Mannan-binding lectin (MBL)-associated serine proteases 1 and 2 (MASP-1 and MASP-2) are homologous modular proteases that each interact with MBL, an oligomeric serum lectin involved in innate immunity. To precisely determine their substrate specificity, human MASP-1 and MASP-2, and fragments from their catalytic regions were expressed using a baculovirus/insect cells system. Recombinant MASP-2 displayed a rather wide, C1s-like esterolytic activity, and specifically cleaved complement proteins C2 and C4, with relative efficiencies 3- and 23-fold higher, respectively, than human C1s. MASP-2 also showed very weak C3 cleaving activity. Recombinant MASP-1 had a lower and more restricted esterolytic activity. It showed marginal activity toward C2 and C3, and no activity on C4. The enzymic activity of both MASP-1 and MASP-2 was specifically titrated by C1 inhibitor, and abolished at 1:1 C1 inhibitor:protease ratio. Taken together with previous findings, these and other data strongly support the hypothesis that MASP-2 is the protease that, in association with MBL, triggers complement activation via the MBL pathway, through combined self-activation and proteolytic properties devoted to C1r and C1s in the C1 complex. In view of the very low activity of MASP-1 on C3 and C2, our data raise questions about the implication of this protease in complement activation.

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Substrate Specificities of Recombinant Mannan-binding Lectin-associated Serine Proteases 1 and -2

Mannan-binding lectin (MBL)1 is an oligomeric C-type lectin that recognizes arrays of neutral carbohydrates such as mannos- and N-acetylglucosamine on the surface of pathogenic microorganisms (2). This selectivity endows MBL with the ability to discriminate self from infectious non-self, and confers this “ante-antibody” a major role in innate immunity, as underlined by numerous clinical reports indicating that MBL deficiency is linked with increased susceptibility to infectious diseases (3–5). In addition to its role as an opsonin (3), MBL has devised the ability to associate to several modular proteases termed MASP (MBL-associated serine proteases) (6–9). A single MASP entity was initially identified, and characterized as a protease with the ability to cleave complement proteins C4, C2, and C3 (7, 10). Further studies by Thiel et al. (6) revealed that MASP was indeed a mixture of two related but distinct proteases, MASP-1 and MASP-2, and that only the latter had the ability to cleave C4. A third protein component Map19, arising from alternative splicing of the MASP-2 gene (11, 12), and very recently a further protease MASP-3 (13) were also shown to be associated with MBL.

MASP-1 and MASP-2 show a domain organization identical to that of C1r and C1s, the enzymatic components of the C1 complex of complement (14), with an N-terminal CUB module (15) followed by an epidermal growth factor-like module, a second CUB module, two contiguous CCP modules (16), and a C-terminal chymotrypsin-like serine protease domain (see Fig. 1). By analogy with human C1s, it may be anticipated that the proteolytic activity and specificity of the MASPs is defined by the two CCP modules together with the serine protease domain (17), and that the latter forms a rigid association with the preceding, second CCP module (18). Comparative analysis of the cDNAs of C1r, C1s, and the MASPs in different animal species reveal that these fall into two groups (19, 20). In the smaller and probably more ancient group comprising MASP-1 and the ascidian MASPs, the active site serine is encoded by a “TCN” codon (where N is A, T, G, or C), and the histidine-loop disulfide bridge is present. In the larger group encompassing C1r, C1s, MASP-2, and most of the known animal MASPs, the active site serine is encoded by an “AGY” codon (where Y is T or C), and the histidine-loop is missing. The only available information dealing with the substrate specificity of MASP-1 and MASP-2 has been obtained on proteases isolated from human serum, and is somewhat controversial. Thus, whereas it is now widely accepted that MASP-2 cleaves both C4 and C2 (6, 21, 22), the observation that MASP-1 can cleave C3 and hence directly trigger complement activation (21, 23) is debated (22, 24). In addition, the relative efficiency of MASP-2 with respect to C4 and C2 cleavage has not been assessed.

The objective of the present work was to produce recombinant human MASP-1 and MASP-2 and catalytic fragments thereof, to precisely determine their substrate specificity on both protein substrates and synthetic esters, and to measure the kinetic parameters of their activity. Our data reveal that...
MASP-2 cleaves C4 much more efficiently than does C1s, emphasizing the physiological relevance of MASP-2 with respect to complement activation. In contrast, recombinant MASP-1 shows only marginal C3 cleaving activity, raising questions about its involvement in complement activation.

