Reactions of [^{14}C]-3,4-Dichloroisocoumarin with Subunits of Pituitary and Spleen Multicatalytic Proteinase Complexes (Proteasomes)†

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ABSTRACT: Exposure to [^{14}C]-3,4-dichloroisocoumarin (DCI) of multicatalytic proteinase complexes (MPC) isolated from bovine pituitary and spleen leads to label incorporation into several β-type subunits, to rapid inactivation of the chymotrypsin-like (ChT-L) activity, and to a slower inactivation of other activities of the MPC. The pituitary and spleen MPCs differ in that the first contains almost exclusively the X, Y, and Z subunits, whereas in the latter these subunits are largely replaced by LMP2, LMP7, and MECL1. Preincubation with two peptidyl aledhyde inhibitors of the ChT-L activity protected the X subunit in the pituitary MPC and unexpectedly the LMP2 subunit in the spleen MPC from label incorporation, despite the greater amino acid sequence homology of the LMP7 subunit to that of the X subunit. Losses in the yield of amino acids in both subunits, shown by amino acid sequencing, and lability of the DCI−protein bond indicated formation of an acyl derivative by reaction of DCI with the threonine OH group. Brief exposure to [^{14}C]-DCI led to preferential incorporation of label into the LMP2 and X subunits, consistent with the high inactivation rate constants of the ChT-L activity. Z-LLF-CHO, an inhibitor of ChT-L activity, but not Z-GPFL-CHO, an inhibitor of the branched chain amino acid preferring component, prevented incorporation of radioactivity into the X subunits, whereas both inhibitors prevented label incorporation into LMP2, indicating differences in susceptibility to inhibition between the two components. These and other data are consistent with involvement of the X and LMP2 subunits in expression of the ChT-L activity in the pituitary and spleen MPC, respectively, and suggest the catalytic functions of two other β-subunits.

The multicatalytic proteinase complex (MPC,[1] proteasome) of eukaryotic cells (*1*−*3*) contains 14 distinct subunits, each represented twice in a cylindrical structure organized in 4 stacked rings, each composed of 7 subunits (*4*). The subunits surround a central tunnel and are grouped into two classes, designated as α and β, based on the amino acid sequence similarity to subunits of the proteasome isolated from *Thermoplasma acidophilum*, a structurally similar complex, having the same number of subunits but only of two distinct types (*5, 6*). The β-subunits, that provide the catalytic activity, form the inner two rings, whereas the outer rings are formed by the α-subunits. Work with the specific proteasome inhibitor lactacystin, and X-ray crystallography of the proteasome from *Thermoplasma acidophilum*, has provided evidence for the involvement of an N-terminal threonine residue in the catalytic mechanism (*7, 8*). Since only four out of the seven β-subunits contain an N-terminal threonine, the possibility cannot be excluded that other catalytic mechanisms might also be involved. Furthermore, the finding that the integrity of the full structure of the MPC is absolutely necessary for preservation of catalytic activity indicates that the interaction between two or more subunits might be necessary for catalysis.

Initial substrate specificity studies have identified the presence in the MPC of three distinct catalytic components designated as chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl-peptide hydrolyzing (PGPH), based on the nature of the amino acid residues providing the carbonyl group of the scissile bond (*9*−*11*). More recent work has identified two additional components designated as branched chain amino acid preferring (BrAAP) and small neutral amino acid preferring (SNAAP) (*12, 13*). Data derived from yeast proteasome mutants have linked the *PRE2* gene, a homologue of the mammalian LMP7 gene, as coding for a subunit necessary for expression of the ChT-L activity (*14*), and the *PRE4* and *PRE3* genes, the latter a homologue

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[1] Abbreviations: conventional one- or three-letter abbreviations are used for amino acids; BrAAP, branched chain amino acid preferring; ChT-L, chymotrypsin-like; DCI, 3,4-dichloroisocoumarin; DMSO, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography; MCA, 7-amino-4-methyl coumarylamide; MPC, multicatalytic proteinase complex; 2NA, 2-naphthylamide; pNA, p-nitroaniline; pAB, p-aminobenzoate; PGPH, peptidylglutamyl-peptide hydrolyzing; peptidyl-CHO, peptidyl aldehyde; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; SNAAP, small neutral amino acid preferring; Suc, succinyl; T-L, trypsin-like; Z, benzyloxycarbonyl.

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of the LMP2 gene, as necessary for expression of the PGPH activity (*15, 16*). Identification, however, of subunits expressing defined activities in mammalian tissues and cells remains uncertain. Two variations of subunit composition can be found in mammalian proteasomes; one, present in most tissues, contains the constitutively expressed X, Y, and Z subunits, whereas in the other these subunits are to a different degree replaced by LMP2, LMP7, and MECL1. Recent work in this laboratory has shown that the MPCs isolated from pituitary and spleen differ with respect to subunit composition in that the pituitary MPC contains almost exclusively the X, Y, and Z subunits, whereas in the spleen MPC these subunits are largely replaced by LMP2, LMP7, and MECL1 (*17*). These replacements are associated with changes in the catalytic properties of the MPC toward both synthetic and natural peptides (*18, 19*), as well as changes in the sensitivity to inhibition by substrate-related inhibitors (*17*), further complicating association of subunits with distinct catalytic properties.

Two catalytic components exhibit ChT-L activity. The first cleaves amide bonds between hydrophobic amino acids and aromatic amines in such substrates as succinyl-Leu-Leu-Val-Tyr-*MCA (asterisks shows the site of cleavage) and Z-Gly-Gly-Leu-*pNA or Z-Gly-Gly-Phe-*pAB. The second cleaves only bonds between adjacent amino acids, with preference of bonds with a branched chain amino acid in the P_1 position. It does not cleave bonds between amino acids and aromatic amines (*12*). The first component can be rapidly inactivated by exposure to 3,4-dichloroisocoumarin (DCI) (*20*), an irreversible inhibitor of serine proteases (*21*−*23*), whereas the second is activated by such treatment. Expression of the full activity of the two components requires the presence of activators such as fatty acids or low concentrations of SDS (*10, 24*), or the presence of a protein activator designated as PA28 or REG (*25, 26*). Both components were shown to represent major factors in the degradation of proteins (*27, 28*).

