Amino acid sequencing of subunits of the multicatalytic proteinase complex (MPC) isolated from bovine spleen showed an almost complete replacement of the X, Y, and Z subunits, constitutively expressed in most tissues, by the interferon-γ-inducible LMP7, LMP2, and MECL1 subunits. A comparison with the pituitary MPC found a decreased chymotrypsin-like activity, a depressed peptidylglutamyl-peptide hydrolyzing activity, and a highly active component with properties similar to, but not identical with, that of the pituitary branched chain amino acid preferring (BrAAP) component. Unlike the pituitary BrAAP component, that of the spleen MPC exhibited a greatly decreased $K_m$, a highly increased catalytic efficiency ($k_{cat}$/$K_m$), and a 80–180 times greater specificity constant ($K_m/K_v$) toward substrates with either branched chain or aromatic amino acid residues in the P$_3$ position. Also, unlike the pituitary BrAAP component, that of the spleen was sensitive to inactivation by 3,4-dichloroisocoumarin and sensitive to inhibition by peptidyl-aldehydes with either phenylalaninal or leucinal residues. Several phenylalanyl peptidyl-aldehydes were identified which selectively inhibited components of the spleen but not of the pituitary MPC. Two of the inhibitors are dipetidyl-aldehydes, two others are tetrapetidyl-aldehydes with a Pro residue in the P$_3$ position. The possibility is discussed that the properties and specificity of the spleen MPC are a consequence of the presence of the interferon-γ-inducible subunits.

The finding that LMP7 and LMP2, products of two genes located within the class II region of the major histocompatibility complex, have amino acid sequences related to those of multicatalytic proteinase complex (MPC) subunits stimulated interest in the possible function of MPC in antigen processing (1–4). This possibility gained support from results of experiments showing that interferon-γ (IFN-γ) increased expression of LMP subunits (4, 5) and that expression of another subunit, referred to as MECL1, and expression of a potent protein activator of catalytic components of the MPC, referred to as PA28 or the 11 S regulator (REG) (6–10), was also stimulated by IFN-γ. Furthermore, inhibitors of the proteasome were reported to block generation of class I antigenic peptides (11), and presentation of these peptides was reported to require a functioning ubiquitin system (12). It was suggested that incorporation of these subunits into the MPC could affect the catalytic properties of the complex in a manner that would favor its function in antigen processing.

Previous work on the catalytic activities of the bovine pituitary MPC identified five distinct catalytic components. They were designated as chymotrypsin-like (ChT-L), trypsin-like (T-L), peptidylglutamyl-peptide hydrolyzing (PGPH), branched chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAAP), based on the nature of the P$_1$ amino acid residue at the scissile bond (13–18). As products of catalytic activity, most class I antigenic peptides are characterized by the presence of hydrophobic or basic amino acids at the carboxyl terminus. Attempts were therefore directed toward determining IFN-γ-induced changes in specificity toward substrates with hydrophobic and basic residues at the P$_3$ position. However, such experiments yielded contradictory results, with some groups reporting increases in ChT-L and T-L activities after IFN-γ treatment (19–21) and others reporting no changes or even decreased activities after IFN-γ (22, 23).

Work in this laboratory demonstrated marked differences in the subunit pattern and catalytic activities of the MPCs isolated from bovine pituitary, lung, and liver (24). Thus, the lung and liver MPCs contained 10 and 5 times, respectively, greater concentrations of LMP7 than the pituitary. Other changes included a decreased concentration of a subunit designated as X (also referred to as MB1 or BO2) (25, 26) and its partial replacement by LMP7, having a closely related amino acid sequence. Examination of the five catalytic activities in MPCs isolated from the lung and liver and a comparison with those of the pituitary exposed additional differences between these preparations. Thus, the ChT-L, PGPH, T-L, and SNAAP activities of the lung and liver MPCs were markedly decreased, whereas the BrAAP activity of lung and liver was activated as evidenced by a 20-fold increase in the specificity constant, $k_{cat}/K_v$ (24).

The increased concentration of LMP7 in lung MPC suggested that the elevated expression of this subunit might reflect an increased immune responsiveness of cellular elements of the lung to exposure to pathogens. This prompted the examination...
of the catalytic activities, subunit composition, and properties of the spleen MPC, in view of the known involvement of this organ in immune responses. Here we report that unlike the pituitary, the spleen MPC shows extensive replacement of the X, Y (also referred to as DELTA), and Z subunits by the LMP7, LMP2, and MECL1 subunits, respectively. Changes are also described in the specificity profile and sensitivity to inhibition by peptidyl-aldehyde inhibitors of the spleen MPC, and peptidyl-aldehydes are identified which specifically inhibit several components of the spleen but not the pituitary proteasome. The possibility is discussed that these changes are related to introduction of the IFN-γ-inducible subunits into the MPC structure.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fresh frozen bovine spleens were obtained from Pel-Freez (Roger, AR). Isolation of the MPC was carried out according to a previously described procedure for the purification of the pituitary MPC (27). After the final purification step, aliquots of the enzyme (0.25 ml) containing 1 mg of protein/ml were kept frozen at −70 °C and thawed just before determining enzyme activities. PAGE and HPLC separation of subunits (24) showed the presence in the final preparation of small amounts of higher molecular weight impurities that were not present in the pituitary enzyme. Integration of the area under the peaks of subunits separated by HPLC indicated that the impurities constituted about 10% of the total protein contents. Additional purification was carried out by rechromatography of the final preparations on a Superose 6 column (2.5 × 50 cm; Pharmacia Biotech Inc.) equilibrated with a Tris-EDTA buffer (0.05 M, pH 7.5) containing 1 mg of protein/ml.