EXPERIMENTAL PROCEDURES

Materials—Disopropyl phosphorofluoridate was from Acros Organics, Noisy le Grand, France. The plasmids containing the full-length MASP-1 and MASP-2 DNAs were obtained as described previously (6, 25). Oligonucleotides were obtained from Oligogene, Paris, France. Trypsin (500 units/mg) was obtained from Sigma, Saint Quentin Fallavier, France. Z-Gly-A2-S-Bzl was from Enzyme Systems Products, Livermore, CA. Ac-Gly-Lys-Bzl, Bz-Arg-OEt, and Tso-Arc-Ome were obtained from Sigma.

Proteins—MBL was isolated from human plasma according to the procedure described by Tan et al. (26), with a further purification step using ion-exchange chromatography, as described in Thielens et al. (27). Recombinant full-length MASP-1 was expressed using a baculovirus insect cell system and purified by ion-exchange chromatography and affinity chromatography on an UltraLink™ MBL column, as described previously (28). Activated C1s and complement proteins C4, C2, and C3 were purified from human plasma according to published procedures (28). 

Fraction of the last C2 purification step on C4b-Sepharose (30). C inhibitor was purified from human plasma essentially as described in Ref. 32, except that cancanavalin A-agarose (Sigma) was used instead of cancanavalin A-Sepharose. Purified human α2-macroglobulin (α2M) was kindly provided by L. Sottrup-Jensen (University of Aarhus).

The concentrations of purified proteins were determined using the following absorption coefficients: C1s, 14.5 and 79,800; C4, 8.3 and 205,000; C2, 8.9 and 102,000; C3, 10.0 and 185,000 (29, 30, 33). The absorption coefficients were used to calculate the concentrations of full-length MASP-1 and MASP-2 and of the CCP1/2-SP fragment of MASP-1. Culture supernatants were collected by centrifugation.

Protein Production and Purification—High Five cells (1.75 x 10^7 cells/175-cm^2 tissue culture flask) were infected with the recombinant viruses at a multiplicity of infection of 2–3 in SF900 II SFM medium (Life Technologies, Inc.) as described in the manufacturer’s protocol. Recombinant virus particles were collected 4 days later, titrated by virus plaque assay, and amplified as described by King and Possee (35).

The supernatant containing MASP-2 was dialyzed against 50 mM NaCl, 1 mM EDTA, 50 mM triethanolamine hydrochloride, pH 8.5, and loaded onto a Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech) (2.8 x 10 cm) equilibrated in the same buffer. Elution was carried out by applying a 800-ml linear gradient from 50 to 500 mM NaCl in the same buffer. Fractions containing the recombinant protein were identified by Western blot analysis, and dialyzed against 50 mM triethanolamine, pH 7.4.

Further purification was achieved by ion-exchange chromatography on a Mono-Q HR 5/5 column (Amersham Pharmacia Biotech) equilibrated in the same buffer. Elution was carried out with a linear NaCl gradient from 50 to 500 mM in 90 min.

The supernatants containing the CCP1/2-SP and CCP2-SP fragments of MASP-2 were dialyzed against 5 mM EDTA, 20 mM NaHPO4, pH 8.6, and loaded onto a Q-Sepharose Fast Flow column (2.9 x 10 cm) equilibrated in the same buffer. Elution was carried out by applying a 700-ml linear gradient from 0 to 350 mM NaCl in the same buffer. Fractions containing the recombinant proteins were identified by Western blot analysis, and dialyzed against 50 mM triethanolamine, pH 7.4, and further purified by high-pressure hydrophobic interaction chromatography on a TSK-Phenyl 5PW column (Beckman) equilibrated in the same buffer. Elution was carried out by decreasing the (NH4)2SO4 concentration from 1.5 M to 0 in 30 min. Both purified recombinant fragments were dialyzed against 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, concentrated up to 0.2 mg/ml by ultrafiltration, and stored at −20°C.

The supernatant containing the CCP1/2-SP fragment of MASP-1 was dialyzed against 50 mM Na acetate, pH 5.1, and loaded on a SP-Sepharose column (Amersham Pharmacia Biotech) (2.8 x 8 cm) equilibrated in the same buffer. Elution was carried out by applying a 600-ml linear gradient from 0 to 600 mM NaCl. Recombinant proteins were dialyzed against 50 mM triethanolamine hydrochloride, 145 mM NaCl, pH 7.4, concentrated by ultrafiltration to 0.05–0.5 mg/ml, and stored at 0°C.

