A Comparative Study of the Chymotrypsin-like Activity of the Rat Liver Multicatalytic Proteinase and the ClpP from Escherichia coli*

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A comparative study of the chymotrypsin-like activity of the purified recombinant ClpP protease and the multicatalytic proteinase from rat liver is presented. The peptidase activity of both enzymes has been analyzed with several synthetic fluorogenic peptides, containing either aromatic or nonpolar amino acids in their P1 position. The respective $V_{max}$, $K_m$, and $V_{max}/K_m$ were calculated from a number of experiments. The substrate specificity of the multicatalytic proteinase, as expressed by $V_{max}/K_m$ values, indicates the following order of preference: N-Suc-IIW-MCA > N-Suc-LY-MCA > N-Suc-LVY-MCA > N-Suc-LLVY-MCA > N-Suc-AAF-MCA > N-Cbz-GGL-β-NA > Glut-GGF-8-NA > FPAM-4-MNA. In the case of the ClpP the order of preference is: N-Suc-LY-MCA > N-Suc-IIW-MCA > N-Suc-LVY-MCA > N-Suc-AAF-MCA > N-Cbz-GGL-β-NA > FPAM-4-MNA (where: N-Suc, N-succinyl-; MCA, 7-amido-4-methyl coumarin; β-NA, β-naphthylamide; N-Cbz, N-benzoxycarbonyl-; 4-MNA, 4-methoxy-β-naphthylamide; Glut, glutaryl. This similar substrate specificity is further supported by the lack of activity of both enzymes against SY-MCA and N-Suc-AAFP-MCA (known substrates of chymotrypsin), by very reduced activity against N-Suc-AAA-MCA and by no significant activity against LG-β-NA. The results of mixed substrate experiments show that all the peptides that are substrates seem to be hydrolyzed by a single class of chymotrypsin-like site in both enzymes. The substrate specificity studies suggest a possible evolutionary relationship between the catalytic component of the ClpP of Escherichia coli and the multicatalytic proteinase chymotrypsin-like catalytic component. This conclusion is further supported by other circumstantial evidence: the fact that affinity-purified anti-ClpP antibodies cross-react with two polypeptide components of the rat liver multicatalytic proteinase complex, presented here and also shown previously; the known resemblance of both structures at the electron microscope level; and their reported role in the degradation of NH₂-end rule substrates.

The multicatalytic proteinase hydrolyzes small peptides whose carboxyl side contains basic, hydrophobic and acid amino acids; the first two specificities are often referred to as the trypsin-like (basic) and chymotrypsin-like (hydrophobic) activities, which does not imply that the mechanism of hydrolysis by the MCP is identical to those of trypsin and chymotrypsin, respectively. Experiments aimed at the identification of subunits responsible for the peptidase activities within the MCP complex by the means of labeling by [3H]diisopropyl fluorophosphate (that should label the putative Ser residue of the active sites), show that either several protein bands of the MCP (1, 2) or a single (the smallest) polypeptide band are labeled (3, 4). The Clp or Ti protease from Escherichia coli is composed of two subunits, 81 kDa (ClpA) and 21 kDa (ClpP), both required for protein degradation in a reaction coupled to ATP hydrolysis (5, 6). The isolated ClpP component is not able to hydrolyze proteins, but it hydrolyzes small hydrophobic peptides, like N-Suc-LY-MCA, in an ATP-independent manner, indicating that its active site has chymotrypsin-like activity (7). Molecular cloning of the ClpP subunit (8, 9) has shown that its deduced amino acid sequence has no clear similarity to either trypsin or chymotrypsin (or the serine protease family); the sequence around the essential Ser residue (labeled with [3H]diisopropyl fluorophosphate) shows homology with ClpP homologues from chloroplasts, and a part of the possible catalytic triad (Ser, His) has been suggested (9). The native ClpP protein has a molecular mass of 240 kDa with a characteristic hexagonal ring-like structure when viewed in the electron microscope that is reminiscent of the multicatalytic proteinase (8), and it has even been reported that antibodies against ClpP recognize two MCP polypeptides from yeast and one polypeptide from rat liver MCP (10).

We decided to explore the possible relationship between the ClpP and the MCP by studying the kinetics of the chymotrypsin-like activity of both enzymes which allowed us to show that they have similar substrate specificity, suggesting a possible evolutionary relationship of their chymotrypsin-like catalytic sites, which is further supported by the fact that affinity-purified anti-ClpP antibodies cross-react with polypeptide components of the rat liver MCP complex.

**EXPERIMENTAL PROCEDURES**

Materials—Enzymes for nucleic acid modification were provided by Boehringer or New England Biolabs. Oligonucleotides with the desired sequence were synthesized in an Applied Biosystems 391...