**Substrates for Determination of Activities of Components of the MPC**—Z-GGL-pNA, Z-GGF-pAB, Z-(D)ALR-2NA, Z-GPALA-pAB, Z-GPGG-pPAB, and Z-GPAFG-ppAB were synthesized as described previously (15, 17, 18, 25). Other reagents were obtained from Sigma.

**Synthesis of Peptidyl-Aldehyde Inhibitors**—Peptidyl-aldehyde inhibitors were synthesized from the corresponding peptidyl-alcohols by oxidation using a modification (18, 29, 30) of the dimethyl sulfoxide-carbodiimide reaction described by Pfister and Molfett (31). The formation of the aldehyde was followed by reacting aliquots of the reaction mixture with 2,4-dinitrophenylhydrazine to form the corresponding dinitrophenylhydrazones as described previously (32).

**Determination of Enzyme Activities**—Activities of the Chk-L, T-L, FPGH, BrAAP, and SNAAP components of the MPC were determined with Z-GGL-pNA (or Z-GGF-pAB), Z-(D)ALR-2NA, Z-GPALA-pAB, Z-GPGG-pPAB, and Z-GPAFG-ppAB, respectively, as described previously (16–18). BrAAP and SNAAP activities were determined in a coupled enzyme assay in the presence of an excess aminopeptidase N as described previously (15). Activity is defined in units as the amount of enzyme causing degradation of 1 μmol of substrate/h. Where indicated specific activities represent the number of μmol of substrate degraded/mg of protein.

**PAGE and Amino Acid Sequencing**—PAGE was done in 10% gels at 70 °C and thawed 20 min to tubes at 37 °C containing the appropriate substrate in Tris-HCl buffer, pH 8.0. The rate of release of the aromatic amine was determined as described previously. Alternatively, aliquots of the preincubation mixtures were withdrawn at 0.5, 10, 20, and 30 min, and the activity was determined as described above. Controls containing the same amount of dimethyl sulfoxide but not DCI were also carried through the procedure.

**Determination of Casein Degradation by the MPC**—Incubation mixtures contained 100 μg of β-casein, 20 μm of MgCl2, 5 μg of DCI dissolved in dimethyl sulfoxide. The dimethyl sulfoxide activity was determined as described above. Controls containing the same amount of dimethyl sulfoxide but not DCI were also carried through the procedure.

**Identification of Degradation Products**—Products of degradation of the substrates Z-GPAFG-pAB, Z-GPGG-pPAB, and Z-GPAFG-pAB were identified after incubation for 30 min of a 1 μm substrate solution with 10 μg of enzyme in Tris-HCl buffer, pH 8.0, and a final volume of 200 μl. Degradation products were separated by HPLC using a Deltapak C18 column (5 μm, 300 Å). Elution was carried out with a linear gradient established between 10 and 50% acetonitrile, each containing 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min. The rate of casein degradation was determined by measuring the peak height of the casein in relation to controls in which the enzyme was omitted.

**Effect of 3,4-Dichloroisocoumarin (DCI) on Activities of Components of the MPC**—The pituitary and spleen MPC were preincubated at 26 °C with 20 μm DCI dissolved in dimethyl sulfoxide. The dimethyl sulfoxide concentration did not exceed 5% of the volume of the preincubation mixture. Aliquots of the preincubation mixtures were mixed after 30 min to tubes at 37 °C containing the appropriate substrate in Tris-

![FIG. 1. Electrophoretic separation of subunits of the MPCs isolated from spleen (lane S), pituitary (lane P), and lung (lane L). Protein bands were visualized by silver staining. For conditions of PAGE see "Experimental Procedures." Subunit identification is based on NH2-terminal sequencing. Protein bands are numbered consecutively in order of their rate of migration, independent of whether they are, or are not, represented in all three preparations. Band 1, LMP2; band 2, X; band 3, LMP7; band 4 spleen MPC, MECL1, DELTA, and C5; band 4 pituitary MPC, DELTA and C5; band 5 spleen, RN3 and C5; band 5 pituitary, RN3; band 6 spleen, MECL1; band 6 pituitary, Z.](http://www.jbc.org/content/379/7/11825/F1.expansion.png)
as in the intensity of their staining. Amino acid sequence analysis of stained protein bands on polyvinylidene difluoride membranes obtained after electrotransfer of MPC subunits separated by SDS-PAGE similar to that shown in Fig. 1 was used to identify those subunits having unblocked amino termini. The \( \beta \)-type subunits identified by this procedure are listed in Table I. The fastest moving discrete protein (band 1, Fig. 1) in the spleen MPC was found by amino acid sequencing to correspond to LMP2. This protein, albeit more faintly stained, is also clearly visible in the lung MPC but is virtually absent in the pituitary enzyme. The second fastest moving protein of the pituitary MPC is identical with X, a subunit found previously in MPC preparations of the pituitary, lung, and liver. Consistent with our previous observations this subunit is most prominent in the pituitary and is much less abundant in the lung MPC (24). It is either absent or is present in only trace amounts in the spleen MPC. The third fastest moving protein is most intensely stained in the spleen MPC but is also present in the lung MPC. Its NH\( _2 \)-terminal amino acid sequence is consistent with identification as LMP7. This subunit is virtually absent in the pituitary MPC. The fourth stained protein, numbered 4 in Fig. 1, is most prominent in the pituitary, but is fainter in the spleen enzyme and was shown by sequencing to contain more than a single subunit. Two sequences, corresponding to subunits Y and C5, were identified in the pituitary MPC, and sequences corresponding to Y, C5, and MECL1 were identified in the spleen MPC. Subunits in band 5 were identified as RN3 and C5 in the spleen MPC, and RN3 in the pituitary MPC. Band 6 was found to contain subunits MECL1 in the spleen preparation, whereas a similar band in the pituitary was identified as subunit Z. There were no amino acid sequences obtained from these preparations which were not from the MPC.