Polyacrylamide Gel Electrophoresis and Immuno blotting—SDS-PAGE analysis was performed as described previously (36). Western blot analysis and immunodetection of the recombinant proteins were carried out as described by Rossi et al. (17), or using the ECL detection procedure of Amersham Pharmacia Biotech. The antibodies used were the mouse monoclonal anti-MASP-2 antibody 1.3B7, a rabbit polyclonal anti-MASP-2 antibody (37), and a rabbit anti-peptide antibody directed against the serine protease domain of MASP-1 (6).

N-terminal Sequence Determination—N-terminal sequence analyses were performed after SDS-PAGE and electrotransfer, using an Applied Biosystems model 477A protein sequencer (Applied Biosystems, USA) as described previously (38).

Proteolytic Assays—The proteolytic activity of MASP-1, MASP-2, and C1s toward C2, C3, C4, C5, and Factor B was measured by incubation at different enzyme:protein ratios for varying periods at 37°C. Kinetic parameters were determined by the Lineweaver-Burk method.
FIG. 1. Modular structure of the recombinant proteins and fragments used in this study. The nomenclature and symbols used for protein modules are those defined by Bork and Bairoch (1). Ser, Pr, serine protease domain. The arrow indicates the Arg-Ile bond cleaved upon activation. The only disulfide bridge shown is that connecting the activation peptide to the serine protease domain. N, N-linked oligosaccharides.

All kinetic analyses were conducted in 150 mM NaCl, 5 mM EDTA, 20 mM sodium phosphate, pH 7.4.

Reactivity Toward Inhibitors—Complex formation between C1 inhibitor and MASP-1 or MASP-2 was assessed by incubation of the proteases with excess molar ratios of C1 inhibitor for 15 min at 37 °C in 50 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, followed by SDS-PAGE analysis under reducing conditions. C1 inhibitor-protease complexes were revealed by Western blot analysis using a rabbit polyclonal antibody directed against full-length MASP-2, and a rabbit anti-peptide antibody directed against the serine protease domain of MASP-1. Titration of the enzymatic activity of C1s, MASP-1, and MASP-2 by C1 inhibitor was performed by preincubating these proteases (at concentrations of 0.25 μM, 20 nM, and 0.25 μM, respectively) with increasing concentrations of C1 inhibitor (10–500 nM) for 15 min at 37 °C. The residual C2 cleaving activity of MASP-2 and C1s was then measured by incubating the enzymes (3.75 and 7.5 nM, respectively) for 15 min at 37 °C in the presence of 2.25 mM C2, followed by SDS-PAGE analysis, as described above. In the case of MASP-1, the residual esterolytic activity was measured on Bz-Arg-OEt, as described above.

The reactivity of C1s and MASP-2 toward α2M was measured by preincubating the enzymes (0.25 μM each) for 30 min at 37 °C in the presence of increasing concentrations (3.7–300 μM) of α2M. Residual C4 cleaving activity was then measured by incubating the enzymes (1.25 nM each) for 15 min at 37 °C in the presence of 2.25 mM C4, followed by SDS-PAGE analysis.

Esterolytic Assays—Esterolytic activities were measured on the synthetic esters Ac-Gly-Lys-OMe and Tos-Arg-OMe using a spectroscopic assay based on the measurement of methanol released upon hydrolysis (39). Assays on the thioester Z-Gly-Arg-S-Bzl and on Bz-Arg-OEt were carried out as described by McRae et al. (40), and Arlaud and Thielens (39). All assays were conducted at 30 °C, at substrate concentrations of 0.1–3 mM (Ac-Gly-Lys-OMe and Tos-Arg-OMe), 0.5–3 mM (Bz-Arg-OEt), or 0.075–0.2 mM (Z-Gly-Arg-S-Bzl).

RESULTS

Production and Characterization of Recombinant MASP-2 and C-terminal Fragments from MASP-1 and MASP-2—The modular structures of MASP-1, MASP-2, and of the truncated fragments used in the present study are depicted in Fig. 1. The recombinant baculoviruses used for expression of each construct were obtained as described under “Experimental Procedures” and used to infect High Five insect cells for various periods at 28 °C. The amount of recombinant material recovered in the culture medium, as estimated by SDS-PAGE and Western blot analysis, ranged from 0.15 (MASP-2) to 2 μg/ml (CCP1/2-SP fragment of MASP-1). Protein purification was performed as described under “Experimental Procedures,” using an initial ion-exchange fractionation step in all cases. Because of the low amounts of material recovered, protein detection and analysis was performed routinely using Western blot analysis rather than Coomassie Blue staining.