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†The abbreviations used are: Mbp, multicatalytic proteinase; proteasome; N-Cbz, N-benzoxycarbonyl-; N-Suc, N-succinyl-; 4-MNA, 4-methoxy-β-naphthylamide; MCA, 7-amido-4-methyl coumarin; β-NA, β-naphthylamide; PAGE, polyacrylamide gel electrophoresis; pNA, p-nitroaniline; HPLC, high performance liquid chromatography; DTDT, dithiothreitol; BSA, bovine serum albumin; Glut, glutaryl.
DNA synthesizer. Oligonucleotide primers for sequencing were from Pharmacia. The following fluorescent peptides (the amino acids were used in this study were bought from Sigma: N-Cbz-ARR-4-MNA, N-Cbz-LLE-2NA, N-Cbz-PHLLVLS-β-NA, HS-4-MNA, N-Cbz-GGTLW-5-MNA, LG-4-MNA, N-Cbz-NA, SY-β-NA, FPAM-4-MNA, N-Suc-LVLY-β-NA, N-Suc-LY-β-NA, N-Suc-AAFP-MCA, N-Suc-AAF-MCA, N-Suc-AAA-MCA, N-Suc-IWW-MCA. All other reagents were of analytical reagent grade or molecular biology grade.

Purification of MCP from Rat Liver—The latent form of the rat liver multicatalytic proteinase was purified as described (11), adding one additional step after the glycerol gradient sedimentation. A pool of the glycerol gradient fractions was applied to an HPLC (System Gold, Beckman) DEAE-SPW column equilibrated with 50 mM Tris-Cl, pH 8.0, 25 mM KC1, 10% glycerol, 1 mM DTT, and 1 mM MgCl2 (buffer A), the multicatalytic proteinase was eluted with a linear gradient from 25 to 500 mM KC1 (buffer B) in the same buffer, and the protein and activity peak eluted at 70% buffer B (0.38 M KCl).

Cloning and Expression of Recombinant ClpP—Bacterial DNA was obtained from 1 ml of an overnight culture of HB101 strain of E. coli (12). The ClpP gene was cloned by an amplification reaction with the following oligonucleotides: 5'-oligonucleotide, 5'-ATCATGGGATCC TACGCGGCGACGAGATAC-3', 3'-oligonucleotide, 5'-CTGCA GTCTAGACGGCCTTGATAGTCGGG-3', derived from the reported ClpP sequence (8). The amplification reaction contained in a final volume of 50 μl: 20 mM Tris-Cl, pH 7.5, 50 mM KC1, 10 mM MgCl2, 0.5 mM of each deoxynucleotide, 0.5 μg of each oligonucleotide primer, 0.1 μg of bacterial genomic DNA, and 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus). The amplification reaction consisted of 30 cycles with the following profile: denaturation at 94°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 2 min with a final extension of 5 min. The amplified DNA band was gel-purified and digested with BamHI and XhoI for cloning into pGEM2 vector, digested with the same restriction enzymes (12). The DNA insert subcloned into pGEM2 was sequenced by the chain termination method (13) to confirm that we obtained the ClpP gene, the construct named pClpP. For expression of the recombinant ClpP protein, the ClpP gene was excised from the plasmid pGClpP by digestion with BamHI and subcloned into the pTT-7 expression vector (14), digested with the same restriction enzyme. This construct containing the full-length ClpP gene under the control of a T7 RNA polymerase promoter, named pTClpP, was used to transform BL21 (DE3) for expression (15) of the recombinant protein.

Purification of Recombinant ClpP—For induction of protein expression, BL21(DE3) cells harboring the pTClpP plasmid were grown overnight in LB (12) + 100 μg/ml ampicillin (LB ampicillin medium) and diluted next day 1:10 in fresh LB ampicillin medium, grown at 37°C to an OD (at 600 nm) of 0.4-0.5. Fifteen minutes of each oligonucleotide primer, 0.1 μg of bacterial genomic DNA, and 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus). The amplification reaction consisted of 30 cycles with the following profile: denaturation at 94°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 2 min with a final extension of 5 min. The amplified DNA band was gel-purified and digested with BamHI and XhoI for cloning into pGEM2 vector, digested with the same restriction enzymes (12). The DNA insert subcloned into pGEM2 was sequenced by the chain termination method (13) to confirm that we obtained the ClpP gene, the construct named pClpP. For expression of the recombinant ClpP protein, the ClpP gene was excised from the plasmid pGClpP by digestion with BamHI and subcloned into the pTT-7 expression vector (14), digested with the same restriction enzyme. This construct containing the full-length ClpP gene under the control of a T7 RNA polymerase promoter, named pTClpP, was used to transform BL21 (DE3) for expression (15) of the recombinant protein.