The yields of X, Y, Z and LMP2, LMP7, and MECL1 subunits obtained by sequencing of the pituitary and spleen MPCs are given in Table I. The average yield exceeded 10 pmol with the limit of detection being about 1 pmol. The data show that of the X, Y, and Z subunits present in the pituitary MPC, the X and Z subunits were below the limit of detection in the spleen MPC and that the Y subunit constituted only about 20% of that present in the pituitary MPC. Furthermore, none of the LMP2, LMP7, and MECL1 subunits present in the spleen MPC could be detected by sequencing in the pituitary MPC. These results indicate that amounts of the X and Z subunits in the spleen MPC and the LMP2, LMP7, and MECL1 subunits in the pituitary MPC were below 1 pmol, or less than 10% of that present in the pituitary and spleen MPCs, respectively. Thus, the spleen and pituitary MPCs are ideally suited for studying differences in properties and specificity which could be attributed to the presence of the IFN-\( \gamma \)-inducible subunits in the MPC structure. The composition of the lung subunits is intermediate between that of the pituitary and spleen MPCs, since the lung MPC contains both IFN-\( \gamma \)-inducible subunits as well as those constitutively expressed in the pituitary. Whether the appearance of both types of subunits results from their expression in a single cell population or from the presence of subpopulations of MPCs with different subunit compositions in different cell types remains to be established. It is notable that the \( \beta \)-type subunits identified in Fig. 1 are characterized by a fast rate of migration in SDS-PAGE compared with the slower migrating \( \alpha \)-type subunits, all of which have blocked NH\( _2 \) termini. The finding that three subunits, MECL1, RN3, and C5, appear with more than one electrophoretic mobility but the same respective NH\( _2 \)-terminal amino acid sequences needs to be studied further. Whether this is the result of post-translational modifications, such as phosphorylation, glycosylation, or limited proteolysis, remains to be established.

The differences in subunit composition between the spleen and pituitary MPC prompted us to examine the catalytic activities of the two preparations, in the expectation that such differences could reveal properties associated with the incorporation into the MPC of IFN-\( \gamma \)-inducible subunits. The results of activity measurements of five catalytic components present in MPCs are summarized in Table II. In comparison with the pituitary MPC, the spleen enzyme is characterized by a low ChT-L activity, a greatly elevated BrAAP activity, and a depressed PGPH activity. The extent of the decrease of the PGPH activity becomes more evident when the two preparations are treated with low concentrations of SDS, a detergent that is well known to activate the PGPH activity (16, 27). Thus, whereas the PGPH activity in the pituitary preparation was elevated by SDS treatment to an activity of close to 100 units/mg of protein, the activity of the spleen enzyme reached only a value of 4.2 units/mg of protein, about 4% of that in the pituitary MPC. The activity of the BrAAP component in the spleen MPC was about

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Subunit} & \text{Tissue}\text{a} & \text{NH}_2\text{-terminal amino acid sequence}\text{b} & \text{PAGE}\text{c} \ (\text{Fig. 1}) \\
\hline
X (11.5) & BP & TTTLAFKF\underline{R}GVID & Band 2 \\
X & BL & TTTLAFKF\underline{R}GVIDVAAD & Band 2 \\
LMP7 (9.5) & BS & TTTLAFKFQGVID & Band 3 \\
LMP7 & BL & TTTLAFKFQGVIDVA & Band 3 \\
Y (2.1) & BS & TTTIMAVQFDGV & Band 4 \\
Y (9) & BP & TTTIMAVQFDG & Band 4 \\
LMP2 (12.3) & BS & TTTIMAVQFDGVVGGDSRNXAGEAVVRNFQDXSLMR & Band 1 \\
Z (16) & BP & TTTIAGVXYK\underline{G}IVG & Band 6 \\
MECL1 (4.5) & BS & TTTIAGVXYK\underline{G}IVG & Band 4 \\
MECL1 (7.5) & BS & TTTIAGVXYK\underline{G}IVGIL & Band 6 \\
C5 & BS & RFS\underline{P}YX\underline{F}NGGT\underline{V} & Band 4 \\
C5 & BS & RFS\underline{P}YX\underline{F}NGTVLANGED & Band 5 \\
C5 & BP & RFS\underline{P}YX\underline{F}N & Band 4 \\
RN3 & BS & TQ\underline{N}PMVGTGS & Band 5 \\
RN3 & BP & TQ\underline{N}PMVGTGTVLGLKEE & Band 5 \\
\hline
\end{array}
\]