Due to its very low recovery, recombinant MASP-2 could not be purified to homogeneity since attempts to completely remove contaminant proteins resulted in a nearly complete loss of material. SDS-PAGE analysis of the partially purified MASP-2 fraction under nonreducing conditions revealed two bands reactive with specific antibodies: (i) a major, 80-kDa species corresponding to the full-length protease, yielding two sequences, Thr-Pro-Leu-Gly-Lys-Trp-Pro-Glu-Pro... and Ile-Tyr-Gly-Gly-Gln-Lys-Ala-Lys-Pro-Gly...; and (ii) a 45-kDa species yielding only the first sequence above, and corresponding to a truncated fragment derived from the N-terminal end of the protein. Analysis under reducing conditions indicated that full-length MASP-2 was recovered in a partially activated form, since only 20–30% of the protein migrated as a single chain, proenzyme species, whereas the remainder yielded two bands at 45 and 28 kDa, corresponding to the N-terminal A chain and the serine protease domain, respectively. Based on Coomassie Blue staining after SDS-PAGE analysis, the relative amount of full-length MASP-2 in the partially purified fraction averaged 10% of the total protein contents.

Both the CCP1/2-SP and CCP2-SP fragments of MASP-2 could be purified to homogeneity. On SDS-PAGE analysis, the CCP1/2-SP fragment migrated under nonreducing conditions as a 44-kDa band, which upon reduction split into two bands corresponding to the serine protease domain (27 kDa) and the CCP2 segment (17 kDa) (Fig. 2, lanes 1 and 2), indicating complete activation of the protease. In contrast, the shorter CCP2-SP fragment migrated as a single band of about 35 kDa both under nonreducing and reducing conditions (Fig. 2, lanes 3 and 4), indicating that this species had retained a single chain, proenzyme structure. N-terminal sequence analysis of the latter fragment yielded a single sequence Asp-Pro-Asp-Cys-Gly-Gly-Pro... corresponding to the expected N-terminal end of the fragment, whereas the activated CCP1/2 SP fragment yielded equivalent amounts of two sequences: Asp-Pro-Gln-Pro-Cys-Pro-Tyr... (N-terminal end) and Ile-Tyr-Gly-Gly-Gln-Lys-Ala... (serine protease domain). Protein staining with Coomassie Blue (not shown) yielded the same pictures as observed by Western blot analysis, indicating that no major fragment or contaminant was present in the purified preparations.
As in the case of full-length MASP-2, the CCP1/2-SP fragment of MASP-1 could not be purified to homogeneity, mainly because of the low amounts of recombinant material available. SDS-PAGE analysis indicated that the fragment migrated under nonreducing conditions as a single band of about 50 kDa. Upon reduction, part of the material (40%) still migrated as a single chain, proenzyme species, whereas the remainder yielded a doublet at 32 and 34 kDa, reactive with antibodies directed to the C-terminal end of the molecule, and corresponding to the serine protease domain. Sequence analysis confirmed these findings, as the proenzyme species yielded a single sequence Asp-Leu-Val-Glu-Leu-Pro-Glu-Leu-Gln-Pro... corresponding to the N-terminal end of the fragment, the doublet at 32–34 kDa yielding the expected sequence Ile-Phe-Asn-Gly-Arg-Pro-Ala-Gln-Lys-Gly... characteristic of the serine protease domain. Thus, as previously observed in the case of full-length MASP-1 expressed in the same system, the recombinant CCP1/2-SP fragment was recovered in a partially activated form. As judged from Coomassie Blue staining, the MASP-1 CCP1/2-SP fragment was estimated at 20–30% of the total protein contents of the partially purified fraction, depending on the preparation.