Here we report experiments with [^{14}C]-3,4-dichloroisocoumarin and peptidyl aldehyde inhibitors aimed at identification of subunits necessary for expression of activities in MPC preparations from bovine pituitaries and spleen. The data indicate that the X subunit of the pituitary MPC and the LMP2 but not the LMP7 subunit of the spleen MPC are necessary for the expression of the ChT-L activity, despite the greater sequence homology of the latter to the X subunit. Other preliminary data suggest the involvement of the Y and LMP7 subunits in two other catalytic activities of the MPC.

MATERIALS AND METHODS

Synthesis of [^{14}C]-3,4-Dichloroisocoumarin. The radioactive compound was synthesized in four steps starting from phthalide and [^{14}C]KCN.

[^{14}C]-o-Carboxyphenylacetonitrile. This compound was synthesized by a modification of the procedure described by Price and Rogers (*29*). One gram of powdered K^{14}CN (10 mCi; 42.4 mCi/mmol; Sigma Chemical Co., St. Louis, MO) was thoroughly mixed with 2 g of phthalide and heated to 180−190 °C for 2.5 h. Ten milliliters of water was then added to the cooled mass, and the mixture was stirred until the solids were dissolved. Any insoluble material was removed by filtration, and 6 N HCl was added in a well-ventilated hood to the dark aqueous solution until it became turbid. The solution was neutralized with a 4% solution of sodium bicarbonate, and an amount of Norit necessary to decolorize the solution was added. The filtrate was acidified with concentrated HCl and cooled in an ice bath to give 1.2 g of [^{14}C]-*o*-carboxyphenylacetonitrile, mp 112−115 °C.

[^{14}C]Homophthalic Acid. [^{14}C]-*o*-Carboxyphenylacetonitrile (1.2 g) was dissolved in 10 mL of 50% H_2SO_4 and heated at 100 °C for 4 h (*30*). The reaction mixture was poured over some ice, and the precipitate was collected and dried to give 1.1 g of product (mp 160−166 °C).

[^{14}C]-3-Chloroisocoumarin. [^{14}C]Homophthalic acid (1.1 g) was heated with PCl_5 (2.7 g) in 2.7 mL of $POCl_3$ at 140−150 °C for 3 h, and then 30 mL of water was added. [^{14}C]-3-Chloroisocoumarin was obtained by steam distillation to yield 75 mg of white needles (mp 85−87 °C).

[^{14}C]-3,4-Dichloroisocoumarin. [^{14}C]-3-Chloroisocoumarin (0.12 g) was dissolved in 15 mL of CCl_4, and Cl_2 was continuously bubbled into the solution for 2.5 h. Thin-layer chromatography in benzene showed complete conversion of 3-chloroisocoumarin to a new product (*31*). CCl_4 was removed under reduced pressure, and the residue was dissolved in a small volume of anhydrous ether. An equivalent amount of anhydrous triethylamine was added, and the mixture was cooled in an ice bath. The precipitate (triethylamine hydrochloride) was removed by filtration, and after evaporation of the ether, the residue was dissolved in methylene chloride. The precipitate that formed after addition of hexane was removed by filtration. The solvents were removed by filtration, and the material was dissolved in a small amount of hexane. Cooling to 4 °C yielded crystalline [^{14}C]-3,4-dichloroisocoumarin (37 mg). TLC in benzene gave a single spot with an R_f of 0.73, identical with authentic 3,4-dichloroisocoumarin; specific activity 1 μCi/μmol.

Isolation of MPCs from Bovine Pituitaries and Spleen. The pituitary MPC was isolated as previously described (*20*). The spleen enzyme was isolated essentially by the same method, except that the centrifuged homogenate before the ammonium sulfate fractionation step was treated with a 30% solution of streptomycin sulfate in order to precipitate and remove nucleic acid which interfered with subsequent purification steps. Aliquots of the enzyme (0.17 mL) containing 170 μg of enzyme protein were stored frozen at −70 °C and thawed before being used for the experiments.

Synthesis of Peptidyl Aldehyde Inhibitors and Substrates and Measurements of Activities. Peptidyl aldehydes were synthesized by oxidation of the corresponding peptidyl alcohols using the dimethyl sulfoxide−carbodiimide method of Pfitzner and Moffat (*32*) as described previously (*27*, *33*). The ChT-L, T-L, PGPH, BrAAP, and SNAAP activities were determined with Z-GGF-*p*AB, Z-(D)ALR-2NA, Z-LLE-2NA, Z-GPALG-*p*AB, and Z-GPAGG-*p*AB, respectively. Methods of activity measurements and substrate syntheses were previously described (*9*, *11*, *12*, *34*).