\text{a} B, bovine; L, lung; P, pituitary; S, spleen.
\text{b} Differences in amino acid sequences are shown in boldface. Data in parentheses represent the number of pmol of peptides obtained by sequencing.
\text{c} Band numbering refers to that given in Fig. 1.
20 times greater than that in the pituitary MPC, reaching values close to 70 units/mg of protein. It should be noted that the pituitary BrAAP component is mostly latent, and its full activity is only expressed after treatment with either DCI or SDS. Under such conditions the activity of the pituitary complex is similar to that found in the spleen. The possibility was considered that the high BrAAP activity in the spleen MPC could be the result of the presence in the preparation of a protein activator similar to that of an activator referred to as PA28 or 11 S regulator (6, 8, 10). However, experiments in which the spleen and pituitary MPCs were mixed in different proportions yielded purely additive activities (data not shown), indicating the absence of a protein activator.

Most class I antigenic peptides are characterized by the presence of either aromatic or branched chain amino acids at the carboxyl terminus. Generation of such peptides could have been expected to require the catalytic action of the ChT-L component, but the involvement of the BrAAP component of the MPC needs to be considered. Because of the decrease of the ChT-L activity in the spleen MPC we have mainly concentrated our efforts on a closer examination of the properties of the BrAAP component. Table III summarizes the results of activity measurements toward three substrates, two of which, Z-GPALA-pAB and Z-GPALG-pAB, were found previously to be cleaved by the pituitary BrAAP component at the Leu-Ala and Leu-Gly bonds, respectively (15). Reactions were carried out both in the presence and absence of aminopeptidase N. In the presence of the aminopeptidase the degradation products Ala-pAB and Gly-pAB are degraded further to the free amino acids and the aromatic amine, which is then quantitated after diazotation. This permits insight into the rates of cleavage of the substrates both between the adjacent two amino acids (Leu-Ala and Leu-Gly) and the amino acid-arylamide bond. Analysis of the amino acid composition of degradation products generated during incubation of the spleen or pituitary MPC with Z-GPALA-pAB and Z-GPALG-pAB showed predominantly cleavage of the Leu-Gly and Leu-Ala bonds, respectively, with little direct cleavage of the amino acid-arylamide bond, the latter reaction being catalyzed by the ChT-L activity (15, 28). Thus, cleavage of the Leu-Ala and Leu-Gly bonds by the pituitary MPC proceeded almost 10 and 90 times faster, respectively, than cleavage of the amino acid-arylamide bonds. Although the spleen MPC cleaved the amino acid-arylamide bond at a rate comparable to that of the pituitary MPC, its rate of cleavage of the Leu-Ala and Leu-Gly bonds was about 15 times greater. This was reflected in a further increase in the ratios of the reaction rates in the presence versus the absence of aminopeptidase N. It should be noted, however, that the identity of the degradation products generated by the spleen and pituitary MPC preparations indicated that the increased reaction rates catalyzed by the spleen MPC were apparently due to the presence in the spleen MPC of a catalytic component with a specificity similar to but not necessarily identical with that of the pituitary BrAAP component.

Markedly different results, however, were obtained with the two MPC preparations when the Leu residue in Z-GPALG-pAB was replaced by a Phe (Table III). Like the Leu-containing substrates the pituitary and the spleen MPCs cleaved Z-GPAG-pAB both at the Phe-Gly bond and the adjacent amino acid-arylamide bond. However, the rate of the overall reaction and the ratio of the reaction rates in the presence versus the absence of aminopeptidase N were markedly lower for the pituitary enzyme than that for the Leu-containing substrates (Table III). This indicated that cleavage of the Phe-Gly bond by the pituitary MPC is less favored than cleavage of either the Leu-Gly or Leu-Ala bond and that the presence of a Phe residue in the P1 position and a Pro residue in the P3 position is not favored by the BrAAP component of the pituitary MPC, a finding consistent with the previously observed results with peptidyl-aldehyde inhibitors having a Pro residue in the P3 position and a phenylalaninal residue in the P1 position (30). The spleen enzyme, however, cleaved the Phe-Gly bonds more than 20 times faster and the Gly-pAB bond almost 20 times slower than the pituitary MPC. As a consequence, the Phe-Gly bond was cleaved at a 1,700-fold faster rate than the Gly-pAB bond, although the rate of cleavage of the Phe-Gly bond was similar to that of the Leu-Gly bond. Thus, the spleen BrAAP-like component readily cleaved bonds after both aromatic and branched chain amino acids, a specificity broader than that of the BrAAP component of pituitary MPC, the latter showing a preference toward bonds on the carboxyl side of branched chain amino acids (13, 28).

The broader specificity of the spleen BrAAP-like component is also evident from kinetic parameters of degradation of the three substrates shown in Table IV. Compared with the pituitary MPC the $K_m$ of the spleen enzyme toward all three substrates was greatly decreased, whereas the turnover number was greatly enhanced. As a result the specificity constants for the three substrates was increased by a factor from about 80 to more than 180. These data show that the catalytic efficiency and specificity constants of the spleen MPC are much greater than its pituitary counterpart for bonds both after branched chain and aromatic amino acids, although some preference toward bonds after branched chain amino acids seems still to be preserved.