Proteolytic Activity of Recombinant MASP-2—Recombinant MASP-2 and its CCP1/2-SP fragment both cleaved C4 very efficiently, as shown by their ability to convert the C4a chain into the smaller C4α’ species (Fig. 3A). In both cases, almost complete cleavage of C4 was achieved upon incubation for about 15 min at 37 °C at an enzyme:protein molar ratio of 1:2000. Both recombinant proteases also readily and specifically cleaved C2, as shown by their ability to split the protein into its characteristic C2a and C2b fragments (Fig. 3B). Again, C2 cleavage was essentially complete after 20 min at 37 °C at an enzyme:protein molar ratio of 1:1000. Under these conditions, no significant C4 or C2 cleavage was observed when these proteins were incubated in the presence of the proenzyme CCP2-SP fragment of MASP-2. The ability of MASP-2 to cleave C3 was also tested using the same methodology. As illustrated in Fig. 3C, incubation of C3 with increasing amounts of either full-length MASP-2 or its catalytic fragment CCP1/2-SP led to an increased production of the C3αa fragment. However, the cleavage reaction was very inefficient, as complete conversion of C3αa into C3α’ required overnight incubation at 37 °C at an enzyme:protein molar ratio of 1:6 (Fig. 3C, lane 5). For comparison, complete cleavage of C3αa by trypsin was achieved at an enzyme:protein molar ratio of 1:13 in only 5 min at 37 °C (Fig. 3C, lane 1). Using partially purified preparations of C5 and factor B as substrates, no significant cleavage of these complement proteins by either MASP-2 or its CCP1/2-SP fragment could be detected after overnight incubation at 37 °C at enzyme:protein molar ratios up to 1:10 (not shown).

The kinetic parameters for C2 and C4 cleavage by MASP-2 were determined using the full-length protease and its CCP1/2-SP fragment, and the values were compared with those obtained with active C1s purified from human serum. In the case of C2, all three enzymes showed comparable kcat values, whereas both MASP-2 species exhibited Km values slightly, but consistently lower than C1s (Table I and Fig. 4). As a result, the C2 cleavage efficiency of both MASP-2 species, as measured by the kcat/Km ratio, was slightly higher than that of C1s, with respect to C4 cleavage, again, all three enzymes exhibited similar kcat values. In contrast, both MASP-2 and its CCP1/2-SP fragment showed Km values in the nanomolar range, i.e. 26–32 times lower than the value of 1.92 ± 0.5 μM determined for C1s. As a result, the kcat/Km ratios for MASP-2 and its CCP1/2-SP fragment were 12- and 23-fold higher, respectively, than that for C1s, indicating a much higher efficiency in the case of C4 cleavage. In this respect, it should be mentioned that the kcat values for MASP-2 were significantly lower than those determined for the CCP1/2-SP fragment. This is likely because of the fact that the concentration of MASP-2 could only be roughly estimated (see “Experimental Procedures”) and, we believe, was probably overestimated rather than the opposite. As a consequence, the kcat values determined for the CCP1/2-SP fragment should be regarded as the most representative. It may be estimated therefore that MASP-2 exhibits C2 and C4 cleaving efficiencies about 3 and 23 times higher, respectively, than C1s (see Table I).
II. MASP-2 and C1s efficiently cleaved the four substrates used for determination of the kinetic constants shown in Table I. MASP-1 and the MASP-2 CCP1/2-SP fragment were routinely compared on various synthetic substrates and compared with that of human C1r and C1s. For both MASP-1 and MASP-2, comparable results were obtained using either the full-length proteases or their catalytic CCP1/2-SP fragment. Full-length MASP-1 and the MASP-2 CCP1/2-SP fragment were routinely used for determination of the kinetic constants shown in Table II. MASP-2 and C1s efficiently cleaved the four substrates tested in this study, and both enzymes exhibited similar $k_{cat}$ and $K_m$ values. However, C1s showed a significantly better efficiency on Ac-Gly-Lys-OMe, Bz-Arg-OEt, and Tos-Arg-OMe, whereas MASP-2 was more efficient on the thioester Z-Gly-Arg-S-Bzl, because of a remarkably low $K_m$ value (Table II). In keeping with previous reports (39), C1r hydrolyzed Ac-Gly-Lys-OMe and Z-Gly-Arg-S-Bzl, but had no activity on Bz-Arg-OEt and Tos-Arg-OMe. Likewise, MASP-1 also exhibited a restricted estero-lytic activity, as it was not reactive toward Ac-Gly-Lys-OMe and Tos-Arg-OMe, and cleaved Z-Gly-Arg-S-Bzl and Bz-Arg-OEt to significant extents. Interestingly, of the four proteases used, MASP-2 showed the least $K_m$ value toward Bz-Arg-OEt (Table II). All of the estero-lytic activities measured with recombinant MASP-1 and MASP-2 were blocked after pretreatment of the proteases with excess C1 inhibitor.