Reactions of the MPC with 3,4-Dichloroisocoumarin (DCI). The rate of inactivation of the pituitary and spleen MPCs by DCI was determined by incubating the enzyme at 26 °C with 50 μM DCI dissolved in dry dimethyl sulfoxide (DMSO). The final DMSO concentration did not exceed 2% of the volume of the incubation mixture. Controls contained the same amount of DMSO without DCI. Aliquots of the enzyme were withdrawn at different time intervals and transferred to tubes at 37 °C containing the appropriate substrate in Tris-HCl buffer (0.05 M; pH 8.0). Activities were determined as indicated above. In reactions with [^{14}C]-

DCI, 30 μL of the pituitary MPC and 50 μL of the spleen MPC (30 and 50 μg of protein, respectively) were incubated under the above conditions for 30 min. The reactions were terminated by addition of 2-mercaptoethanol (final concentration 5%) and SDS (final concentration of 1%) before subjecting the samples to SDS−PAGE under dissociating conditions. Variations in the time of exposure to DCI are indicated in the legends to figures. To study the effect of inhibitors on incorporation of [^{14}C]-DCI, samples of the MPC were preincubated for 20 min with different concentrations of peptidyl aldehyde inhibitors at 37 °C before exposure to [^{14}C]-DCI for 30 min. The samples were then prepared for SDS−PAGE by addition of 2-mercaptoethanol and SDS as described above.

SDS−PAGE and Radioautography. PAGE was done in 10% gels at a gel length of 27 cm and a thickness of 1.5 mm in the presence of SDS, using a Hoeffer Scientific vertical slab gel apparatus as described previously (*35*). A current of 30 mA was applied for 12−13 h. The protein bands were transferred after electrophoretic separation to Immobilon P membranes using a current of 70 V for 150 min. The membranes were then rinsed with distilled water and after overnight drying exposed in a Hypercassette (18 × 24 cm) to Hyperfilm-ECL (Amersham), for 30 days at −70 °C. The films were then processed and scanned on an Arcus II Agfa color scanner using the Adobe Photoshop 3.0 software. Prints of radio autographs were obtained using a Tektronix printer.

Amino Acid Sequencing. Protein bands separated by SDS−PAGE as described above were transferred to poly(vinylidene difluoride) membranes (*36*) and stained with Coomassie Blue (*37*). Under the above conditions, β-subunits having free amino termini migrate ahead of the α-subunits that have blocked N-termini and generally higher molecular weights. It was therefore convenient to obtain N-terminal amino acid sequences from protein bands separated by one-dimensional gel electrophoresis. Furthermore, a single SDS−PAGE run provided sufficient material for sequencing. Occasionally, more than one sequence was associated with a single protein band. In such cases, assignment was done on the basis of distinctly different amino acid yields during successive sequencing cycles. Amino acid sequencing was done using a Beckman Instruments Model 2090E gas-phase sequencer.

RESULTS

The subunit patterns of the spleen and pituitary MPCs obtained by SDS−PAGE followed by electrotransfer to PVDF membranes and staining with Coomassie Blue are shown in Figure 1. Amino acid sequencing was used to identify β-subunits with free N-termini. The two fastest moving bands in the spleen MPC were found to correspond to LMP2 and LMP7. The other bands of the spleen MPC corresponded to subunits Y (also referred to as Delta), C5, RN3, and MECL1 listed in the order of their migration rate. The fastest moving band in the pituitary MPC corresponds to subunit X (also referred to as MB1). It is followed by a band containing two subunits, Y and C5, and then by RN3 and subunit Z. This subunit pattern indicates that the X, Y, and Z subunits present in the pituitary MPC are largely replaced in the spleen enzyme by LMP2, LMP7, and MECL1. Occasionally the C5 subunit was found migrating with both the RN3 and Y bands, and some MECL1 was also found migrating with subunit Y in the spleen MPC. The presence of the same subunit in more than one band could result from posttranslational modification such as limited proteolysis, phosphorylation, and/or glycosylation.

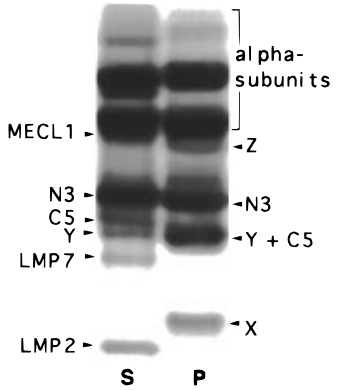

FIGURE 1: Subunits of the spleen (S) and pituitary (P) MPC separated by one-dimensional SDS−PAGE, electrotransfer to a PVDF, and staining with Coomassie Blue. The identification of subunits based on amino acid sequencing is marked in the figure. For details, see the text.

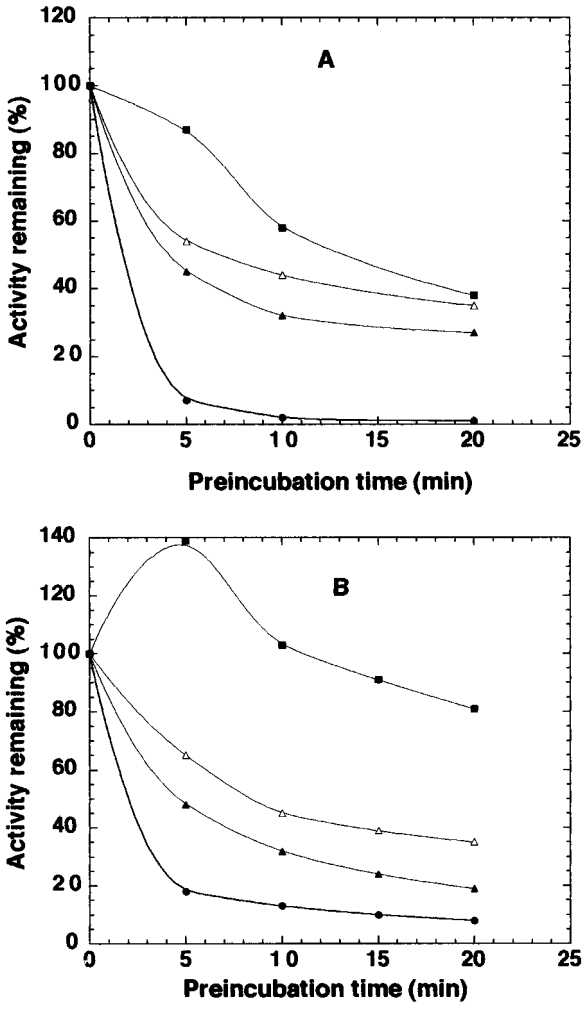

FIGURE 2: Inactivation of components of the pituitary (A) and spleen (B) MPC by exposure to 3,4-dichloroisocoumarin. Filled squares, open triangles, filled triangles, and filled circles correspond to PGPH, T-L, SNAAP, and ChT-L activities, respectively. For details, see Materials and Methods.