### Table II

**Comparison of the five catalytic components of the pituitary and spleen MPC**

Data represent specific activities in $\mu$mol/mg of protein/h. Activities in the spleen enzyme represent mean values obtained from four separate preparations. Value in parentheses is in the presence of SDS (0.02–0.029%).

| Component | Substrate concentration | Specific activity |
|-----------|-------------------------|-------------------|
|           |                         | Spleen  | Pituitary |
|           |                         | $\mu$m | $\mu$m |
| BrAAP     | Z-GPALA-pAB (1.0)       | 65.6   | 3.37   |
| PPGPH     | Z-LLE-2NA (0.64)        | 1.08   | (4.25) |
| ChT-L     | Z-GGL-pNA (0.4)         | 3.2    | 8.8    |
| T-L       | Z-nAP-L-2NA (0.4)       | 6.21   | 5.46   |
| SNAAP     | Z-GPAGG-pAB (1.0)       | 2.7    | 2.6    |

### Table III

**Activity of the spleen and pituitary MPC toward substrates of the BrAAP component**

Data are mean values obtained from three or four separate determinations $\pm$ S.E. Where indicated 20 $\mu$g of aminopeptidase N (AP-N) was present in the incubation mixture.

| Substrate   | Concentration | Spleen MPC activity | Pituitary MPC activity |
|-------------|---------------|---------------------|------------------------|
|             |               | $\mu$mol/mg protein/h | $\mu$mol/mg protein/h |
|             |               | No additions $^a$ | +AP-N | Ratio | No additions $^a$ | +AP-N | Ratio |
| Z-GPALA-pAB | 1.0           | 0.49 ± 0.046       | 65.9 ± 6.9             | 124   |
| Z-GPALG-pAB | 1.0           | 0.078 ± 0.015      | 58.4 ± 1.4             | 768   |
| Z-GPAGF-pAB | 1.0           | 0.04 ± 0.010       | 70.1 ± 6.6             | 1750  |

$^a$ No additions indicates the absence of AP-N. Activities were determined as described under “Experimental Procedures.”
The pituitary and spleen MPC were incubated for 30 min with 20 μM DCI at 26 °C. Activities were determined in aliquots withdrawn at different incubation times as described under “Experimental Procedures.” Controls contained the same incubation mixtures with the enzyme preincubated in the absence of DCI. Values in parentheses indicate percent of control activity.

The results of a more detailed study of the effect of DCI treatment on the BrAAP and ChT-L components in both the spleen and pituitary MPC preparations are shown in Fig. 2. Whereas treatment of the pituitary MPC with DCI caused a 10-fold activation of the BrAAP component toward Z-GPALG-pAB (inset, Fig. 2) and a smaller activation of the activity toward Z-GPAFG-pAB, the same treatment caused a slow inactivation of the BrAAP-like spleen component. The loss of activity proceeded at a similar rate when followed with either Z-GPALG-pAB or Z-GPAFG-pAB as substrates, suggesting that both are cleaved by the same catalytic component. To exclude the possibility that these two substrates are cleaved by the ChT-L activity of the spleen MPC rather than by a BrAAP-like component similar to that found in the pituitary MPC, we compared the time course of inactivation of activity toward these substrates with the time course of inactivation of the ChT-L activity toward Z-GGF-pAB (a substrate resistant to degradation by the BrAAP component). As shown in Fig. 2, the activity in both the spleen and pituitary enzyme preparations toward Z-GGF-pAB was much more sensitive to inactivation by DCI than the activity toward the respective BrAAP substrates. Thus, the pituitary ChT-L activity lost more than 90% of activity after a 30-min exposure to DCI, and the rate of inactivation of the ChT-L component in the spleen MPC proceeded at a similar, although somewhat slower rate (about 80% of this activity was lost after a 30-min exposure to DCI). These results indicated that degradation of Z-GPALG-pAB and Z-GPAFG-pAB is catalyzed at active sites that are distinct from those catalyzing degradation of the ChT-L substrate Z-GGF-pAB. Collectively the data indicate that the spleen BrAAP-like

### Table IV

| MPC          | Substrate          | Range of concentration | \( K_m \) (μM) | \( k_{cat} \) (s⁻¹) | \( k_{cat}/K_m \) (s⁻¹μM⁻¹) |
|--------------|--------------------|------------------------|----------------|---------------------|-----------------------------|
| Pituitary    | Z-GPALA-pAB        | 1.6–8.0                | 6.7            | 5.2                 | 780                         |
| Spleen       | Z-GPALA-pAB        | 0.1–1.0                | 0.10 ± 0.015   | 14.1 ± 1.5          | 141,000                     |
| Pituitary    | Z-GPALG-pAB        | 1.6–8.0                | 5.2            | 3.75                | 720                         |
| Spleen       | Z-GPALG-pAB        | 0.1–1.0                | 0.14 ± 0.005   | 12.9 ± 0.31         | 92,000                      |
| Pituitary    | Z-GPAFG-pAB        | 0.5–8.0                | 3.3 ± 0.55     | 1.98 ± 1.35         | 600                         |
| Spleen       | Z-GPAFG-pAB        | 0.1–2.0                | 0.38 ± 0.06    | 18.8 ± 0.02         | 49,500                      |