Purification of ClpP gene was excised from the plasmid pGClpP by digestion with ClpP was purified from the induced bacterial culture continued for 3 h. The purified recombinant ClpP protein was subjected to 15% SDS-PAGE, Western-blotted, with the anti-ClpP antiserum (0.5 ml) in blocking buffer. After washing, the anti-ClpP specific antibodies were eluted with 0.2 M glycine, pH 2.4. The eluted antibody solution was neutralized with 1 M Tris-Cl, pH 7.5, diluted 3-fold with TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), dialyzed against TBS at 4°C overnight, and concentrated to 0.5 ml on a Centricon ultrafilter (30-kDa cut-off). The purified recombinant ClpP protein was subjected to SDS-PAGE, Western-blotted, stained with Ponceau Red, and a horizontal strip containing the purified ClpP protein excised, blocked in blocking buffer (see above), and incubated with the anti-ClpP antiserum (0.5 ml) in blocking buffer. After washing, the anti-ClpP specific antibodies were eluted with 0.2 M glycine, pH 2.4. The eluted antibody solution was neutralized with 1 M Tris-Cl, pH 7.5, diluted 3-fold with TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), dialyzed against TBS at 4°C overnight, and concentrated to 0.5 ml on a Centricon ultrafilter (30-kDa cut-off). The purified recombinant ClpP protein was subjected to SDS-PAGE, Western-blotted, stained with Ponceau Red, and a horizontal strip containing the purified ClpP protein excised, blocked in blocking buffer (see above), and incubated with the anti-ClpP antiserum (0.5 ml) in blocking buffer. After washing, the anti-ClpP specific antibodies were eluted with 0.2 M glycine, pH 2.4. The eluted antibody solution was neutralized with 1 M Tris-Cl, pH 7.5, diluted 3-fold with TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), dialyzed against TBS at 4°C overnight, and concentrated to 0.5 ml on a Centricon ultrafilter (30-kDa cut-off). The purified recombinant ClpP protein was subjected to SDS-PAGE, Western-blotted, stained with Ponceau Red, and a horizontal strip containing the purified ClpP protein excised, blocked in blocking buffer (see above), and incubated with the anti-ClpP antiserum (0.5 ml) in blocking buffer. After washing, the anti-ClpP specific antibodies were eluted with 0.2 M glycine, pH 2.4. The eluted antibody solution was neutralized with 1 M Tris-Cl, pH 7.5, diluted 3-fold with TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), dialyzed against TBS at 4°C overnight, and concentrated to 0.5 ml on a Centricon ultrafilter (30-kDa cut-off). The purified recombinant ClpP protein was subjected to SDS-PAGE, Western-blotted, stained with Ponceau Red, and a horizontal strip containing the purified ClpP protein excised, blocked in blocking buffer (see above), and incubated with the anti-ClpP antiserum (0.5 ml) in blocking buffer. After washing, the anti-ClpP specific antibodies were eluted with 0.2 M glycine, pH 2.4. The eluted antibody solution was neutralized with 1 M Tris-Cl, pH 7.5, diluted 3-fold with TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), dialyzed against TBS at 4°C overnight, and concentrated to 0.5 ml on a Centricon ultrafilter (30-kDa cut-off). The purified recombinant ClpP protein was subjected to SDS-PAGE, Western-blotted, stained with Ponceau Red, and a horizontal strip containing the purified ClpP protein excised, blocked in blocking buffer (see above), and incubated with the anti-ClpP antiserum (0.5 ml) in blocking buffer. After washing, the anti-ClpP specific antibodies were eluted with 0.2 M glycine, pH 2.4. The eluted antibody solution was neutralized with 1 M Tris-Cl, pH 7.5, diluted 3-fold with TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), dialyzed against TBS at 4°C overnight, and concentrated to 0.5 ml on a Centricon ultrafilter (30-kDa cut-off).
protein, mass standards used to calibrate the HPLC column are under "Experimental Procedures." Together with the previous sequencing of the cloned DNA step of purification (DEAE-HPLC) was subjected to HPLC gel filtration of an aliquot of the corresponding protein peak, processed as described assay.

Both results are in perfect agreement with those reported used for the expression, certainly assure that the protein we bovine pituitary enzyme (19). Further on, although peptides the rat liver MCP substantiates the previous report on the amino acids in their P1 position: R (N-Cbz-ARR-4-MNA), E and P (GP-CMNA, N-Cbz-PAGP-P-NA). None of these peptides are substrates for the ClpP protease, whereas the ClpP are in perfect agreement with those reported previously by many different groups. The new result the specific activity of MCP (assayed at 500 μM peptide concentration) against these two peptides is 10 times (GP-4-MNA) or 100 times (N-Cbz-PAGP-β-NA) lower than the activity observed with N-Suc-LY-MCA (500 μM). These results assure us that there is no significant contamination by other proteases in our ClpP preparation.

The chymotrypsin-like activity of both ClpP and rat liver MCP is based on the hydrolysis of fluorogenic peptides that have a hydrophobic amino acid in the P1 position. In order to compare the two fundamental kinetic parameters of an enzymatic reaction, namely $V_{\text{max}}$ (maximal rate in steady-state kinetics) and $V_{\text{max}}/K_m$ (a measure of catalytic efficiency and specificity), both enzymes were prepared under very similar conditions; the kinetic analysis was performed with the enzymes obtained after the last step of purification (DEAE-HPLC, see under "Experimental Procedures") and approximately at the same protein concentration (within a factor of 2 or 3). All these precautions are specially relevant in the present case, where oligomeric proteins are used and have likely (MCP) and surely (ClpP) several substrate sites per oligomer. All the kinetic data were obtained by continuous recording of the released fluorochrome, and no significant deviation from linearity was detected at any substrate concentration (no significant lag period was detected that could have been an indication of hysteretic behavior). The initial velocity was always calculated after a 5- or 10-min reaction, when less than 10% of the substrate has been consumed. All kinetic data were obtained from at least four different experiments, in which six different peptide concentrations were used, covering the range from 10% of the apparent $K_m$ value reported herein to 4-fold the $K_m$ value (except where indicated), using two or even four different preparations of both enzymes. The values reported for $V_{\text{max}}$ and $K_m$ (summarized in Table I) for the different substrates were obtained by adjusting the corresponding double-reciprocal or Eadie-Hofstee plots by linear regression (usual regression coefficient, 0.93–0.98), and no significant deviation from linearity of the corresponding plots with any of the substrates was observed. The $V_{\text{max}}$ values are expressed in nanomoles/min/μg of enzyme, instead of the $k_{\text{cat}}$, because: 1) most activities reported in the literature are expressed in a similar way, so direct comparison with our data is possible; 2) the calculation of $k_{\text{cat}}$ or turnover number of the enzymes will imply an estimation of the molecular mass of the active subunit and the number of catalytic sites per protomer of the ClpP (possible) and MCP (uncertain). When we say that a certain peptide is not a substrate, we actually mean that the hydrolysis rate of that particular peptide, at a concentration of 500 μM, is less than 2 pmol/min/μg of the corresponding protease. A control of each peptide at the maximal concentration used was always run, without enzyme, in parallel to subtract any possible spontaneous hydrolysis. To discard the possibility that the peptide substrates are cleaved at some other position apart from the peptide-fluorochrome bond, we checked the hydrolysis by MCP and ClpP (20 μg/ml) of the peptides: N-Suc-IIW-MCA, N-Suc-LLVY-MCA, N-Suc-AAP-MCA, N-Cbz-GGL-β-NA, Glut-GGF-β-NA, and FPAM-4-MNA, at 2 mM final concentration. Except for Cbz-GGL (100 μM), after incubation for 3 h at 25 °C we do not detect by reverse phase HPLC any other peptide peak (in amounts bigger than 2% of the input peptide amount) except for the peptide without the fluorochrome (confirmed by amino acid analysis).