Reactivity of MASP-1 and MASP-2 Toward Inhibitors—After incubation with a molar excess of C1 inhibitor, full-length MASP-1 and MASP-2, as well as their CCP1/2-SP fragments, all specifically reacted with this inhibitor, as shown by the formation of stable complexes migrating on SDS-PAGE analysis under reducing conditions as species of over 100 kDa, corresponding to C1 inhibitor-serine protease domain complexes (not shown). To determine the stoichiometry of the reaction between MASP-2 and C1 inhibitor, the CCP1/2-SP fragment of MASP-2 was preincubated with increasing molar ratios of C1 inhibitor:MASP-2 ratio led to a linear decrease of enzyme activity, with complete inhibition at a molar ratio of 1:10 (Fig. 6A, lane 3). This faint activity was nevertheless clearly attributable to MASP-1, since it was blocked by pretreatment of the enzyme:substrate molar ratio (Fig. 5A). In a control experiment, C3 (1 μM) was incubated for 10 min at 37 °C in the presence of 0.1 μM full-length MASP-1 (lane 3), in the presence of MASP-1 plus 0.5 μM C1 inhibitor (lane 4), or in the presence of MASP-1 plus 5 mM DFP (lane 5). In a control experiment, C3 (1 μM) was incubated for 10 min at 37 °C in the presence of 0.1 μM trypsin (lane 6). Lane 1, control, nonincubated C3, B, C2 (2 μM) was incubated overnight at 37 °C alone (lane 2), in the presence of 0.2 μM MASP-1 (lane 3), in the presence of MASP-1 plus 1 μM C1 inhibitor (lane 4), or in the presence of MASP-1 plus 5 mM DFP (lane 5). In a control experiment, C2 (1 μM) was incubated for 1 h at 37 °C in the presence of 0.1 μM C1s (lane 6). Lane 1, control, nonincubated C2 sample. Cleavage reactions were monitored by SDS-PAGE analysis of the samples under reducing conditions.

![Graph](image.png)

**Fig. 4.** Lineweaver-Burk plots of C2 cleavage by C1s and the CCP1/2-SP fragment of MASP-2. Kinetic analyses were performed as described under “Experimental Procedures,” using C2 concentrations ranging from 1 to 4 μM, and fixed enzyme concentrations of 2 nM. •, cleavage by C1s; O, cleavage by the CCP1/2-SP fragment of MASP-2.

![Graph](image.png)

**Fig. 5.** SDS-PAGE analysis of the C3 and C2 cleaving activities of recombinant MASP-1. A, C3 (1 μM) was incubated overnight at 37 °C alone (lane 2), in the presence of 0.1 μM full-length MASP-1 (lane 3), in the presence of MASP-1 plus 0.5 μM C1 inhibitor (lane 4), or in the presence of MASP-1 plus 5 mM DFP (lane 5). In a control experiment, C1 inhibitor-serine protease domain complexes responding to C1 inhibitor-serine protease domain complexes under reducing conditions as species of over 100 kDa, corresponding to C1 inhibitor-serine protease domain complexes (not shown). To determine the stoichiometry of the reaction between MASP-2 and C1 inhibitor, the CCP1/2-SP fragment of MASP-2 was preincubated with increasing molar ratios of C1 inhibitor and then the residual protease activity was measured using the C2 cleavage assay. As shown in Fig. 6B, increasing the C1 inhibitor:MASP-2 ratio led to a linear decrease of enzymatic activity, with complete inhibition at a molar ratio of about 1:1. In a comparative experiment, the same stoichiome-