The effect of exposure of the pituitary MPC to 50 μM DCI is shown in Figure 2, panel A. The ChT-L activity loses about 90% of activity after 5 min, and inactivation is virtually complete after 10 min. The T-L, PGPH, and

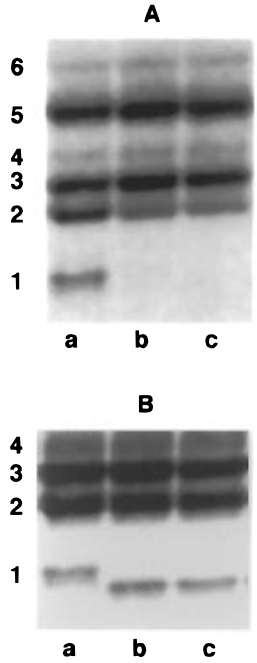

FIGURE 3: (Panel A) Radioautographs of pituitary MPC subunits separated by SDS−PAGE after exposure to [^{14}C]-DCI. Lane a, control MPC sample, shows four intensely labeled bands numbered 1, 2, 3, and 5, corresponding to subunits X, Y and C5, RN3, and Z, respectively. Two other faintly labeled bands (4 and 6) have not yet been identified. Lanes b and c contain the enzyme after preincubation with 50 and 100 μM Z-LLF-CHO (20 min at 37 °C), respectively, before treatment with labeled DCI. For details, see the text. (Panel B) Coomassie blue staining of pituitary MPC subunits after exposure to labeled DCI, separation by SDS−PAGE, and transfer to PVDF membranes. Lane a, enzyme exposed to DCI; lanes b and c, MPC preincubated with 50 and 100 μM Z-LLF-CHO (20 min at 37 °C), respectively, before treatment with DCI. For interpretation, see the text.

SNAAP activities are more resistant to inactivation under the same conditions. The BrAAP activity in the pituitary MPC not only is resistant to inactivation but as reported previously is initially activated up to 10-fold by the same treatment followed by a slow decline of activity probably due to autolysis (*12*). As in the pituitary, the spleen ChT-L activity (Figure 2, panel B) is most sensitive to exposure to DCI, but some residual activity persists in the spleen enzyme even after 20 min. The T-L, SNAAP, and PGPH activities are only partially inactivated under the same conditions, the latter showing a transient activation, apparently due to exposure of the latent activity of this component. The same treatment with DCI causes a slow decrease rather than activation of the BrAAP activity, as seen in the pituitary; nevertheless, this activity is more resistant to inactivation than the other catalytic components (*17*).

Because of the high sensitivity to DCI inactivation of the ChT-L activity, we have initially concentrated our efforts to identify the subunit necessary for expression of this activity in both the pituitary and spleen MPC preparations. Incubation of the pituitary enzyme with [^{14}C]-DCI showed incorporation of label into six protein bands (Figure 3, panel A) of which the intensely labeled bands 1, 2, 3, and 5 contained the β-subunits X, Y and C5, RN3, and Z, respectively. Two faintly labeled bands (4 and 6) have not yet been identified. This labeling pattern indicated that DCI reacts primarily with the β-type subunits since the slower moving α-subunits (Figure 1), with higher molecular weights, remain unlabeled. Preincubation of the pituitary enzyme with 50 and 100 μM

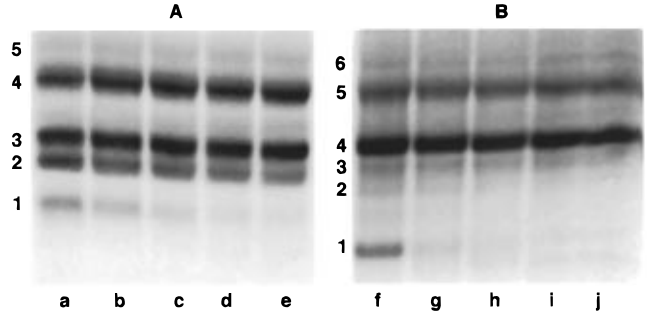

FIGURE 4: Effect of preincubation with different concentrations of Z-LLF-CHO on incorporation of labeled DCI into subunits of the pituitary and spleen MPCs. Panels A and B correspond to labeled subunits of the pituitary and spleen MPC, respectively, after exposure to [^{14}C]-DCI followed by SDS−PAGE. Bands 1 in panels A and B correspond to subunits X and LMP2, respectively. Band 2 in panel B corresponds to LMP7. Bands 2 and 3 in the pituitary MPC correspond to subunits Y and C5, and RN3, respectively. Band 3 in the spleen MPC corresponds to subunit Y. Lanes a and f represent MPC samples treated with [^{14}C]-DCI only; lanes b−e and g−j show the effect of preincubation (20 min at 37 °C) of the pituitary and spleen MPC with increasing concentrations (5, 10, 20, and 50 μM) of Z-LLF-CHO. For interpretation, see the text.