*Values were obtained from Orlowski et al. (15).*

### Table V

| Component | Substrate Concentration | Specific Activity | Control | After DCI |
|-----------|-------------------------|-------------------|---------|-----------|
| ChT-L     | Z-GGL-pNA 0.4           | 2.87              | 0.54 (19) |
| SNAAP     | Z-GPAGG-pAB 1.0         | 2.9               | 1.28 (44) |
| T-L       | Br-dALR-2NA 0.4         | 6.78              | 5.49 (81) |
| PGPH      | Z-LLE-2NA 0.64          | 1.82              | 1.59 (87) |
| BrAAP     | Z-GPALG-pAB 1.0         | 72                | 69 (96)  |

**Effect of DCI on activities of the catalytic components of the spleen MPC activities**

Data are average values obtained from two separate preparations. The spleen MPC (1 mg/ml) was preincubated with 20 μM DCI for 30 min at 26 °C. Aliquots of the enzyme were then transferred to incubation mixtures at 37 °C containing substrate, 0.05 M Tris-HCl buffer, pH 8.0, and excess aminopeptidase N (20 μg of protein). Reactions were terminated after a 30-min incubation, and the activities were determined as described under “Experimental Procedures.” Controls contained the same incubation mixtures with the enzyme preincubated in the absence of DCI. Values in parentheses indicate percent of control activity.

**Fig. 2. Effect of DCI on activities of the BrAAP and ChT-L activities of the pituitary and spleen MPC.** The pituitary and spleen MPC were incubated for 30 min with 20 μM DCI at 26 °C. Activities were determined in aliquots withdrawn at different incubation times as described under “Experimental Procedures.” Open squares and filled squares are BrAAP activities toward Z-GPALG-pAB and Z-GPAFG-pAB in spleen MPC. Filled circles show pituitary MPC activity toward Z-GPAFG-pAB. Open and filled triangles represent pituitary MPC activities toward Z-GGF-pAB in pituitary and spleen MPC, respectively. The inset shows the effect of DCI on the activity of the pituitary BrAAP component toward Z-GPALG-pAB under the same conditions.

Exposure of the pituitary MPC to DCI, an irreversible inhibitor that reacts with the serine residue in the active site of serine proteases and apparently also with the threonine residues in the active sites of MPC subunits (36, 38), leads to inactivation of the ChT-L, T-L, PGPH, and SNAAP components, albeit with different inactivation rate constants. By contrast, the putatorial BrAAP component is rapidly activated up to 10-fold by the same treatment (inset, Fig. 2) (15), although this activation is followed by a slowly progressing inactivation. The low initial activity of the putatorial BrAAP component and its activation by DCI treatment were interpreted as reflecting the presence of a large latent BrAAP activity whose full expression requires activation. As in the pituitary, treatment of the spleen MPC with DCI leads to partial inactivation of the ChT-L, T-L, PGPH, and SNAAP components (Table V). However, the same treatment caused a small decrease rather than activation of the spleen BrAAP-like activity. Nevertheless, the spleen BrAAP-like activity was more resistant to DCI inactivation than the ChT-L, T-L, PGPH, and SNAAP activities. The failure of DCI to activate the spleen BrAAP-like activity could be interpreted as indicating that, unlike the predominantly pituitary BrAAP component, this activity is already activated and has no latent component or that the properties of the spleen BrAAP component are quite different from those of its pituitary counterpart.

The results of a more detailed study of the effect of DCI treatment on the BrAAP and ChT-L components in both the spleen and pituitary MPC preparations are shown in Fig. 2. Whereas treatment of the pituitary MPC with DCI caused a 10-fold activation of the BrAAP component toward Z-GPALG-pAB (inset, Fig. 2) and a smaller activation of the activity toward Z-GPAFG-pAB, the same treatment caused a slow inactivation of the BrAAP-like spleen component. The loss of activity proceeded at a similar rate when followed with either Z-GPALG-pAB or Z-GPAFG-pAB as substrates, suggesting that both are cleaved by the same catalytic component. To exclude the possibility that these two substrates are cleaved by the ChT-L activity of the spleen MPC rather than by a BrAAP-like component similar to that found in the pituitary MPC, we compared the time course of inactivation of activity toward these substrates with the time course of inactivation of the ChT-L activity toward Z-GGF-pAB (a substrate resistant to degradation by the BrAAP component). As shown in Fig. 2, the activity in both the spleen and pituitary enzyme preparations toward Z-GGF-pAB was much more sensitive to inactivation by DCI than the activity toward the respective BrAAP substrates. Thus, the pituitary ChT-L activity lost more than 90% of activity after a 30-min exposure to DCI, and the rate of inactivation of the ChT-L component in the spleen MPC proceeded at a similar, although somewhat slower rate (about 80% of this activity was lost after a 30-min exposure to DCI). These results indicated that degradation of Z-GPALG-pAB and Z-GPAFG-pAB is catalyzed at active sites that are distinct from those catalyzing degradation of the ChT-L substrate Z-GGF-pAB. Collectively the data indicate that the spleen BrAAP-like
component is characterized by a higher sensitivity to inactivation by DCI than its pituitary counterpart and by the ability to cleave efficiently bonds after both branched chain and aromatic amino acids.