**Conditions of Kinetic Experiments**—Before studying the chymotrypsin-like activity, we analyzed the activity of ClpP and MCP against fluorogenic peptides with the following amino acids in their P1 position: R (N-Chz-ARR-4-MNA), E (N-Chz-LLE-2NA), S (HS-4-MNA and N-Chz-PFHLVY-β-NA), and P (GP-4-MNA, N-Cbz-PAGP-β-NA). None of these peptides are substrates for the ClpP protease, whereas those containing R, E and P, but not S, in the P1 position are substrates of the multicatalytic protease. The results obtained with the ClpP are in perfect agreement with those reported previously (7). MCP is able to hydrolyze peptides containing basic and acidic amino acids in the P1 position, as reported previously by many different groups. The new result that peptides with S in the P1 position are not substrates of the rat liver MCP substantiates the previous report on the bovine pituitary enzyme (19). Further on, although peptides having proline in the P1 position are substrates of the MCP, the specific activity of MCP (assayed at 500 μM peptide concentration) against these two peptides is 10 times (GP-4-MNA) or 100 times (N-Cbz-PAGP-β-NA) lower than the activity observed with N-Suc-LY-MCA (500 μM). These results assure us that there is no significant contamination by other proteases in our ClpP preparation.

The chymotrypsin-like activity of both ClpP and rat liver MCP is based on the hydrolysis of fluorogenic peptides that have a hydrophobic amino acid in the P1 position. In order to compare the two fundamental kinetic parameters of an enzymatic reaction, namely $V_{\text{max}}$ (maximal rate in steady-state kinetics) and $V_{\text{max}}/K_m$ (a measure of catalytic efficiency and specificity), both enzymes were prepared under very similar conditions; the kinetic analysis was performed with the enzymes obtained after the last step of purification (DEAE-HPLC, see under "Experimental Procedures") and approximately at the same protein concentration (within a factor of 2 or 3). All these precautions are specially relevant in the present case, where oligomeric proteins are used and have likely (MCP) and surely (ClpP) several substrate sites per oligomer. All the kinetic data were obtained by continuous recording of the released fluorochrome, and no significant deviation from linearity was detected at any substrate concentration (no significant lag period was detected that could have been an indication of hysteretic behavior). The initial velocity was always calculated after a 5- or 10-min reaction, when less than 10% of the substrate has been consumed. All kinetic data were obtained from at least four different experiments, in which six different peptide concentrations were used, covering the range from 10% of the apparent $K_m$ value reported herein to 4-fold the $K_m$ value (except where indicated), using two or even four different preparations of both enzymes. The values reported for $V_{\text{max}}$ and $K_m$ (summarized in Table I) for the different substrates were obtained by adjusting the corresponding double-reciprocal or Eadie-Hofstee plots by linear regression (usual regression coefficient, 0.93–0.98), and no significant deviation from linearity of the corresponding plots with any of the substrates was observed. The $V_{\text{max}}$ values are expressed in nanomoles/min/μg of enzyme, instead of the $k_{\text{cat}}$, because: 1) most activities reported in the literature are expressed in a similar way, so direct comparison with our data is possible; 2) the calculation of $k_{\text{cat}}$ or turnover number of the enzymes will imply an estimation of the molecular mass of the active subunit and the number of catalytic sites per protomer of the ClpP (possible) and MCP (uncertain). When we say that a certain peptide is not a substrate, we actually mean that the hydrolysis rate of that particular peptide, at a concentration of 500 μM, is less than 2 pmol/min/μg of the corresponding protease. A control of each peptide at the maximal concentration used was always run, without enzyme, in parallel to subtract any possible spontaneous hydrolysis. To discard the possibility that the peptide substrates are cleaved at some other position apart from the peptide-fluorochrome bond, we checked the hydrolysis by MCP and ClpP (20 μg/ml) of the peptides: N-Suc-IIW-MCA, N-Suc-LLVY-MCA, N-Suc-AAP-MCA, N-Cbz-GGL-β-NA, Glut-GGF-β-NA, and FPAM-4-MNA, at 2 mM final concentration. Except for Cbz-GGL (100 μM), after incubation for 3 h at 25 °C we do not detect by reverse phase HPLC any other peptide peak (in amounts bigger than 2% of the input peptide amount) except for the peptide without the fluorochrome (confirmed by amino acid analysis).
reported previously (1.3 mM); so data are available for MCP. The first specificity coincidence we report is that peptide SY-
-NA is not a substrate for either MCP or ClpP, whereas it is a substrate for chymotrypsin (7). These results confirm a
previous observation reported with ClpP (7) and may indicate that both enzymes cannot tolerate a free α-NH₂ group in the
P2 position (N-Suc-LY-MCA versus SY-MCA). Increasing the length of the hydrophobic amino acids, but keeping Y in the
P1 position, substrate N-Suc-LLVY-MCA produces a drastic decrease in the $K_m$ of ClpP for this substrate (75 μM)
and in the $V_{max}$ (40 times reduced compared with N-Suc-LY-
MCA hydrolysis). In the case of MCP, there is an increase of the $V_{max}$ (3-fold) and an increase of the $K_m$ (130 μM). The decreased activity of ClpP against N-Suc-LLVY-MCA com-
pared with N-Suc-LY-MCA has already been reported (7).