### Table I

| Enzyme                      | $k_{cat}$ | $K_m$   | $k_{cat}/K_m$ |
|-----------------------------|----------|---------|---------------|
| C1s                         |          |         |               |
|                             | $3.3 \pm 0.1$ | $12.3 \pm 3.0$ | $4.3 \times 10^4$ |
| MASP-2 CCP1/2-SP fragment   |          |         |               |
|                             | $7.4 \pm 1.8$ | $6.6 \pm 1.5$ | $11.2 \times 10^4$ |
| MASP-2                      |          |         |               |
|                             | $4.9 \pm 1.2a$ | $6.5 \pm 1.5$ | $7.5 \times 10^4$ |

| Enzyme                      | $k_{cat}$ | $K_m$   | $k_{cat}/K_m$ |
|-----------------------------|----------|---------|---------------|
| C1s                         |          |         |               |
|                             | $4.7 \pm 1.2$ | $1920 \pm 500$ | $2.45 \times 10^6$ |
| MASP-2 CCP1/2-SP fragment   |          |         |               |
|                             | $3.4 \pm 0.9$ | $60 \pm 15$ | $56.7 \times 10^6$ |
| MASP-2                      |          |         |               |
|                             | $2.2 \pm 0.6a$ | $74 \pm 19$ | $29.7 \times 10^6$ |

* $k_{cat}$ values determined for MASP-2 are likely underestimated, due to overestimation of the enzyme concentration.
try was determined in the case of C1s, in agreement with previous reports (32). The stoichiometry of the reaction between MASP-1 and C1 inhibitor was determined in a similar way, by measuring the esterolytic activity of the protease on Bz-Arg-OEt. As shown in Fig. 6C, preincubation with increasing concentrations of C1 inhibitor led to a linear decrease of MASP-1 esterolytic activity, with again complete inhibition at a C1 inhibitor:MASP-1 molar ratio of about 1:1. Thus, as determined earlier in the case of C1r and C1s (32, 41), both MASP-1 and MASP-2 reacted with C1 inhibitor in a 1:1 stoichiometry.

As mentioned above, the proteolytic activity of MASP-1 toward C3 and C2 was prevented in the presence of 5 mM DFP. Because of the very low activity of MASP-1 on these substrates, it was not possible to further analyze its sensitivity with respect to DFP. In the case of MASP-2, preincubation of the protease with 2 mM DFP for 30 min at 37 °C resulted in 86% inhibition of its C4 cleaving ability, and nearly complete inhibition was obtained when this treatment was performed twice in a row. Thus, MASP-2 exhibited a DFP sensitivity comparable with that previously determined in the case of C1r and C1s (42). We also checked the effect of α2M on the C4 cleaving activity of MASP-2 and C1s. Both proteases were used at a concentration of 0.5 μM in the presence of increasing molar ratios of α2M up to 120:1. Under these conditions, this protein exerted no detectable inhibitory effect on the proteolytic activ-

![C1 inhibitor / Enzyme molar ratio](image)

**Fig. 6. Stoichiometry of the reaction of MASP-1, MASP-2, and C1s with C1 inhibitor.** MASP-1 (20 nM), MASP-2 (0.25 μM), and C1s (0.25 μM) were preincubated for 15 min at 37 °C in the presence of increasing concentrations of C1 inhibitor to achieve C1 inhibitor:protease ratios up to 2.0, as indicated. The residual C2 cleaving activity of C1s (A) and MASP-2 (B), and the residual esterolytic activity of MASP-1 on Bz-Arg-OEt (C) were measured as described under "Experimental Procedures."

| Enzyme | Ac-Gly-Lys-OMe | Bz-Arg-OEt | Z-Gly-Arg-S-Bzl | Tos-Arg-OMe |
|--------|----------------|------------|----------------|-------------|
| kcat |      |     |        |      |
| Km | 1 mM |     |        |      |
| kcat/Km | 1 s^-1 |     |        |      |
| kcat/Km | 1 s^-1 |     |        |      |

**Table II Kinetic constants for the esterolytic activity of MASP-1, MASP-2, Clr and Cls on various synthetic substrates**

| Enzyme | Ac-Gly-Lys-OMe | Bz-Arg-OEt | Z-Gly-Arg-S-Bzl | Tos-Arg-OMe |
|--------|----------------|------------|----------------|-------------|
| kcat |      |     |        |      |
| Km | 1 mM |     |        |      |
| kcat/Km | 1 s^-1 |     |        |      |
| kcat/Km | 1 s^-1 |     |        |      |

*Values not measurable due to the lack of activity.*
ity of either protease, indicating that, unlike MASP-1 (43), and like C1s (39), MASP-2 is not sensitive to $\alpha_M$. 

