05 M Tris-HCl buffer (pH 8.5). A minimal amount of 1 M Tris base was then added to adjust the pH to 8.0, and the solution was brought to a final volume of 1 mL (final B chain concentration of 1 mM). In a typical incubation mixture, 4.5 μL of the B chain solution (final concentration of 100 μM), 4.5 μL of 0.2 M $MgCl_2$ (final concentration of 20 mM), either 1.0 μL of DMSO or 1.0 μL of peptidyl−aldehyde inhibitor, 33 μL of Tris-HCl (0.05 M, pH 8.0), and 2 μL of MPC (2 μg of protein) were incubated at 37 °C. Preliminary experiments showed that $MgCl_2$ markedly accelerated the degradation of the B chain. Aliquots (10 μL) of the solution were withdrawn at time zero and after 30 and 60 min and treated with 5 μL of glacial acetic acid. Ten microliters of the mixture was subjected to HPLC on a Deltapak C18 column (5 μm, 3.9 mm × 150 mm, 300 Å). To determine the rate of degradation of the B chain of insulin, elution was carried out with a linear 30 min gradient established between 2 and 40% acetonitrile each containing 0.1% trifluoroacetic acid. The flow rate was 1.0 mL/min, and the absorbance of emerging peaks was monitored at 210 nm. In addition to the native enzyme, degradation of the B chain of insulin was also examined using the MPC treated with 50 μM DCI as described above. To determine the concentration of the B chain at half-maximal velocity, reactions were started by adding 5 μL of the enzyme to tubes containing the oxidized B chain of insulin [2−12 μL of B chain in 50 mM Tris-HCl (pH 8.0)], 9 μL of 0.1 M $MgCl_2$, and 50 mM Tris-HCl buffer (pH 8.0) to a final volume of 90 μL. Aliquots (20 μL) were removed after 0 and 10 min and mixed with 10 μL of glacial acetic acid. Degradation of the oxidized B chain was followed by HPLC using 20 μL of the sample and the conditions described above. Reaction rates were determined on the basis of the decrease in the height of the peak of the oxidized insulin B chain over time. The concentration at half-maximal velocity for the degradation of the oxidized B chain of insulin by the DCI-treated MPC was determined at five substrates concentrations between 22 and 133 μM and was found to be about 20 μM.

To identify the major cleavage sites of the insulin B chain, reaction mixtures containing 40 μL of insulin B chain (final concentration of 440 μM), 4.5 μL of 0.2 M $MgCl_2$ (final concentration of 10 mM), 35.5 μL of 0.05 M Tris-HCl (pH 8.0), and 10 μL of MPC preincubated with either 50 μM DCI or DMSO for 2 h were incubated overnight at 37 °C. The reactions were then stopped by the addition of 5 μL of glacial acetic acid. Degradation products were separated by HPLC as described above and were collected manually in glass tubes. The molecular masses of peptides contained within these peaks were determined by mass spectrometry (Peptidogenics, Livermore, CA). Peptides were identified by comparison of the measured molecular mass to the molecular masses of all possible peptides resulting from fragmentation of the B chain of insulin using the mass spectrometry data analysis program Paws (version 6.6.1 for Macintosh computers).

Determination of Inhibition Constants. K_i values were determined by the method of Dixon (*29*) (plots of $1/V$ versus [I]) by starting the reaction with addition of the substrate to a mixture of the enzyme, inhibitor, and buffer. Three different substrate concentrations and six different inhibitor concentrations were used. The nature of the plots was consistent with predominantly competitive inhibition.

Table 1: Effect of Peptidyl−Aldehyde Inhibitors on the Chymotrypsin-like and Branched Chain Amino Acid Preferring Component of the Pituitary MPC[a]

	inhibitor	[I] (μM)	ChT-L activity K_i (μM)	[I] (μM)	BrAAP activity K_i (μM)
1	Z-LGF-CHO	0.5−2.5	1.1 ± 0.16 (6)	50−250	NI[b]
2	Z-LAF-CHO	0.5−2.5	1.5 ± 0.17 (6)	50−250	NI
3	Z-LVF-CHO	0.5−2.5	1.4 ± 0.07 (3)	50−250	NI
4	Z-LLF-CHO	1−5	1.4 ± 0.28 (3)	50−250	110 ± 9.2 (4)[c]
5	Z-LGL-CHO	0.25−1.25	1.76 ± 0.56 (3)	20−100	76.4 ± 1.2 (3)
6	Z-LAL-CHO	0.5−2.5	2.14 ± 0.21 (6)	5−25	12 ± 0.17 (3)
7	Z-LLL-CHO	0.2−2.5	6.9 ± 0.84 (6)	2−10	6.8 ± 0.9 (12)

[a] The ChT-L and BrAAP activities were determined as described in Experimental Procedures with Z-GGF-PAB and Z-GPALG-pAB as substrates, respectively. Three different substrate concentrations (0.5, 1.0, and 2.0 mM) and six different inhibitor concentrations were used to obtain the K_i values. [b] Neither the native nor the DCI-activated BrAAP activity was sensitive to inhibition. [c] Data from ref *30* indicate IC_{50} values, inhibitor concentrations that decrease the reaction rate by 50%.