The peptide N-Suc-AAPF-MCA is hydrolyzed by both en-
zymes with apparent $K_m$ of 300 μM (MCP) and 80 μM (ClpP).
In contrast, the peptide N-Suc-AAF is not a substrate for ei-
ther of the two enzymes. Furthermore, 500 μM N-Suc-
AAFP-MCA added to the assay mixture, prior (preincubation for 10 min) or after, the addition of either N-Suc-AAF-MCA, N-Suc-LY-MCA, or N-Suc-LLVY-MCA (50 μM), does not change the velocity of the reaction observed with either of the
enzymes. These results indicate that N-Suc-AAFP-MCA is not only a poor substrate, but that it is unable to compete with similar substrates, so it may not bind at all to the catalytic site of both enzymes; N-Suc-AAFP-MCA is an ex-
cellent substrate for chymotrypsin (20, 21). These results clearly indicate that $P_1$ in the P2 position of a peptide con-
taining $P_1$ in its P1 position, makes of the corresponding peptide a poor substrate for the chymotrypsin-like activity of both, ClpP and MCP. When the activity of both enzymes was assayed with the peptide Ght-GGF-β-NA, whose main difference with N-Suc-AAFP-MCA is the change of amino acids in
P2 and P3 position for less hydrophobic amino acid (G versus A), the estimated $K_m$ of MCP for this peptide is 275 μM and significant hydrolysis (at 500 μM) was detected with the ClpP, 12 pmol/min/μg, but kinetic analysis was not possible as the $K_m$ of the ClpP for this substrate is likely to be higher than the peptide solubility in water (3 mM). These results suggest that MCP (and likely ClpP) seems to prefer more hydrophobic residues in the P2 and P3 positions. Another interesting point with these substrates can be made by comparison of other groups’ results. Rivett (22) reported a $K_m$ of 1.2 mM for AAF-
MCA and similar $V_{max}$ for AAF-MCA and N-Suc-AAF-MCA with the rat liver MCP. The $K_m$ for N-Suc-AAF-MCA is 290
μM (see Table I), so it seems that just the presence of a charged blocked group or the simple blocking of the free α-
NH₂ of the first amino acid, makes of the same peptide a better substrate (increasing the affinity of the enzyme). Ac-

### Table 1

| Peptide substrate         | $K_m$ (μM) | $V_{max}$ (pmol/min/μg) | $V_{max}/K_m$ ( μM⁻¹) | $K_m$ (μM) | $V_{max}$ (pmol/min/μg) | $V_{max}/K_m$ ( μM⁻¹) |
|---------------------------|------------|------------------------|-----------------------|------------|------------------------|-----------------------|
| N-Suc-IW-MCA              | 24 ± 3     | 269 ± 30               | 10.8                  | 24 ± 5     | 7.5 ± 0.6              | 0.3                   |
| N-Suc-LY-MCA              | 25 ± 3     | 175 ± 20               | 7                     | 660 ± 40   | 543 ± 30              | 0.82                  |
| N-Suc-LLVY-MCA            | 130 ± 20   | 560 ± 60               | 4.3                   | 75 ± 8     | 13 ± 1                | 0.17                  |
| N-Suc-AAF-MCA             | 290 ± 30   | 820 ± 80               | 2.83                  | 80 ± 10    | 11.5 ± 0.7            | 0.14                  |
| Ght-GGF-β-NA              | 275 ± 30   | 280 ± 30               | 1.18                  | ND         | ND                    | ND                    |
| N-Cbz-GGL-β-NA            | 90 ± 15    | 170 ± 25               | 1.88                  | 30 ± 6     | 4.6 ± 0.3             | 0.15                  |
| FPAM-4MNA                 | 140 ± 20   | 80 ± 15                | 0.57                  | 55 ± 8     | 4.5 ± 0.4             | 0.082                 |

Chymotrypsin-like Specificity of the MCP and the ClpP

Kinetic parameters of the chymotrypsin-like activity of the rat liver multicatalytic protease and ClpP from E. coli

Kinetische Parameter der chymotrypsinähnlichen Aktivität des Leber-Mehl-Zell-Katalysatorproteins und ClpP aus E. coli

( $n$ = number of different experiments) as described under “Experimental Procedures” and “Results,” ND, not determined.