Z-LLF-CHO (lanes b and c), a rather specific slow binding inhibitor of the ChT-L activity of the pituitary MPC (K_i 0.46 μM; ref *33*), abolished completely labeling of the fastest moving band previously identified as subunit X (band 1, lane a, Figure 3A), suggesting the importance of this subunit for expression of the ChT-L activity. Furthermore, previous experiments have shown that treatment of the MPC with DCI slows somewhat migration of the X band in the pituitary MPC (*35*). This change in migration was mainly attributed to the covalent binding of DCI to the X subunit and a commensurate small increase of its molecular weight. Figure 3B shows a scan of a Coomassie Blue stained PVDF membrane used to generate the autoradiogram shown in Figure 3A. It shows migration of the X subunit in SDS−PAGE after treatment with [^{14}C]-DCI (lane a) compared with the migration of the same subunit after preincubation with 50 and 100 μM Z-LLF-CHO (lanes b and c), respectively. As can be seen, covalent modification by DCI causes a decrease in the migration rate of X. Preincubation, however, with Z-LLF-CHO before exposure to DCI completely abolishes this change of migration, further indicating that the inhibitor prevents covalent modification by DCI of the X subunit. The same changes in migration were obtained for the LMP2 subunit of the spleen MPC after the same treatment (not shown).

The effect of different concentrations of Z-LLF-CHO on incorporation of [^{14}C]-DCI into both the pituitary and spleen MPC subunits is shown in Figure 4. Preincubation of the MPC for 20 min at 37 °C with as little as 5 μM of the peptidyl aldehyde causes a marked decrease in incorporation of the radioactive ligand into the X subunit of the pituitary MPC (band 1 in lane b; Figure 4), and at a concentration of 20 μM (lane d), the inhibitor virtually prevents label incorporation. The same treatment of the spleen MPC (K_i of Z-LLF-CHO toward the ChT-L activity of the spleen MPC is without preincubation with the inhibitor about 6 μM) prevents virtually completely labeling of the LMP2 subunit at a concentration of 5 μM (band 1, lane g of Figure 4). Preincubation of the MPC from the two organs under the same condition with Z-LLL-CHO, another inhibitor of the ChT-L activity (K_i about 7 μM for both the spleen and

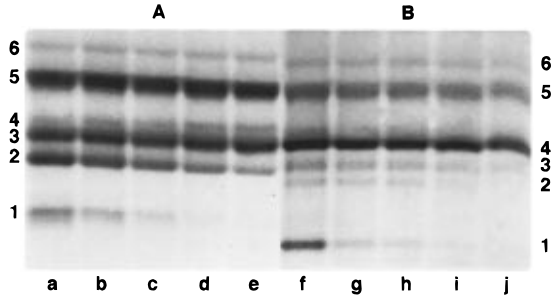

FIGURE 5: Effect of preincubation with different concentrations of Z-LLL-CHO on incorporation of labeled DCI into subunits of the pituitary and spleen MPCs. Conditions of the experiments and identification of radioactive bands in the two panels, and concentrations of the inhibitors are the same as in Figure 4.

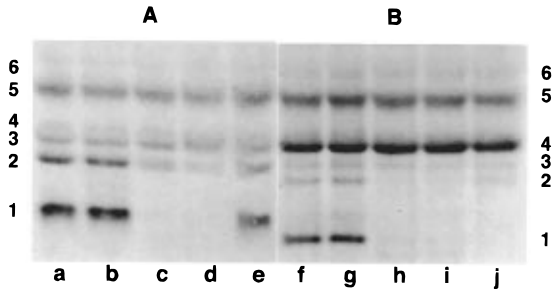

FIGURE 6: Radioautographs of subunits of the pituitary (panel A) and spleen MPCs (panel B) after short exposure (10 min) to [^{14}C]-DCI. Bands 1 correspond to X and LMP 2 subunits, bands 2 correspond to subunits Y, C5, and LMP7 in the pituitary and spleen MPCs, respectively, and band 3 in the spleen MPC corresponds to subunit Y. Lanes a, b and f, g are controls treated for 10 min with [^{14}C]-DCI; lanes c, d and h, i were preincubated (20 min) with 50 μM Z-LLF-CHO and Z-LLL-CHO before exposure to labeled DCI; lanes e and j are samples preincubated with Z-GPFL-CHO. For interpretation, see the text.

pituitary MPC), had a similar effect (Figure 5) with marked protection at a concentration of 5 μM (bands 1 in lanes b and g of Figure 5) and almost complete protection at higher inhibitor concentrations. It is notable that preincubation with the latter inhibitor also interferes with incorporation of label into the LMP7 subunit in the spleen MPC (band 2 of the spleen MPC, lanes g−j in Figure 5) although this becomes more evident at higher inhibitor concentration. Both inhibitors seem to decrease label incorporation, though more moderately, into band 2 of the pituitary MPC and bands 2 and 3 of the spleen enzyme. Band 2 contains subunits Y and C5 in the pituitary MPC, and band 3 contains the Y subunit in the spleen enzyme. It is possible that subunit Y might be necessary for expression of the PGPH activity, and that subunit LMP7 might be important for expression of the spleen BrAAP-like activity (see Discussion). Additional experiments, however, are needed to confirm these possibilities.

We have examined the time course of [^{14}C]-DCI incorporation into the two MPC preparations in order to determine whether the rapid inactivation of the ChT-L activity is reflected in the intensity of label incorporation. Figure 6 shows a radioautograph obtained after a 10 min treatment of the pituitary and spleen MPCs with 50 μM [^{14}C]DCI. The results show preferential incorporation of the radioactivity into the X subunit (band 1, lanes a and b in panel A of Figure 6) of the pituitary MPC and into the LMP2 subunit of the spleen MPC (bands 1 in lanes f and g of panel B), a finding suggesting that these subunits are involved in expression of the ChT-L activity. It is notable that short exposure to the