Microsequencing of MPC. Forty-nine microliters of MPC was mixed with either 1 μL of DMSO or 1 μL of 2.5 mM DCI in DMSO (final concentration of 50 μM) and incubated at room temperature for 2 h. Samples of MPC (30 μL) were denatured by the addition of 3 μL of 10% SDS and 1.5 μL of β-mercaptoethanol, and incubation at room temperature for 10 min. Sucrose (3 grains) and bromphenol blue (3 μL of a solution containing 4 mg/mL) were then added, and 30 μL of this mixture was applied to lanes of a 30 cm long SDS−polyacrylamide gel (12% polyacrylamide). After separation by electrophoresis, proteins were electrophoretically transferred to Immobilon P polyvinylidine fluoride (PVDF) membranes in 0.01 M TAPS buffer (pH 8.4) containing 10% methanol as described previously (*8*). Proteins were visualized by staining with Coomassie Blue, and membranes were then washed with double-deionized water and air-dried. Bands containing the Y subunit were excised and subjected to microsequencing by Edman degradation.

RESULTS

A series of tripeptidyl aldehydes differing with respect to the nature of the aldehyde moiety and the amino acid residues in the P_2 position were tested for inhibition of the ChT-L and BrAAP activities of the pituitary MPC. Data obtained from the experiments are summarized in Table 1. Consistent with the finding that the ChT-L activity is capable of cleaving peptidyl−arylamide bonds in substrates containing either an aromatic or branched chain amino acid residue in the P_1 position, peptidyl−aldehyde inhibitors containing either a phenylalaninal or a leucinal residue were effective inhibitors of this activity. The nature of the amino acid residue in the P_2 position did not seem to have any significant effect on the K_i values of the phenylalaninal group of inhibitors (inhibitors 1−3), but increasing the hydrophobicity of the P_2 residue in the leucinal inhibitors seemed to cause some increase in the K_i values (inhibitors 5−7). By contrast, three of the peptidyl−aldehyde inhibitors containing a phenylalaninal group (inhibitors 1−3) had no significant inhibitory effect on the cleavage of the Leu−Gly bond in the BrAAP substrate Z-GPALG-pAB at concentrations of up to 250 μM. Similarly, the fourth inhibitor (inhibitor 4, Z-LLF-CHO) was a good inhibitor of the ChT-L activity but a weak inhibitor of the BrAAP activity. These results are consistent with previous conclusions that the presence of a Phe rather than of a Leu residue in the P_1 position does not favor binding of the substrate to the active site of the BrAAP component of the pituitary enzyme (XYZ-MPC) (*24*, *28*, *30*). The finding that several potent phenylalaninal inhibitors of the ChT-L activity do not inhibit the activity of the BrAAP component indicates that the ChT-L activity does not contribute significantly to the cleavage of the Leu−Gly bond of the BrAAP substrate. Unlike the phenylalaninal inhibitors, however, the degradation of the BrAAP substrate is significantly inhibited by the peptidyl−aldehyde inhibitors containing a leucinal residue (inhibitors 5−7 in Table 1), confirming the selectivity of this component toward branched chain amino acids in the P_1 position. The distinguishing properties of the leucinal inhibitors for the two components are much less prominent than those of the phenylalaninal inhibitors, and inhibitors such as Z-LLL-CHO inhibit the ChT-L, T-L, and PGPH activities in addition to inhibiting the BrAAP component, and can therefore be regarded as general, nonspecific proteasome inhibitors. It is interesting that the potency of inhibition of the BrAAP activity increases with an increase in hydrophobicity of the amino acid residue in the P_2 position of the leucinal group of inhibitors. This was also indicated by the potency of the previously synthesized BrAAP inhibitor Z-GPFL-CHO ($K_i = 1.5$ μM), which also contains a Phe in the P_2 position, and is so far the best selective BrAAP inhibitor (*24*).

Table 2: Inactivation Rate Constants k_{obs}/[I] of Components of the Pituitary Multicatalytic Proteinase Complex for Inactivation by Lactacystin[a]

component	substrate	lactacystin (μM)	$t_{1/2}$ (min)	k_{obs}/[I] (M^{-1} s^{-1})
ChT-L	Z-GGL-pNA	10	5.7 ± 0.36 (3)	204 ± 12 (3)
BrAAP	Z-GPALG-pAB	100	31.3 ± 0.9 (4)	3.7 ± 0.11 (4)
PGPH	Z-LLE-2NA	100	20.9 ± 0.59 (3)	5.53 ± 0.16 (3)

[a] The pituitary MPC was preincubated at 26 °C with lactacystin at the concentrations indicated in the table. Aliquots of the enzyme were withdrawn for the determination of activity at time zero and subsequently at different time intervals for the determination of residual activity. The pseudo-first-order inactivation rate constants was determined from plots of $\ln(v_t/v_0)$ vs time. Data are mean values ± the standard error with the number of determinations given in parentheses. Statistical evaluation of the results for the BrAAP and PGPH activities (*t* test) gave a *p* value of <0.0005.

Lactacystin was previously shown to be a selective inhibitor of proteasomal activity (*6*). To gain further information about whether the ChT-L or PGPH activities contribute to hydrolysis of the BrAAP substrate, we used this inhibitor to examine its effect on components of the MPC that could be involved in the cleavage of bonds after hydrophobic amino acids. Lactacystin irreversibly inhibited the ChT-L, PGPH, and BrAAP activities. The inactivation rate constants k_{obs}/[I] for inactivation by lactacystin of three components of the MPC are summarized in Table 2. Plots of $\ln(v_t/v_0)$ gave straight lines with a correlation coefficient of 0.99 or better. The ChT-L activity was the most sensitive to inactivation with a k_{obs}/[I] that is about 55 times higher than that for the BrAAP activity. Although the k_{obs}/[I] values for the PGPH were only about 50% higher than those for the BrAAP activity, statistical evaluation (*t* test) showed significant differences between the two values with $p < 0.0005$.