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Chymotrypsin-like Activity of ClpP and MCP: Substrates with Hydrophobic, Not Aromatic, Amino Acids in P1 Position—Let us consider the results obtained with peptides containing hydrophobic, not aromatic amino acids, in the P1 position. We have analyzed two substrates of this type, FPAM-4MNA and N-Cbz-GGL-β-NA (see Table I). It is noteworthy that both the MCP and the ClpP are able to hydrolyze after methionine, like chymotrypsin, although with much less catalytic efficiency than after Y or F. Besides, P in the P2 position seems to make a bulky P1 residue (F) insensitive to both enzymes, but does not prevent the hydrolysis at a less bulky residue, like methionine, when P is present in the P3 position. N-Cbz-GGL-β-NA is a well known substrate of MCP (19, 24). The $K_m$ and $V_{max}$ data for this peptide are subject to some criticism in the case of the MCP, as the estimated $K_m$ and $V_{max}$ are based on initial velocity with five different concentrations, up to 100 μM (estimated $K_m$ 90 μM), due to insolubility of the peptide. This $K_m$ value is similar to the one reported before (65 μM) by Chu-Ping et al. (25), who also recognized the same problem of solubility of the peptide. When the P1 position of the synthetic fluorogenic peptide contains A, as in the peptide N-Suc-AAA-MCA, significant hydrolysis is detected with both enzymes, at 500 μM, which proved to be too low for kinetic studies. When it contains G, as in the peptide LG-β-NA (500 μM), we detect no significant hydrolysis with either of the two proteases.

Fig. 2 shows a graphic representation of the relative values of $V_{max}/K_m$ of ClpP and MCP for each of the peptides presented in Table I, considering the values obtained for N-Suc-IW-MCA as 1. These results accompanied by those on peptides that are not substrates (SY-β-NA, N-Suc-AAFP-MCA, LG-β-NA) or poor substrates (N-Suc-AAA-MCA) presented above, allow us to conclude that the specificity of the chymotrypsin-like activities of MCP and ClpP are similar.
Mixed Substrate Experiments—Another interesting issue is whether or not all the substrates presented are hydrolyzed at the same chymotrypsin-like site. It can also be argued, in the case of the ClpP, that we have a contamination by another chymotrypsin-like enzyme, due to the low V\text{max} of all peptides compared to N-Suc-LY-MCA.

To rule out possible contamination of the ClpP preparation, the activity with all the substrates presented in Table I was checked in an experiment like the one presented in Fig. 1. The hydrolysis of the different peptides presented in Table I was perfectly coincident with the N-Suc-LY-MCA hydrolytic activity and the same result was obtained in a similar experiment with rat liver MCP (data not shown). To address the issue of the number of chymotrypsin-like sites, we conducted mixed substrate experiments as shown in Table II, but all the combinations were not possible, since the substrates with MCA and NA cannot be mixed together due to interference in the fluorometric detection. The conclusion from these mixed substrate experiments is that the MCP and ClpP activities, assayed in the presence of two of the chymotrypsin-like substrates, is always lower than that obtained by adding up the velocities observed with each polypeptide when present separately (when MCP and ClpP are added together in the same reaction the summing up of activities is effectively observed, as predicted for independent catalytic sites). These results indicate that N-Suc-AAF-MCA, N-Suc-LLVY-MCA, N-Suc-LY-MCA, and N-Suc-IIW-MCA are hydrolyzed in the same catalytic site and that Glu-GGF-\(\beta\)-NA, N-Cbz-GG-\(\beta\)-NA, and FPAM-4-MNA are hydrolyzed in the same catalytic site. As it is highly unlikely that Glu-GGF-\(\beta\)-NA and N-Suc-AAF-MCA are hydrolyzed in different catalytic sites, one can conclude that all the peptides having in their P1 position either W, Y, F, L, or M are hydrolyzed in one unique type of catalytic sites in both proteases. This conclusion is further supported by the fact that heat inactivation of MCP and ClpP by incubation at 65 °C leads to a parallel decrease in the activities of both enzymes when assayed with N-Suc-LY-MCA, N-Suc-IIW-MCA, N-Cbz-GG-\(\beta\)-NA, Glu-GGF-\(\beta\)-NA, and FPAM-4-MNA (data not shown).