Table 1: Yield of Amino Acids in the First Six Sequencing Cycles of the X and LMP2 Subunits before and after Treatment with [^{14}C]-DCI[a]

		cycle					
subunit	treatment	1	2	3	4	5	6
X	residues:	T	T	T	L	A	F
	none	23	25.5	26	35	32	34
	[^{14}C]-DCI	*b*					
	Z-LLF-CHO + [^{14}C]-DCI	15	18	18	28	27	20
LMP2	residues:	T	T	I	M	A	V
	none	22	23	31	30	30	30
	[^{14}C]-DCI	trace	1.4	2.2	1.7	2.2	1.6
	Z-LLF-CHO + [^{14}C]-DCI	19.4	19.6	21.7	7.4	15.4	12

[a] N-Terminal amino acid sequencing was carried out after separation of subunits by SDS−PAGE as described under Materials and Methods, electrotransfer of proteins to PVDF membranes, and visualization of proteins by staining with Coomassie Blue. Data show the number of picomoles of each of the amino acids obtained from two bands of the proteins visualized after PAGE separation. [b] No sequence.

radioactive ligand causes a marked decrease in label incorporation into several bands otherwise intensely labeled after longer (30 min) exposure to DCI (Figures 3−5). These results are consistent with slower rates of label incorporation and therefore slower inactivation rates compared with those of the X and LMP2 subunits. It is also notable that the LMP7 band (band 2 in panel B of Figure 6) is only lightly labeled, suggesting that this subunit binds DCI slower. Like in the previous experiments, preincubation with Z-LLF-CHO and Z-LLL-CHO (50 μM for 20 min), the two inhibitors of the ChT-L activity, prevents incorporation of the label into the X (lanes c and d in panel A) and LMP2 bands (lanes h and i in panel B). By contrast, Z-GPFL-CHO, a specific and potent inhibitor of the pituitary BrAAP activity (*27*), but a weak inhibitor of the ChT-L activity, does not interfere with label incorporation into the X subunit of the pituitary MPC (lane e). This inhibitor, however, prevents labeling of the LMP2 subunit in the spleen MPC, consistent with the recent finding that Z-GPFL-CHO is a potent inhibitor of both the BrAAP and ChT-L activities in the spleen MPC (*17*). The results obtained with Z-GPFL-CHO provide additional evidence that although both the ChT-L and BrAAP components of the pituitary cleave bonds after hydrophobic amino acids, they are expressed by different components, a finding consistent with previous conclusions (*12*, *13*). They also suggest the presence of differences in susceptibility to inhibition by peptidyl aldehydes of the catalytic sites of the X and LMP2 subunits.

It was important to determine the nature of the reaction of [^{14}C]-DCI with the X and LMP2 components of the pituitary and spleen MPCs, respectively. Amino acid sequencing (Table 1) showed that exposure of the two MPC preparations to DCI caused loss of the sequence for the X subunit of the pituitary MPC and only about a 5% recovery of the sequence of the LMP2 subunit in the spleen MPC when related to the yield of amino acids in the control samples (not treated with DCI). By contrast, preincubation for 20 min with 50 μM Z-LLF-CHO caused preservation of the bulk of the amino acid sequences in both subunits. This result is consistent with the prevention of labeling observed in the radioautographs. Loss of the amino acid sequences after reaction with DCI could have resulted by formation of an amide bond with the amino group of the N-terminal threonine residues or by formation of an ester bond with the

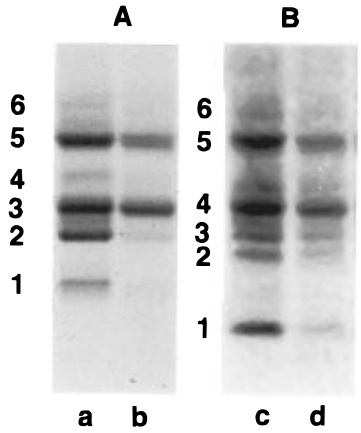

FIGURE 7: Effect of boiling on retention of the [^{14}C]-DCI label in subunits of the pituitary (A) and spleen (B) MPCs. Lanes a and c in panels A and B, respectively, are samples treated with 50 μM [^{14}C]-DCI for 30 min before SDS−PAGE. Lanes b and d contain samples boiled for 3 min after treatment with radioactive DCI and then subjected to SDS−PAGE. For interpretation, see the text.

hydroxyl group of threonine. Support for a reaction of the hydroxyl group of threonine with DCI was obtained from experiments in which the MPC samples were subjected to 3 min of boiling after exposure to DCI and SDS but before PAGE. Such treatment led to almost complete loss of label from the X and LMP2 subunits, suggesting interference with the Edman degradation by formation of a labile ester rather than an amide bond (lanes b and d of panels A and B of Figure 7). It is notable that this treatment caused to a different extent loss of label from other β-subunits, suggesting formation of ester bonds between DCI and residues of other β-subunits.

DISCUSSION

Several lines of evidence indicate that the X subunit of the pituitary is necessary for the expression of the ChT-L activity. First, incorporation of [^{14}C]-DCI into this subunit proceeds at a faster rate than incorporation into any of the other β-type subunits, a finding consistent with the high rate of inactivation of the ChT-L activity compared with the other MPC activities. Second, labeling of the X subunit is prevented by preincubation of the MPC with Z-LLF-CH0, a rather specific inhibitor of the ChT-L activity (*33*), and also by Z-LLL-CHO, another, albeit less specific, inhibitor of the same activity. Third, preincubation of the MPC with Z-LLF-CHO, and also Z-LLL-CHO, prevents covalent modification of the X subunit as manifested by prevention of the decrease in the electrophoretic mobility in SDS−PAGE, induced by incorporation of DCI. Fourth, preincubation of the pituitary MPC with Z-GPFL-CHO, a weak inhibitor of the pituitary ChT-L activity but a potent inhibitor of the BrAAP component, a chymotrypsin-like activity with a preference toward bonds after branched chain amino acid, does not interfere with label incorporation into the X subunit. Furthermore, the rapid inhibition of the ChT-L activity by treatment with DCI and the concomitant covalent modification of the N-terminal threonine residue of the X subunit are consistent with involvement of this residue in the catalytic process, thereby confirming the conclusions derived from experiments with lactacystin, a specific inhibitor of the proteasome (*7*). The same conclusion was also reached from X-ray diffraction studies of the proteasome from *Thermoplasma acidophilum* (*8, 38*).