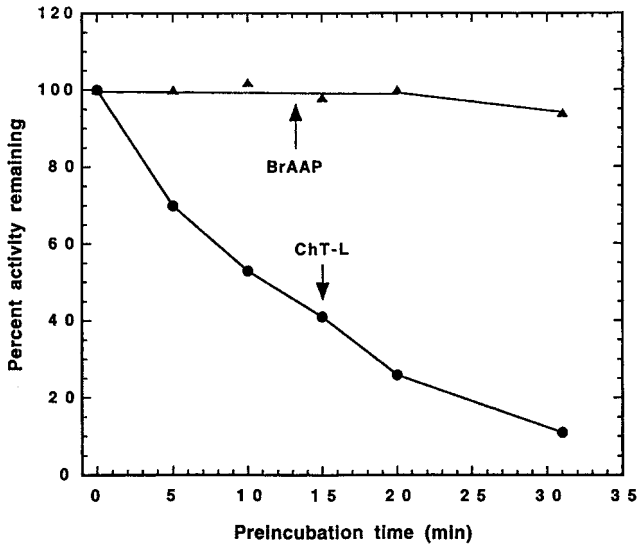

FIGURE 1: Effect of lactacystin on the ChT-L and BrAAP activities of the pituitary MPC. The pituitary MPC was preincubated with 10 μM lactacystin at 26 °C. Aliquots of the enzyme were withdrawn at time zero and at the indicated time intervals for determination of the ChT-L and BrAAP activities using substrates Z-GGF-PAB and Z-GPALG-pAB, respectively (final concentrations of 1 mM).

The high inactivation rate for inactivation of the ChT-L activity by lactacystin (Table 2) compared with that of the BrAAP activity made it possible to examine the question of whether the ChT-L activity contributes in any significant way to the degradation of the BrAAP substrate Z-GPALG-pAB. Exposure of the pituitary MPC to 10 μM lactacystin caused an about 90% loss of the chymotrypsin-like activity during the 30 min preincubation without a significant change during that time period in the BrAAP activity (Figure 1.). It should be noted that if the ChT-L activity were responsible for 60−80% of the degradation of the BrAAP substrate (*31*), a marked decrease of the ChT-L activity should have been observed under the conditions used in the experiment. The results shown in Figure 1 provide further evidence that the ChT-L activity plays no part in cleaving substrates of the BrAAP component.

It was interesting therefore to compare the effects of lactacystin to those of DCI, another irreversible inhibitor of the MPC, previously shown to inactivate the ChT-L, T-L, and PGPH activities of the MPC. Previous studies with [^{14}C]-DCI have shown that, like lactacystin, [^{14}C]DCI inactivation of the ChT-L activity is caused by acylation of the hydroxyl group of the active site N-terminal threonine of the X subunit (*8*). We have therefore examined the effect of exposure of the pituitary MPC to 50 μM DCI and determined the rate of inactivation of this activity. As shown in Figure 2, the ChT-L activity is virtually completely inactivated within 15 min under conditions that cause an up to 10-fold activation of the BrAAP activity. These findings are again inconsistent with involvement of the ChT-L activity in hydrolysis of bonds after Leu residues in substrates cleaved by the BrAAP component. Inactivation of the PGPH activity proceeded at a much slower pace; nevertheless, more than 90% of this activity was lost after 2 h of preincubation. By contrast, treatment with DCI caused an up to 10-fold activation of the BrAAP activity, and there was no apparent time-dependent relationship between the level of activation of the BrAAP activity and the level of inactivation of the PGPH

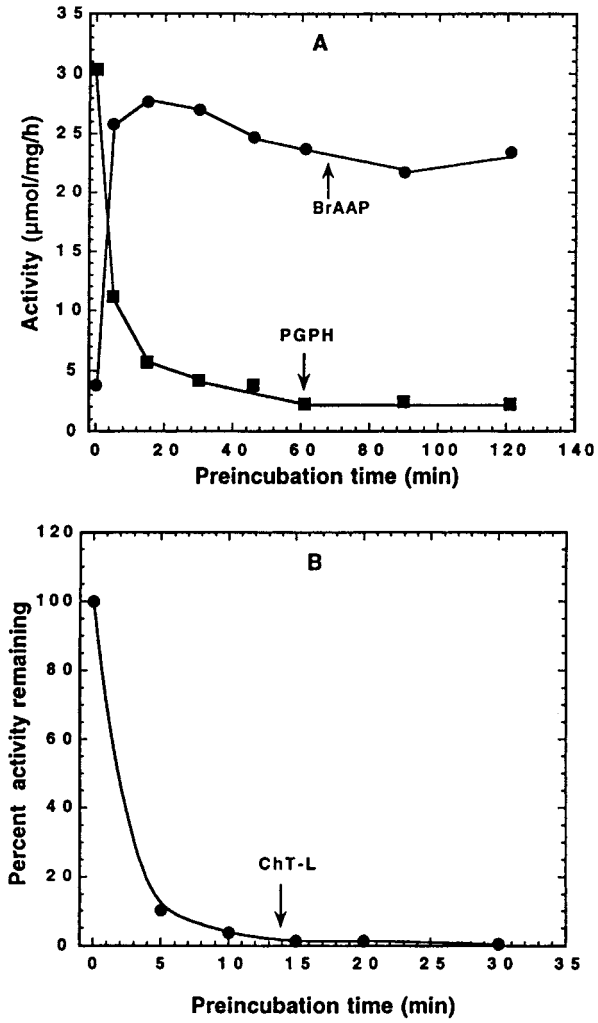

FIGURE 2: Effect of preincubation with 3,4-dichloroisocoumarin (DCI) on the PGPH, BrAAP (A), and ChT-L (B) activities of the pituitary MPC. The pituitary enzyme (49 μL, 1 mg of protein/mL) in 0.01 M Tris-EDTA buffer (pH 7.5) was exposed to 3,4-dichloroisocoumarin (1 μL of a 2.5 mM solution in dimethyl sulfoxide; final concentration of 50 μM) at 26 °C. Aliquots of the preincubation mixture (1−2 μL) were withdrawn at different time intervals and used for determination of the PGPH, BrAAP, and ChT-L activities as described in Experimental Procedures. Data for the PGPH and BrAAP activities are averages from three separate determinations, except for the points taken at times 0, 30, 60, and 120 min, which are averages obtained from four determinations. Because of the rapid inactivation of the ChT-L activity under the conditions that were used, residual activities were determined only up to 30 min.

activity. Thus, for example, the activation of the BrAAP activity was complete within the first 10−15 min, whereas the inactivation of the PGPH activity proceeded more slowly.