**Affinity-purified Anti-ClpP Antibodies Cross-react Mainly with Two Subunits of the Rat Liver MCP Complex**—The substrate specificity of ClpP and MCP suggests that both chymotrypsin-like activities are related, and decided to check if this relationship is extended to polypeptide structure. As a probe, rabbit polyclonal antibodies against purified ClpP were obtained. Direct immunoblot with the anti-ClpP serum of rat liver subcellular fractions showed several cross-reacting polypeptides, similar to what has been reported previously (9), while reacting with a single polypeptide of 21 kDa in *E. coli*

![Graphical representation of the relative Vmax/Km of the ClpP and MCP for different peptide substrates.](image)

**TABLE II**

Mixed substrate experiments for the chymotrypsin-like activities of MCP and ClpP

Results are average from two different experiments. When two peptides are present in the assay, each one was at the concentration indicated when assayed alone.

| Substrates                      | MCP activity | ClpP activity |
|---------------------------------|--------------|---------------|
|                                | Observed     | Expected*     | Observed     | Expected*     |
|                                | pmol/min/\(\mu\)g | pmol/min/\(\mu\)g | pmol/min/\(\mu\)g | pmol/min/\(\mu\)g |
| N-Suc-LLVY-MCA, 500 \(\mu\)M   | 440          | 10            |
| N-Suc-LY-MCA, 20 \(\mu\)M      | 75           | 150           |
| N-Suc-LLVY-MCA + N-Suc-LY-MCA  | 350          | 517           | 8            | 160           |
| N-Suc-IIW-MCA, 200 \(\mu\)M    | 230          | 6             |
| N-Suc-LY-MCA, 20 \(\mu\)M      | 77           | 150           |
| N-Suc-IIW-MCA + N-Suc-LY-MCA   | 200          | 317           | 5            | 156           |
| N-Suc-AAF-MCA, 500 \(\mu\)M    | 500          | 10            |
| N-Suc-LY-MCA, 20 \(\mu\)M      | 77           | 150           |
| N-Suc-AAF-MCA + N-Suc-LY-MCA   | 450          | 577           | 7            | 160           |
| Glut-GGF-\(\beta\)-NA, 500 \(\mu\)M | 170         | 12            |
| FPAM-4MNA, 200 \(\mu\)M         | 45           | 3             |
| Glut-GGF-\(\beta\)-NA + FPAM-4MNA | 100       | 215           | 2.6          | 15            |
| Glut-GGF-\(\beta\)-NA, 500 \(\mu\)M | 170       | 12            |
| N-Cbz-GG-\(\beta\)-NA, 100 \(\mu\)M | 79         | 3.5           |
| Glut-GGF-\(\beta\)-NA + N-Cbz-GG-\(\beta\)-NA | 110 | 249 | 3.5 | 15.5 |
| N-Cbz-GG-\(\beta\)-NA, 100 \(\mu\)M | 79         | 3.5           |
| FPAM-4MNA, 200 \(\mu\)M         | 45           | 3             |
| N-Cbz-GG-\(\beta\)-NA + FPAM-4MNA | 40         | 124           | 2            | 6.5           |

* Expected values for independent catalytic sites are obtained by the addition of the observed values for each peptide assayed alone.
crude extracts. As a consequence, the anti-ClpP antiserum was affinity-purified against purified recombinant ClpP, as described under "Experimental Procedures." The affinity-purified anti-ClpP antibodies were used to probe possible cross-reaction with MCP polypeptides by immunoblot. The reaction was weak when 4-chloro-1-naphthol was used as the developing reagent, so we turned to a more sensitive developing system based on bioluminescence (ECL, Amersham), and the results of one of such experiments are presented in Fig. 3. The affinity-purified anti-ClpP antibodies showed cross-reaction with two polypeptides components of the rat liver MCP with apparent molecular masses of 23 and 22 kDa. The same two polypeptides were detected in early steps of the purification of rat liver MCP, so it is unlikely that one is derived from the other by proteolysis during the purification. These affinity-purified anti-ClpP antibodies show weaker cross-reaction with other polypeptide components of the MCP of higher molecular mass (see Fig. 3), and we did the reverse, trying to see if our anti-MCP polyclonal antibody (26) could cross-react with the ClpP, but no cross-reaction was detected. The anti-ClpP antibodies immunoprecipitate the ClpP protein, but do not immunoprecipitate the MCP complex, nor do they inhibit the chymotrypsin-like activity of either ClpP or MCP (data not shown). These results suggest that the anti-ClpP affinity-purified antibodies recognize linear epitope(s), not exposed by the MCP subunits in their native conformation.

**DISCUSSION**

Based on the kinetic experiments presented, one can conclude that the specificity of the chymotrypsin-like activity of the ClpP and MCP is similar. The substrate specificity of MCP, as dictated by the $V_{max}/K_m$ values, indicates the following preference: N-Suc-IWW-MCA > N-Suc-LYY-MCA > N-Suc-LVVY-MCA ≥ N-Suc-AAF-MCA > N-Chz-GGL-β-NA > Glut-GGF-β-NA > FPAM-4-MNA; in the case of the ClpP the order of preference is: N-Suc-LYY-MCA > N-Suc-IWW-MCA > N-Suc-LVVY-MCA ≥ N-Suc-AAF-MCA ≥ N-Chz-GGL-β-NA ≥ FPAM-4-MNA. The mixed substrate experiments in the case of the MCP (as well as, of the ClpP), clearly point out that all these substrates are hydrolyzed in a unique type of catalytic site. This conclusion based on kinetic experiments is also supported by the fact that thermoplasma MCP catalyzes the hydrolysis of N-Suc-LVVY-MCA, N-Suc-AAF-MCA (27), and N-Chz-GGL-MCA (28), but not of N-Chz-LLE-2NA and N-Chz-ARR-4-MNA, and the by the description that the MCP (yeast) purified from yeast with mutations in the PRE1 and PRE2 genes, shows reduced activity against N-Suc-LVVY-MCA, N-Suc-FLP-pNA, and N-Chz-GGL-pNA (29), not affecting the peptidease activity against N-Chz-ARR-4-MNA and N-Chz-LLE-2NA peptides. The poor hydrolysis of N-Suc-AAA-MCA and LG-β-NA by the two proteases is supported, in the case of the MCP, by the kinetic analysis with substrates containing G or A in their P1 position reported by Orłowski et al. (30), which appeared when this manuscript had already been submitted, and led them to designate this new site as the small neutral amino acid preferring component of the MCP.