The broader specificity of the spleen BrAAP-like component induced us to examine the possibility that this specificity is also reflected in the susceptibility of the spleen MPC to peptidyl-aldehyde inhibitors with substrate-related structures. The results of experiments testing this possibility are summarized in Table VI. Four of the inhibitors (inhibitors 1–4) specifically inhibited both the BrAAP and ChT-L activities of the spleen MPC but were ineffective or inhibited only weakly the same components of the pituitary MPC. Two of these inhibitors (Z-FF-CHO and Z-LF-CHO) are dipeptidyl-aldehydes containing a phenylalaninal as the aldehyde group and either Leu or Phe residues as the amino acid moieties. The other two inhibitors (Z-GPACF-CHO and Z-GPPF-CHO) are characterized by the presence of a phenylalanyl group and a Pro residue in the P2 position. It is of interest that replacement of the phenylalaninal in Z-GPFF-CHO with a leucinal group leads to loss of this specificity. Thus, Z-GPFCCHO (inhibitor 5 in Table VI) inhibited the BrAAP activity of both the pituitary and spleen MPC and also the spleen ChT-L activity, albeit with a somewhat higher Ki. Z-LLF-CHO (inhibitor 6 in Table VI), a potent inhibitor of the pituitary ChT-L activity (29) and a weak inhibitor of the pituitary BrAAP activity, was the most potent inhibitor of the spleen BrAAP-like component but a weaker inhibitor of the spleen MPC ChT-L activity. The inhibition of the spleen BrAAP-like activity by peptidyl-aldehydes having either a phenylalaninal group and a Pro residue in the P2 position. Identification of peptidyl-aldehyde inhibitors that effectively inhibit the BrAAP and ChT-L components of the spleen MPC but not that of the pituitary should be helpful in examining the functional importance of the INF-γ-inducible subunits.

Previous work on the effect of Z-GPFF-CHO on casein degradation by the pituitary MPC indicated that the BrAAP component is an important factor in degradation of this protein (30). Furthermore, exposure of intact cells in culture to this inhibitor caused accumulation of ubiquitin-protein conjugates, indicating inhibition of the ubiquitin-dependent pathway of intracellular proteolysis. To examine the effect of peptidyl-aldehydes on the caseinolytic activity of the pituitary and spleen MPC we tested the effect of two peptidyl-aldehydes on degradation of this protein by the two enzymes (Table VII). Z-FF-CHO (inhibitor 1 in Table VI), an inhibitor that selectively inhibited both the BrAAP and ChT-L activities of the spleen MPC, markedly inhibited the caseinolytic activity of the spleen MPC but not that of the pituitary. By contrast, Z-GPFF-CHO (inhibitor 5 in Table VI), an inhibitor of the BrAAP activity of both the pituitary and spleen MPCs, effectively inhibited casein degradation by both preparations. The results indicate that selectivity of the inhibitors deduced from their effect on degradation of synthetic substrates extends toward the caseinolytic activity of the two enzyme preparations.

**DISCUSSION**

Incorporation of the three IFN-γ-stimulated subunits into the proteasome structure leads to replacement and down-regulation of three constitutively expressed subunits designated as X, Y, and Z. It was suggested that such replacement generates “immunoproteasomes” having properties facilitating the generation of the 8–9 amino acid-containing class I antigenic peptides (39). Since most class I antigenic peptides contain at the carboxyl terminus either aromatic, branched chain, or basic amino acid residues (40), the expectation was that immunoproteasomes would have to exhibit increased ChT-L and T-L activities. Attempts to test this hypothesis through experiments in which different cell lines were exposed to IFN-γ yielded contradictory results, with some groups reporting increases in ChT-L activity (19–21) and others reporting no changes or even decreased activities (22, 23). Furthermore, transfection experiments leading to overexpression of LMP2 and LMP7 subunits in a murine fibroblast cell line and a human lymphoblastoid T2 cell line led to a marked decrease of the ChT-L activity measured with succinyl-Leu-Leu-Val-Tyr-MCA as the substrate and to a depression of the PGPH activity measured with Z-Leu-Leu-
Glu-2NA (41, 42).

The presence of LMP7, LMP2, and MECL1 subunits in the spleen MPC, makes it an excellent system for determining changes in catalytic activities which could be associated with the presence of these subunits, without resort to transfection experiments or exposure of cells to IFN-γ. Our results showing that the spleen MPC is characterized by a lower ChT-L activity and by a depression of the PGPH activity are consistent with results of transfection experiments cited above. The decreased Ch-T-L activity, however, would seem to be inconsistent with the expected increased efficiency of immunoproteasomes in generating antigenic peptides having aromatic or branched chain amino acid residues at the carboxyl terminus, unless other factors such as, for example, activation of the latent portion of this component by intracellular activators is of importance in this MPC function. Furthermore, a study of degradation products generated from a 25-amino acid-containing peptide that included in its sequence a 9-amino acid peptide epitope from murine cytomegalovirus-infected mouse cells led to the conclusion that there is no correlation between changes in specificity induced by incorporation of LMP subunits into the MPC and activity toward synthetic substrates such as succinyl-L-L-L-L-V-MCA (41, 42).