Low concentrations of sodium dodecyl sulfate (SDS) markedly increase the PGPH and BrAAP activities of the MPC, and increase the ChT-L activity toward the synthetic substrate suc-LLVY-MCA by changing kinetic parameters such as V_{max} and K_m. The effect, however, of SDS on inhibitor binding and inhibitor selectivity has not been properly examined. This question took on importance given the fact that when the BrAAP activity of rabbit muscle MPC was assayed in the presence of low concentrations of SDS, kinetic analysis of inhibition by tripeptide−aldehyde inhibitors suggested an important role for the ChT-L activity in cleaving a substrate of the BrAAP component (*31*). We

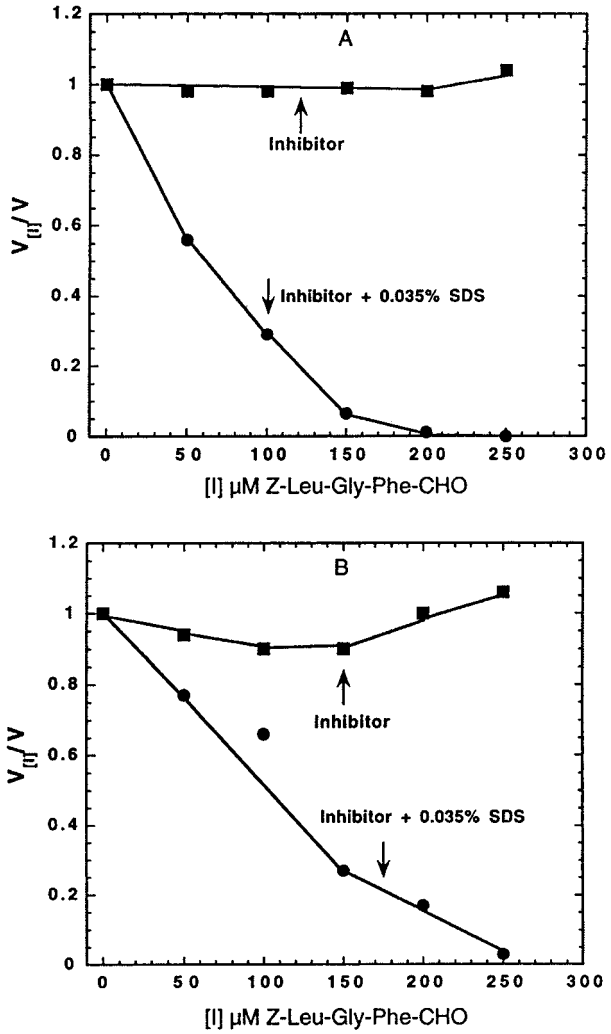

FIGURE 3: Effect of a tripeptidyl−aldehyde inhibitor of the chymotrypsin-like activity on the degradation of the BrAAP substrate by the native and SDS-treated MPC. Incubation mixtures contained MPC, purified aminopeptidase N (15 μg), the ChT-L activity inhibitor Z-LGF-CH0 (from 0 to 250 μM), 0.05 M Tris-HCl buffer (pH 8.0), 0.035% SDS where indicated, and 1 mM BrAAP substrate [(A) Z-GPALA-pAB and (B) Z-GPALG-pAB] in a final volume of 0.2 mL. Incubations were carried out for 30 min at 37 °C. The rate of the reaction was measured by the rate of release of pAB as described in Experimental Procedures. Controls in which the MPC and the aminopeptidase were omitted were also carried through the procedure. Rates of reaction are expressed as ratios obtained in the presence of inhibitor to those of controls in the absence of inhibitor ($V_{[i]}/V$). Data are mean values obtained from two (A) and three (B) independent determinations.

therefore examined the inhibition of the BrAAP activity by tripeptidyl−aldehyde inhibitors of the ChT-L activity in the absence and presence of 0.035% SDS. The results are presented in Figure 3. Consistent with the inhibitor studies (Table 1), Z-LGF-CHO, a potent inhibitor of the ChT-L activity, did not significantly affect the degradation of two BrAAP substrates (Z-GPALA-pAB and Z-GPALG-pAB) in the absence of SDS at concentrations as high as 250 μM. However, in the presence of SDS, the inhibitor exhibited marked inhibition of degradation of both BrAAP substrates, suggesting that the presence of the detergent causes great changes in the kinetic parameters of response to the inhibitor. Similar results were also obtained with two other inhibitors of the ChT-L activity, Z-LAF-CHO and Z-LVF-CHO (not shown). In addition, introduction of SDS (0.035%) to an incubation mixture containing the DCI-treated enzyme completely abolished the activation of degradation of the BrAAP substrate (not shown), further pointing to marked changes in the catalytic properties of the proteasome induced by the presence of this detergent.