Experiments suggestive of the involvement of the smallest subunit of chicken liver MPC, probably identical with the X subunit, in expression of the ChT-L activity have been reported (*39*). This was supported by the finding that the ChT-L-like activity is the only MPC activity inhibited by high concentrations (10 mM) of diisopropyl fluorophosphate (DFP), and the finding that treatment with radiolabeled DFP followed by SDS−PAGE resulted in incorporation of the label into the smallest subunit. Although the amino acid residue reacting with DFP has not been identified, this led to the opinion that the MPC might belong to the class of serine proteases. The unusually high concentrations of DFP needed for inhibition, however, and the resistance of other catalytic components of the MPC to DFP treatment brought into question this conclusion. Furthermore, treatment with high concentrations of [^{3}H]-DFP was reported to result in label incorporation into several MPC subunits rather than in selective labeling of a single subunit (*40*), further questioning the selectivity of the DFP reaction at these concentrations.

Replacement of the X, Y, and Z subunits in the spleen MPC by the LMP7, LMP2, and MECL1 subunits poses the question which of the latter subunits is involved in expression of the ChT-L activity in the resulting modified structure. The finding that exposure of the spleen MPC to [^{14}C]-DCI induces changes in the LMP2 subunit similar to those in the X subunit of the pituitary MPC argues for the involvement of the former in expression of the ChT-L activity in the spleen MPC. This is supported by the rapid rate of label incorporation and covalent modification of LMP2 by DCI, prevention of labeling by preincubation with relatively low concentrations of both Z-LLF-CHO and Z-LLL-CHO, and by association of the activity loss with modification of the N-terminal threonine residue which is prevented by Z-LLF-CHO. By contrast, the LMP7 subunit is only weakly labeled under the same conditions, and protection from labeling, especially by Z-LLL-CHO, seemed to be less pronounced than that of the LMP2 subunit (Figures 4 and 5). It is notable that incorporation of DCI into the LMP2 subunit of the spleen MPC was prevented not only by inhibitors of the ChT-L activity but also by Z-GPFL-CHO, a good inhibitor of the pituitary BrAAP component but a weak inhibitor of the ChT-L activity (*27*). This finding, however, is consistent with recent data from our laboratory (*17*) showing that the spleen ChT-L and BrAAP-like activities show a broader specificity than those of the pituitary MPC, and also a broader susceptibility to inhibition by peptidyl aldehydes.

Mutational studies of the proteasome from yeast led to the conclusion that the PRE2 and PRE3 subunits are necessary for expression of the ChT-L and PGPH activities, respectively (*14*, *15*). Because of the amino acid sequence similarity of PRE2 to both the X and LMP7 subunits, it is generally assumed that these subunits may be necessary for expression of the ChT-L activity in the two classes of mammalian proteasomes, one containing the X, Y, and Z and the other containing the LMP2, LMP7, and MECL1 subunits. By contrast, the marked homology between the amino acid sequences of PRE3 and LMP2 was considered supporting the involvement of the latter subunit in the PGPH activity of the mammalian proteasomes. However, the labeling experiments described here provide support for the involvement of the X subunit in expression of the ChT-L activity in the pituitary MPC, whereas the weak labeling of the LMP7 subunit and the low protection provided by preincubation with Z-LLL-CHO (Figure 5) argue against the

same function of this subunit in the spleen MPC. It is rather the LMP2 subunit which responds to exposure to DCI in a manner expected from the component expressing the ChT-L activity. Furthermore, the involvement of the LMP2 subunit in expression of the PGPH activity (rather than the ChT-L activity) is brought into doubt by the finding that incorporation of LMP2 into lymphoblastoid and murine fibroblast cell lines causes repression of the PGPH activity (*19, 41*), and a repressed PGPH activity is also characteristic of the MPC isolated from the spleen (*17*). The presence of a low PGPH activity in the spleen MPC is therefore more compatible with the residual amount of the Y (Delta) subunit after its replacement with LMP2. Since the Y subunit shows marked amino acid homology to both PRE3 and LMP2, the above conclusions are not in conflict with linking the Y subunit with the PGPH activity in proteasomes expressing the X, Y, and Z subunits.

The partial protection from labeling of the Y bands by the peptidyl aldehydes in both MPC preparations is markedly weaker than that of either the X subunit or the LMP2 subunit, and becomes generally more evident at high inhibitor concentrations (Figures 4 and 5). At these concentrations, the protection of the Y subunits is probably caused by lack of absolute specificity of the two peptidyl aldehydes toward the ChT-L activity. When 10 min labeling was used (Figure 6), incorporation of label into the Y subunit was both in the pituitary and in the spleen MPC much weaker than into the X or LMP2 subunits, consistent with the higher resistance of activities other than the the ChT-L activity to DCI inactivation (the spleen enzyme has no X subunit). The protection from labeling of the LMP7 subunit would not be surprising if this subunit were to express the BrAAP activity, considering the inhibitory effect of both peptidyl aldehydes on both the ChT-L and BrAAP-like activities in the spleen MPC (see reference *17*). This would be consistent with the low labeling of the LMP7 subunit (this study), and the slow rate of inactivation by DCI of the BrAAP-like activity of spleen MPC (*17*). The proposal that subunits Y and LMP7 could be necessary for expression of the PGPH and BrAAP-like activities, respectively, should nevertheless be regarded as tentative, and its confirmation requires further experimental evidence.