Activity measurements reported here indicate that the most prominent difference between the spleen and pituitary MPCs is the presence of an activated BrAAP-like component in the spleen enzyme. This component is characterized by a high specificity constant toward substrates having either aromatic or branched chain amino acid residues at the P1 position. Because of this specificity the catalytic activity of this component needs to be considered as a potential factor contributing to the nature of amino acids at the COOH terminus of antigenic peptides. There are two properties of the pituitary and spleen BrAAP-like components which differ markedly from those of the Ch-T-L activity. First, both are more resistant to inactivation by DCI than the Ch-T-L activity, and indeed the pituitary BrAAP component is actually activated by exposure to this reagent. Second, the BrAAP component is characterized by an inability to cleave amino acid-arlylamide bonds. Whereas most studies of activities of MPC preparations from various tissues and cell cultures are based on measuring the rate of hydrolysis of peptidyl-arylamide bonds, determination of the BrAAP activity is based on measuring the rate of cleavage of bonds between two adjacent amino acids. In this respect such measurements reflect more closely the type of reactions catalyzed by the MPC with natural peptide substrates. Examination of reaction products derived from degradation of the 25-amino acid polypeptide cited above showed that the predominant cleavage occurs after a Leu residue that contributes the COOH terminus of the naturally produced antigenic epitope and that generation of two other peptides requiring cleavage after hydrophobic residues was stimulated by incorporation of LMP7 into the MPC (42).

Furthermore, in the presence of the protein activator PA28 and after incorporation of the LMP subunits into a murine fibroblast cell line, an accelerated cleavage after a Phe residue was observed (Fig. 4 in Ref. 41). All of these cleavage sites occurred after a Leu residue that contributes the COOH terminus of the naturally produced antigenic epitope and that generation of two other peptides requiring cleavage after hydrophobic residues was stimulated by incorporation of LMP7 into the MPC (42). Furthermore, in the presence of the protein activator PA28 and after incorporation of the LMP subunits into a murine fibroblast cell line, an accelerated cleavage after a Phe residue was observed (Fig. 4 in Ref. 41). All of these cleavage sites could have therefore resulted from the action of either the Ch-T-L or the BrAAP component of the MPC. It is therefore notable in this respect that the BrAAP component was reported to be activated by PA28 to a much higher degree than any of the other catalytic components of the MPC (43), and therefore the participation of the BrAAP component in cleavage of natural peptides needs to be considered. It should be emphasized that none of the above findings excludes the possibility that after an initial fragmentation of the epitope-containing protein by the MPC, generation of antigenic peptides could require further processing by aminopeptidases and/or oligopeptidases, enzymes that are abundant in the cell cytoplasm.

It is tempting to associate the differences in catalytic activities between the spleen and pituitary MPCs solely with the presence of LMPs and MECL1 subunits in the spleen MPC. Indeed, the changes in the Ch-T-L and PGPH activities observed after transfection experiments cited above would seem to support such conclusion at least with respect to these two activities (41, 42). However, a comparison of the subunit patterns of the two MPC preparations shows differences in the presence and absence of certain bands, as well as differences in intensity of staining. Furthermore, amino acid sequencing showed that three of the β subunits migrated with more than one electrophoretic mobility. Such differences in mobility could have resulted from post-translational modifications, such as phosphorylation or glycosylation, but could have also resulted from limited proteolysis that caused a change of the subunit molecular weight. Both phosphorylations and glycosylations of MPC subunits have been described, although either no changes or only small catalytic changes have been reported as a result of phosphorylation (44–46). Accordingly, linkage with post-translational modifications of catalytic differences between the spleen and pituitary MPCs described here would require evidence that post-translational modifications are restricted to only one of the enzymes studied. No evidence of such restriction is available. Nevertheless, none of the above factors could be excluded as potentially contributing to the changes in the catalytic activities described here.

The inhibitory profile of peptidyl-aldehydes on the BrAAP activity of the spleen MPC is consistent with substrate studies indicating a broad specificity toward peptide bonds after both aromatic and branched chain amino acids in the P1 position. It is of particular interest that the specificity constant (k_cat/K_m) toward substrates with both branched chain and aromatic amino acids is for the spleen BrAAP component up to more than a hundred times greater than for the corresponding component in the pituitary MPC. It remains to be established whether these changes are functionally significant for the antigen-processing function of the MPC. The role of different catalytic components of the MPC in reactions catalyzed by the MPC needs further examination. In view of the importance of the MPC in fundamental cell functions including the mitotic cycle, antigen processing, and regulation of the half-life of regulatory proteins and transcription factors (14, 47–49), general inhibition of MPC activity would be expected to cause cell toxicity. Accordingly, examination of the involvement of distinct catalytic components in selected functions of the MPC would require knowledge of exact specificities of these components and the use of specific inhibitors capable of modulating their activities. The specificity differences between MPCs from pituitary and spleen reported here and the associated differences in sensitivity to aldehyde inhibitors could constitute a basis for exploration of the functional roles of the two types of proteasomes, one containing the X, Y, and Z subunits, the other containing the LMP2, LMP7, and MECL1 subunits.

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