Table 3: Inhibition of the PGPH and BrAAP Activities by Proteins[a]

protein	range of concentrations (μM)	IC_{50} (μM) PGPH	IC_{50} (μM) BrAAP
BSA	0.18−1.8	0.79	NI[b]
β-casein	0.086−0.652	0.4	NI
ubiquitin	0.472−2.83	1.6	NI
cytochrome *c*	0.154−1.54	0.74	NI
ovalbumin	0.454−4.54	3.9	NI
insulin	6.67−40	13	NI

[a] Effects of proteins on peptidase activities were determined in assays with 0.64 mM Z-Leu-Leu-Glu-2NA and 1 mM Z-GPALG-pAB as substrates for the PGPH and BrAAP activities, respectively, as described in Experimental Procedures. Incubation mixtures contained 1.43×10^{-8} M MPC. IC_{50} values for the PGPH activity were estimated by interpolation of plots of activity vs protein concentration for each of 10 protein concentrations within the indicated range. Effects of proteins on BrAAP activity were tested only at the highest concentrations indicated for each protein. BSA is bovine serum albumin. [b] NI means no inhibition.

The results showed marked differences in the sensitivity to inactivation of the BrAAP and PGPH activity by DCI and lactacystin. Other distinguishing properties between these activities were suggested by previous studies showing that the PGPH activity is quite sensitive to inhibition by low concentrations of various proteins (*32*). It was therefore interesting to examine whether the same proteins have a similar effect on the BrAAP activity. As shown in Table 3, low micromolar concentrations of different proteins were sufficient to cause a 50% inhibition of the PGPH activity, whereas the highest protein concentrations used in experiments with the PGPH activity had no effect on the BrAAP activity. The inhibitory effect of low protein concentrations on the PGPH activity is also responsible for the observation that pure MPC preparations from the pituitary have generally a specific PGPH activity of 20−30 μmol of substrate degraded mg of MPC^{-1} h^{-1}, whereas those with even minimal protein impurities have specific activities that are markedly lower (not shown). By contrast, the specific activity of the BrAAP component in purified MPC preparations is not materially affected by such protein impurities. These results suggest that low concentrations of a variety of proteins selectively interfere with the access of the Z-LLE-2NA substrate to the active site of the Y component, whereas the access of the BrAAP substrate to its active site is not affected by this interaction.

The finding that exposure of the MPC to DCI caused activation of degradation of several natural peptides, including the oxidized B chain of insulin and casein, made it important to determine whether differences in sensitivity to inhibitors between the BrAAP and ChT-L components extended beyond reactions with model synthetic substrates to naturally occurring peptides. We have therefore examined the effect of inhibitors of the ChT-L and BrAAP components on the degradation of the oxidized B chain of insulin by the native and DCI-treated enzyme. The MPC was exposed to 50 μM DCI for 120 min, conditions which virtually completely inactivated the ChT-L and PGPH activities

Table 4: Inhibition by Z-LGF-CHO and Z-GPFL-CHO of Degradation of the Oxidized B Chain of Insulin by the Native and DCI-Treated Enzyme[a]

enzyme	inhibitor	[I] (μM)	activity (nmol mg^{-1} h^{-1})	inhibition (%)
native	none	−	967	−
	Z-LGF-CHO	50	0	100
	Z-GPFL-CHO	50	0	100
DCI-treated	none	−	2440	−
	Z-LGF-CHO	50	2330	4
	Z-GPFL-CHO	50	852	65

[a] The extent of degradation of the oxidized B chain of insulin was determined using the native and DCI-treated enzyme under conditions described in Experimental Procedures. Incubations were carried out for 30 min at 37 °C and a B chain concentration of 100 μM. Data are mean values obtained from two independent experiments. The differences between two determinations did not exceed 10%.

FIGURE 4: Major sites of cleavage of the oxidized B chain of insulin by the native and DCI-treated MPC. The insulin B chain was incubated overnight with either the native pituitary MPC or enzyme preincubated for 2 h with 50 μM DCI. Degradation products were separated by HPLC and identified by mass spectrometry. Arrows indicate major cleavage sites. Identical peptide products were found for both enzyme forms.

(Figure 2). The rates of degradation of the oxidized B chain of insulin by either the DCI-treated or the native MPC were then determined in the absence or presence of Z-LGF-CHO, an inhibitor of the ChT-L activity, or Z-GPFL-CHO, an inhibitor of the BrAAP component. The results are summarized in Table 4. As reported previously (*22*), treatment of MPC with DCI caused activation of degradation of the insulin B chain. The degradation of the B chain by the native MPC was almost completely inhibited by both the inhibitor of the ChT-L activity and that of the BrAAP component. One interpretation of these findings is that binding of any one of these inhibitors interferes with binding of the extended peptide to either of the two active sites, either by spatial hindrance or by structural changes that prevent binding. The inhibitor of the ChT-L activity, however, had no effect on the degradation of the B chain of insulin by the DCI-treated enzyme. By contrast, Z-GPFL-CHO, an inhibitor of the BrAAP component, strongly inhibited the degradation of the B chain. These findings strengthen the conclusion that the ChT-L and BrAAP activities are catalyzed at two different sites, and suggest that the substrate specificities identified with model substrates and synthetic inhibitors extend as well to degradation of natural peptides by the MPC.