The recently published crystal structure of the 20S proteasome from yeast (*42*) allows only limited inferences to its mammalian counterpart, if only because the yeast enzyme does not contain the LMP2, LMP7, and MECL1 subunits present in the spleen proteasome. The assignment of the ChT-L and PGPH activities to PRE2 and PRE3, respectively, is consistent with the homology to the X and Y subunits, respectively, of the mammalian enzyme. However, assignment of trypsin-like activity also to the PRE2 subunit seems to contradict the mutational experiments in yeast showing that mutation of the N-terminal Thr residue in the yeast PUP1 subunit (a homolog of human Z subunit) eliminates trypsin-like activity (*43*).

The linkage of the PGPH activity in the spleen MPC with Y but not the LMP2 subunit would indicate that relatively small changes in the amino acid sequences of these subunits are sufficient to change the selectivity for defined peptide bonds. In summary, the data presented here argue for the involvement of the X and LMP2 subunits in expression of the ChT-L activity of the pituitary and spleen MPC, respectively, and suggest the involvement of the Y (Delta) subunits in both MPC preparations in expression of the

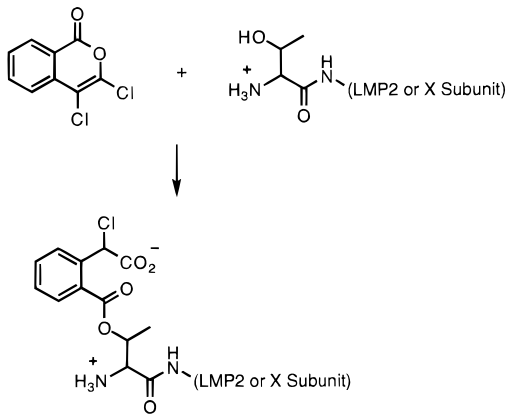

FIGURE 8: Reaction of 3,4-dichloroisocoumarin with the N-terminal threonine residue of X and LMP2 subunits.

PGPH activity. The above assignments leave uncertain the role of the LMP7 subunit. It is possible that the weak incorporation of the DCI label into this subunit indicates its involvement in expression of the BrAAP-like activity of the spleen MPC since this activity, while being more susceptible to inactivation by DCI than the pituitary BrAAP component, is still more resistant to inactivation than the other activities (*17*).

DCI inhibits most serine proteases and is therefore considered to be a mechanism-based general serine protease inhibitor (*21, 22*). Other isocoumarins such as 3-alkoxy-7-amino-4-chloroisocoumarins are more specific for individual proteases. In addition, DCI will inhibit a few cysteine proteases and has been shown to acylate glycogen phosphorylase (*44*). The mechanism of inhibition was derived from X-ray structure studies of five complexes of various isocoumarin inhibitors with porcine pancreatic elastase or bovine trypsin (*23, 45, 46*). Isocoumarins react initially with active site serine residues to form acyl enzymes which can deacylate to regenerate the active enzyme. Alternatively, the acyl enzyme can react with an enzyme nucleophile such as the histidine residue of the catalytic triad to give an alkylated enzyme (*22, 47*).

The data presented here indicate that, as with serine proteases, exposure of the MPC to DCI leads to the formation of an acyl enzyme with the hydroxyl groups of the N-terminal active site threonine residues in β-subunits of the MPC (Figure 8). Formation of an ester bond is indicated by the rather facile removal of the DCI label by brief boiling, and by loss of the amino acid sequences of the X and LMP2 subunits after reaction with DCI. Acylation of the N-terminal group, while preventing the Edman degradation, would have produced derivatives stable to short boiling. It also seems reasonable to assume that DCI forms ester bonds with the hydroxyl groups of the other β-subunits containing an N-terminal threonine residue. However, only four out of seven β-subunits contain a threonine at the N-terminus, but label incorporation was observed into more than four subunits. Thus, it is possible that labeling of other not yet identified residues might be involved.

The finding that subunits X and LMP7 could not be sequenced after treatment with DCI was attributed to interference with the Edman degradation due to the formation of ester bonds. The formation of such bonds was assumed on the basis of the lability of the adduct to short boiling, immediately after the labeling experiment. It is, however, not excluded that the long exposure to the buffer during the

electrophoresis experiments at pH above 8.0 and electrotransfer could have favored alkylation of the amino group of the N-terminal threonine. Indeed, in reactions of serine proteases with DCI, it was concluded that after initial formation of an acyl enzyme with the hydroxyl group of serine, the acyl enzyme can during longer incubations eliminate chlorine in an alkylation reaction with an enzyme nucleophile, such as the imidazole ring of the histidine residue in the catalytic triad (*22*, *47*). It is therefore possible that a similar mechanism could be responsible for the losses of amino acid sequences described here.

Our results support the involvement of the N-terminal threonine residue of the X and LMP2 subunits in the catalytic process, as postulated previously from studies with lactacystin (*7*), a specific inhibitor of the proteasome. The same conclusion was reached from X-ray diffraction studies of the proteasome from *Thermoplasma acidophilum* (*8*). The catalytic mechanism could be similar to that of penicillin acylase, an enzyme in which the hydroxyl group of an N-terminal serine residue, activated by proton transfer to the amino group of the same residue, attacks the carbonyl carbon of the peptide bond undergoing cleavage (*48*). Such a mechanism involves the formation of a tetrahedral intermediate, and compounds which can form tetrahedral addition products with the active center nucleophile are expected to act as inhibitors. Since aldehydes tend to react with catalytic hydroxyl groups in the active sites of proteases and form hemiacetals which are analogs of the transition state for substrate hydrolysis (*49*, *50*), the protection of the MPC from DCI-labeling, provided by aldehyde inhibitors of the ChT-L activity, points to the formation of such adducts with the hydroxyl group of the N-terminal threonine.

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