The results with the different peptide substrates in the case of the ClpP deserve an special comment. All the peptide substrates show a very reduced $V_{max}$ compared with that of N-Suc-LYY-MCA, whereas the $K_m$ for N-Suc-LYY-MCA is 1 order of magnitude higher than the $K_m$ for the other substrates. The fact that simple increase of the peptide substrate length with more amino acids (N-Suc-LVVY-MCA, N-Suc-AAF-MCA) greatly reduces both the $K_m$ and the $V_{max}$ (with respect to N-Suc-LYY-MCA), indicates that the catalytic site of the ClpP has higher affinities for more hydrophobic peptides, having less effect on the $K_m$, the type of hydrophobic amino acid present in P1 position. Based on the general model of catalysis by serine proteases (31), one can postulate that the substrate pocket of the ClpP preferentially binds a stretch of hydrophobic amino acids, and the specificity pocket can accommodate either aromatic (W, Y, F) or hydrophobic (L and M) amino acids. This increased affinity of ClpP for hydrophobic substrates makes them poorly hydrolyzed (low $V_{max}$, compare to N-Suc-LYY-MCA), so low, that they appear as strong competitors for N-Suc-LYY-MCA hydrolysis (see Table II). This could be due either to a slow rate of ES to EP transformation or to slow dissociation of the EP complex with these substrates. These kinetic results could explain why the ClpP subunit alone is unable to hydrolyze proteins. It is known that the presence of the ClpA subunit and ATP (5–7) is required for proteolysis, but the mechanism is still unclear.

A still unresolved critical issue is which subunits of the MCP contain the catalytic activities for the three different specificities, particularly in the present discussion, the chymotrypsin-like activity. Thermoplasma MCP actually seems to be the most promising enzyme to approach this problem. The work developed with this enzyme with the cloning (32, 33) of the two subunits α and β has led to the proposal that thermoplasma MCP α and β subunits are the precursors of the two families of eukaryotic MCP subunits (32), but the putative active site is still elusive (27). Several studies have used different proteases inhibitors as probes of the catalytic sites of the eukaryotic MCP (34–37). In this sense, Tanaka et al. (1) reported labeling studies of the putative Ser of the active site of the rat liver MCP with $[^3H]$disopropyl fluorophosphate and the cross-reaction of the anti-ClpP antibodies (10) with two subunits of the yeast MCP complex YC1 α and β and one polypeptide of the rat liver MCP. Here we present the cross-reaction of affinity-purified anti-ClpP antibodies

![Fig. 3. Immunoblot analysis of purified rat liver MCP and ClpP from E. coli with affinity-purified anti-ClpP antibodies.](image-url)
with two components of 22 and 23 kDa and a minor cross-
reaction with higher molecular mass polypeptides of the rat liver MCP. The molecular mass of the anti-ClpP cross-reacting polypeptides (Fig. 3) is consistent with the size of the smallest polypeptides labeled in the MCP by [3H]diisopropyl fluorophosphate in the chicken MCP (3) and bovine lens MCP (4) that are actually the same major labeled polypeptides obtained by Tanaka et al. (1) with the rat liver enzyme. Our pattern of cross-reacting polypeptides are similar (compare Fig. 8 of Ref. 1 with Fig. 3 of the present report) to the one presented by Tanaka et al. (1) by labeling MCP with [3H] diisopropyl fluorophosphate. This circumstance evidence (similar substrate specificity and antibody cross-reaction) suggest that the ClpP may be one of the ancestral catalytic proteins of the chymotrypsin-like activity of the eukaryotic MCP. Definitive proof of a structural relationship must await the isolation of the cDNAs encoding the two anti-ClpP cross-reacting (22 and 23 kDa) subunits of eukaryotic MCP, as no clear sequence similarity has been found between the ClpP and the protein sequence of MCP subunits already cloned.

The ClpP protein (like the MCP in yeast) has been shown to be implicated in the degradation of substrates of the NH2-end rule pathway (38) in E. coli. Based on the kinetic experiments presented here, we want to emphasize the partial coincidence of the ClpP specificity (and reduced peptidase activity, see discussion above) with the NH,-end rule in E. coli. The specificity pocket of the ClpP can accommodate W, Y, F, L, all destabilizing residues in E. coli (the only exception is M that is not destabilizing in E. coli and that can enter the active site of ClpP). It is possible that the ClpP is somehow involved in the recognition of the NH2 terminus of those proteins that are targeted for degradation in E. coli. Similarly the possible role of the multiscatalytic proteinase in the recognition of NH2 terminus of proteins that are going to be ubiquitinated has also been suggested, as a possible interpretation of the results of the experiments with NH2-end rule substrates in yeast PRE1 and PRE2 mutants (40, 41).

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