As shown above, exposure of the MPC to DCI causes inactivation of the ChT-L, T-L, and PGPH activities, but also causes activation of degradation of the insulin B chain and degradation of the synthetic BrAAP substrates. It was therefore important to compare the reaction products generated from the insulin B chain by the native and DCI-treated MPC to identify the cleavage sites attacked by the two enzyme forms. Separation of reaction products by HPLC revealed that the overall pattern of peptide fragments produced by the two MPCs was quite similar, and involved formation of three major products (Figure 4). To identify the peptides contained within the three peaks, we collected the HPLC fractions corresponding to these peaks and the molecular masses of the peptides were measured by mass spectrometry. A single peptide was found in each HPLC peak. Comparison of the measured mass for each peptide to the masses for all possible peptide fragmentation products of the insulin B chain revealed, in each case, a single matching peptide. Two major cleavages were observed, one after Glu[13] and a second after cysteic acid[19] (Figure 4). Surprisingly, identical cleavage sites were observed in reactions with both the DCI-treated and native MPC, indicating that cleavages after acidic residues in longer peptides are unaffected by exposure to DCI.

Table 5: Effect of DCI Treatment on Yields during Amino Acid Sequencing of the Y Subunit[a]

sample	cycle	1	2	3
control	residue found	Thr	Thr	Ile
	yield (pmol)	8.4	7.5	4.0
DCI-treated	residue found	Thr	Thr	Ile
	yield (pmol)	5.7	5.1	2.5

[a] Amino acid sequencing of the Y subunit was performed after incubation of the pituitary MPC with 50 μM DCI or DMSO followed by separation of subunits by SDS−PAGE, electrophoretic transfer of proteins to PVDF membranes, and excision of bands containing the Y subunit. Equal amounts of the Y subunit from control (DMSO-treated) and DCI-treated MPC were subjected to amino acid sequencing by Edman degradation, and the yields of amino acids from each cycle were calculated.

The above finding raised questions about the mechanisms by which DCI inactivates the PGPH component that is responsible for hydrolysis of bonds after glutamate residues in synthetic substrates such as Z-LLE−2NA, generally used for measuring this activity. For example, although previous experiments indicated that the Y subunit undergoes acylation by DCI, the findings raised the possibility that DCI covalently modifies an amino acid residue close to, but distinct from, the active site N-terminal threonine in the Y subunit. Such modification could selectively block binding of the synthetic substrate Z-LLE-2NA, perhaps by interfering with access of the bulky 2-naphthylamide group to the active site, but may not interfere with binding of natural peptide chains, such as the insulin B chain. Of interest, acylation of the N-terminal threonine of the X subunit, involved in the ChT-L activity, is associated with a decreased yield of threonine in the first amino acid sequencing cycle and complete loss of ChT-L activity. (*8*, *16*).

To determine whether exposure of the MPC to DCI led to acylation of the N-terminal threonine of the Y subunit, we incubated the MPC for 120 min with 50 μM DCI as described in Experimental Procedures. We then subjected the Y subunit to amino acid sequencing after separation of subunits by SDS−PAGE and electrotransfer of proteins onto PVDF membranes. The yields observed during amino acid sequencing of the Y subunit are shown in Table 5. Preincubation of the MPC with DCI caused no apparent decrease in the yields of threonines in the first or second cycle. In addition, overall yields during sequencing of the Y subunit were similar for the sequences from DCI- and DMSO-treated MPC. Thus, the findings indicate that acylation of the Y subunit by DCI involves an amino acid residue other than the N-terminal threonine.

DISCUSSION

Several lines of evidence indicated that the catalytic activity of the MPC is not limited to the three initially identified components. Thus, for example, treatment of the MPC with *N*-acetylimidazole, an acetylating agent (*33*), under conditions that led to inactivation of the ChT-L, T-L, and PGPH activities, was shown to accelerate rather than inhibit the degradation of β-casein. This led to the postulate that the caseinolytic activity is due to a catalytic component distinct from the ChT-L, T-L, and PGPH activities (*34*). Similar conclusions were derived from experiments with DCI, an irreversible inhibitor of the above three activities that activated the caseinolytic activity (*23*). Furthermore, inactivation by DCI of the three activities led to an almost 10-fold activation of a component cleaving preferentially bonds after branched chain amino acids (BrAAP) (*13*). The same treatment also accelerated the degradation of a series of natural peptides, including neurotensin, proinsulin, and the oxidized B chain of insulin (*22*).

In view of these findings, the possibility must also be considered that since each of the subunits is represented twice in the MPC, and the structural integrity of the complex is absolutely necessary for expression of activity, subunit interactions might markedly modify substrate binding properties and therefore the specificity of each of the six, generally accepted, catalytically active subunits. There is a tendency to assume that substrate binding properties, and therefore specificity of a pair of identical catalytically active subunits, are the same. This is contradicted by several published observations. Thus, experiments led to the conclusion that two ChT-L activities can be distinguished on the basis of differential susceptibility to peptidyl chloromethyl ketones. For example, Ala-Ala-Phe-CH_2Cl inhibits the ChT-L activity measured with suc-Leu-Leu-Val-Tyr-AMC but not with Ala-Ala-Phe-AMC (*35*, *36*). Also, several studies have reported evidence for the presence of two PGPH components due to the presence of two binding sites, one having a high affinity and the other having a low affinity. The relationship between substrate concentration and reaction velocity gave sigmoidal kinetics, indicating the presence of positive cooperativity and the presence of two components giving different responses to metal ions and also peptidyl aldehydes (*32*, *37*, *38*). Significantly, treatment of the native 20S MPC with low concentrations of SDS was shown to suppress cooperativity observed in studies of the PGPH activity (*32*, *38*). A drastic increase in V_{max} and a decrease in substrate concentration at half-maximal velocity, with elimination of the S-shaped substrate-reaction velocity dependence, was described. These changes are apparently an expression of detergent-induced disruption of subunit interactions that modify the specificity of the native enzyme as reflected by differences in substrate binding properties of the six generally accepted catalytic sites.

Our results are inconsistent with any significant participation of the ChT-L activity in the hydrolysis of BrAAP substrates. Thus, for example, several tripeptidyl−aldehyde inhibitors with a phenylalaninal group had no effect on the BrAAP activity at concentrations more than 150 times higher than the K_i for the ChT-L activity (Table 1). Similar inhibitors with a leucinal moiety, while inhibiting both activities, showed different affinities for the two components depending on the nature of the amino acid residue in the P_2 position (Table 1). Other findings support the conclusions derived from the inhibitor experiments. Thus, lactacystin, an inhibitor that inactivates the ChT-L activity, had no effect on the BrAAP activity at concentrations that caused more than 90% inhibition of the ChT-L activity (Figure 1). Similarly, complete inactivation of the ChT-L activity by DCI, rather than inhibiting the BrAAP component, caused an up to a 10-fold activation of this activity toward model synthetic BrAAP substrates and significant activation of degradation of the insulin B chain (Figure 2 and Table 4). Also, previous studies indicate that exposure to DCI causes activation of degradation of other natural peptides and proteins such as casein and proinsulin (*22*, *23*). It should be again emphasized that treatment with DCI that activates the BrAAP activity causes a complete acylation of the hydroxyl group of the N-terminal Thr residue in the active site of the X subunit involved in expression of the ChT-L activity (*8*, *20*, *21*). It is also notable that unlike Z-LLF-CHO and Z-LLL-CHO, both potent inhibitors of the ChT-L activity, Z-GPFL-CHO, an inhibitor of the BrAAP and PGPH components, did not protect the hydroxyl group of the N-terminal residue of the X subunit from acylation by [^{14}C]DCI (*8*). This indicates that Z-GPFL-CHO does not bind to the active site of the subunits involved in the expression of the ChT-L activity. The fact that the ChT-L activity is not a factor in the degradation of the BrAAP substrates was also evident from studies on the degradation of the oxidized B chain of insulin by both the native and the DCI-treated enzyme. Thus, exposure of the MPC to DCI (120 min), which eliminates the ChT-L, PGPH, and T-L activities, accelerates the degradation of the B chain of insulin by a BrAAP activity that is resistant to Z-LGF-CHO, a selective inhibitor of the ChT-L activity, but is sensitive to inhibition by Z-GPFL-CHO, a rather selective inhibitor of the BrAAP and PGPH components (Table 4). Cleavage of the oxidized B chain of insulin could therefore be due to either the BrAAP or PGPH activity provided that the Y subunit of the enzyme treated with DCI cannot cleave amino acid arylamide bonds but can still function in cleavage after acidic residues of bonds between two amino acids in natural peptides.

Our findings differ from those obtained with a rabbit muscle MPC which, on the basis of kinetic experiments, reported that 60−80% of the BrAAP activity can be attributed to the ChT-L component and that the remaining BrAAP activity can be attributed to the PGPH component (*31*). These results, however, were obtained in experiments in which a different BrAAP substrate, enzyme source (rabbit muscle MPC), and buffer system were used, all known to affect the catalytic properties of the proteasome. Furthermore, rather than using the native 20S MPC in the kinetic experiments, data were obtained with an enzyme treated with SDS, a detergent that was previously shown to eliminate cooperativity phenomena observed in preparations of the native 20S MPC (*32*, *37*, *38*). These factors dramatically change the catalytic properties of the subunits by affecting both V_{max} and K_m values toward substrates as well as the K_i values of inhibitors, changes that result in as yet poorly charted effects on the specificity of the subunits. Moreover, concentrations of SDS (0.02%), even lower than those used in these kinetic studies (0.035−0.05%), were reported to change the sedimentation velocity of the enzyme and showed

evidence of some dissociation of the complex and formation of aggregates (*39*). It is noteworthy that the BrAAP component activated by exposure to DCI loses almost completely its activity in the presence of SDS (0.035%), and that the presence of the detergent makes this activity responsive to the phenylalaninal inhibitors (Figure 3). This result can lead to the erroneous conclusion that the ChT-L activity contributes significantly to the expression of the BrAAP activity.

Insights into the relationship between the PGPH and BrAAP activities are also suggested from the results presented above. The two activities differ significantly in that whereas the PGPH activity is inactivated by DCI and is exquisitely sensitive to inhibition by proteins, the BrAAP activity is activated by DCI and is not affected by proteins. Previous studies have documented that exposure of the pituitary MPC to [^{14}C]DCI results in incorporation of the radiolabel into a protein with the SDS−PAGE mobility of the Y subunit (*8*), and have provided evidence of acylation of an amino acid within this protein. Direct evidence that labeling involves primarily the Y subunit was also provided by the finding that replacement in the spleen MPC of the Y subunit by an LMP subunit was associated with a marked decrease in the extent of incorporation of the radiolabel (*8*). It seems likely that a covalently modified residue is located near the active site of the Y subunit because Z-LLL-CHO, a peptide aldehyde inhibitor with a K_i for the PGPH activity of about 5 μM, is effective in blocking incorporation of [^{14}C]-DCI (*8*). However, amino acid sequencing of the Y subunit showed no evidence of incorporation of DCI into the N-terminal threonine thought to be involved in catalysis (Table 5). Thus, the data indicate that covalent modification by DCI involves a side chain of an amino acid positioned near the active site distinct from the N-terminal threonine. Crystallographic studies of the MPC from the yeast *S. cerevisiae* (*18*) have identified several amino acids in the Pre3 subunit (responsible for the PGPH activity in yeast) that have side chains containing hydroxyl groups positioned close to the active site. Each is conserved in the Y subunit, the mammalian homologue of Pre3, and thus represents a potential target for acylation. They include Ser^{129} and Ser^{169}, located near the hydroxyl group of the active site threonine and postulated to be involved in autocatalytic processing, and Thr^{20}, Thr^{31}, and Thr^{35} which contribute to the walls of the S1 substrate binding pocket. Acylation by DCI of any one of these might affect binding of substrates to the active site. However, identification of the acylated residue was not possible because of the labile nature of the bonds between [^{14}C]DCI and the hydroxyl groups of Thr or Ser.

Covalent modification by DCI of an amino acid near the active site of the Y subunit could explain our finding that, under conditions causing near-complete loss of PGPH activity measured with Z-LLE-2NA, exposure to DCI had little effect on cleavage of bonds after acidic amino acids in the B chain of insulin. The presence of the isocoumarin-derived acyl group near the active site could limit access of substrates to the catalytic center with selective exclusion of the bulky 2-naphthylamide group of Z-LLE-2NA, while allowing cleavage of peptide bonds between two adjacent amino acids in natural peptides or proteins.

Our results therefore do not exclude the possibility that the BrAAP substrates, which are cleaved between two amino acids and are significantly longer than Z-LLE-2NA, could be degraded by the Y subunit. Findings to be considered in this regard are as follows: Z-GPFL-CHO, the most potent inhibitor of the BrAAP activity, inhibited the PGPH activity with a K_i similar to that of the BrAAP activity (*24*), and substrate studies suggested that the BrAAP activity is capable of cleaving bonds after glutamate residues (*40*). In addition, exposure to DCI had no effect on the cleavage of bonds after acidic residues in the insulin B chain. However, the possibility that the BrAAP activity is expressed by a subunit distinct from Y, or that it is an expression of differences in specificity between the two Y subunits, each located in a different β-ring, cannot be excluded. The increased activity of the DCI-treated enzyme toward synthetic (Z-GPALG-pAB) and natural substrates would have to be assumed to be the result of increased accessibility of the two active sites to both types of substrates. Some confirmation of this possibility is provided by the finding that the increased V_{max} of the DCI-treated enzyme toward BrAAP substrates is associated with a marked decrease in K_m values (*13*, *41*). However, because of experimental evidence of the presence of two distinct PGPH components differing in substrate binding properties (low and high K_m and V_{max} values) (*32*, *37*, *38*), one can also speculate that one of the sites is more readily accessible than the other to synthetic peptides and protein substrates or that interaction with neighboring subunits differentially affects the catalytic properties of the two β-subunits involved in expression of this activity. While the data suggest that the PGPH component of the proteasome could be responsible for both the PGPH and BrAAP activities, this conclusion would require the assumption that the two Y subunits of the proteasome differ in substrate accessibility and specificity due to different subunit interactions that are required for proteasomal function, or that DCI treatment limits the accessibility to the active site of substrates containing bulky arylamide bonds but not substrates cleaved between two adjacent amino acids.

A report on the specificity of the MPC from *S. cerevisiae* and the effect of mutations on the subunit specificity of the enzyme toward synthetic substrates and natural peptides also addressed the question of the active site involved in cleavages of substrates of the BrAAP component (*42*). Mutants containing a defective Pre2 subunit were deficient in the ChT-L activity, while mutations in the Pre3 subunit were defective in expression of the PGPH and BrAAP activity. The Pre2 and Pre3 subunits of the yeast enzyme are homologues of the X and Y subunits of the mammalian enzyme, respectively. Accordingly, extrapolation of these data to the mammalian proteasome would suggest that the X subunit is involved in expression of the ChT-L activity but not in expression of the BrAAP activity, a conclusion consistent with our findings. Similar extrapolations also suggest that the Y subunit is involved in cleavage of substrates after both acidic and branched chain amino acids, provided that there are no catalytic differences between the yeast and mammalian proteasomes. It is noteworthy, however, that the yeast proteasome is much more resistant to inactivation by DCI than its mammalian counterpart, and that the yeast enzyme is not responsive to PA28, a protein activator that greatly affects the activities and cooperativity phenomena of the mammalian enzyme (*42*). Crystallographic analysis of the yeast proteasome suggested the possibility

of the presence in this complex of catalytic centers other than those provided by N-terminal threonine residues in β-subunits (*18*). Since few if any proteolytic enzymes exhibit absolute specificity, particularly when studied with model synthetic substrates, the finding of some similarities between the BrAAP and PGPH components inhibitors does not exclude the possibility that the catalytic mechanism of the BrAAP component could be different from that provided by the N-terminal nucleophile mechanism (*43*, *44*). Confirmation, however, of the existence of distinct subunits catalyzing the BrAAP activity of the pituitary MPC, separate from the X, Y, and Z subunits, awaits a positive identification of a subunit responsible for its expression.

It should be noted that replacement in the spleen MPC of the X, Y, and Z subunits by the LMP subunits is associated with an almost complete repression of the PGPH activity and the appearance of a highly active BrAAP-like activity (*30*) which, while demonstrating a preference for cleaving bonds after hydrophobic amino acids, displays a substrate specificity different from that of the BrAAP activity of the pituitary MPC. Radiolabeling studies suggested that the BrAAP-like activity of the spleen MPC is associated with the LMP7 subunit, and that the LMP2 subunit, which replaces the Y subunit, expresses the ChT-L activity of the spleen MPC (*8*). In addition, regardless of the subunit ultimately found to be responsible for its catalysis, it should be noted that the BrAAP component of the pituitary MPC, through its distinct substrate specificity, including a strong preference for cleaving bonds after branched chain amino acids, represents a potentially important factor in numerous processing functions of the MPC, including the formation of MHC class I-restricted peptides.

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