**DISCUSSION**

Full-length MASP-2, as well as fragments from the C-terminal catalytic regions of MASP-1 and MASP-2 were expressed using a baculovirus/insect cell system. As observed previously in the case of full-length MASP-1 expressed in the same system (27), the recombinant material was produced at very low yields (0.15–2.0 $\mu$g/ml). This rendered isolation of the recombinant material difficult, but nevertheless allowed us to achieve complete purification of MASP-2 fragments CCP1/2-SP and CCP2-SP, and partial purification of the MASP-1 CCP1/2-SP fragment and of full-length MASP-2. As judged from all functional assays performed in this study, the latter two preparations were functionally pure, *i.e.* devoid of detectable contaminant esterolytic or proteolytic activities. Thus, full-length MASP-1 on the one hand, and full-length MASP-2 on the other hand, exhibited esterolytic and proteolytic activities comparable with those of their CCP1/2-SP fragments, both in terms of efficiency and specificity. In addition, these activities were all blocked by C1 inhibitor, providing further evidence that they were not due to contaminant proteases arising from the insect cells. The recombinant material used in this study was therefore appropriate for a precise assessment of the enzymic properties and specificity of MASP-1 and MASP-2.

As previously observed for the full-length recombinant protease (27), the CCP1/2-SP fragment of MASP-1 was recovered in a partially (about 60%) activated form, suggesting that either autolytic activation or extrinsic proteolytic cleavage occurred during the synthesis process. Similarly, in the case of MASP-2, the full-length species was also recovered in a partially activated form. Interestingly, the short catalytic fragment CCP2-SP of MASP-2 was recovered in a fully proenzyme state, whereas the larger fragment CCP1/2-SP was secreted by the insect cells as a partially activated form, and underwent complete activation during the purification process. These latter findings appear not consistent with an activation process mediated by an extrinsic protease, as access to the Arg$^{444}$-Ile$^{445}$ activation site of MASP-2 is not expected to be made easier in the larger CCP1/2-SP fragment than in the shorter CCP2-SP fragment. On the other hand, this strikingly different behavior of the two fragments would be consistent with an autolytic activation mechanism of MASP-2 involving interaction between two molecules through their C-terminal catalytic region, if this process requires the first CCP module. This latter hypothesis appears plausible, in light of the observation that dimerization of the catalytic domains of the homologous protease C1r involves its first CCP module (44). In the same way, the fact that recombinant full-length MASP-2 only undergoes partial activation may be explained by analogy with previous findings on C1r (48, 49) indicating that the N-terminal, Ca$^{2+}$-binding CUB-EGF-CUB region of C1r exerts a negative control on the activation of the C-terminal catalytic region of the protease. The above observations support the hypothesis of an intrinsic ability of MASP-2 to self-activate, in full agreement with previous reports by Vorup-Jensen *et al.* (45) indicating that reconstitution of MBL with MASP-2 alone is sufficient to trigger complement activation.

Although recombinant MASP-2 and its CCP1/2-SP fragment displayed a detectable C3 cleaving activity, this was only observed upon overnight incubation at elevated enzyme:substrate ratios. It is likely therefore that this faint activity is nonspecific and has no biological relevance. In contrast, in agreement with previous reports (6, 21, 45), this work confirms that MASP-2 specifically cleaves and activates C4 and C2 and provides the first detailed analysis of the kinetic parameters of these reactions. These data indicate that (i) MASP-2 cleaves C2 slightly more efficiently (2–3 times) than does C1s; (ii) MASP-2 exhibits a C4-cleaving efficiency that is 20–25 times higher than that of C1s, arising mainly from a much lower $K_m$ value (about 60 nM versus about 2 $\mu$M). These findings have direct functional implications, since C4 cleavage, the initial event in the formation of the classical pathway C3-convertase C4b,2a, occurs in the fluid phase and is the limiting step of this process. Thus, the relative efficiency of C4 cleavage by MASP-2 and C1s in vivo will directly depend on their respective $K_m$ values for C4 (about 60 nM and 2 $\mu$M) relative to the C4 concentration in serum (about 3 $\mu$M) (46). It can be deduced from these figures that, whereas C1s will cleave C4 in serum at a rate well below the $V_m$ value, MASP-2 will always function at or close to maximal velocity. C2 cleavage, the second step of the assembly of the C4b,2a-convertase, is postulated to take place after prior binding of C2 to membrane-bound C4b, in close vicinity of either C1 or the MBL-MASP complex. Therefore the C2 cleavage efficiency of MASP-2 or C1s is not directly related to their $K_m$ values for this substrate, which in both cases are well above the C2 concentration in serum, *i.e.* 0.13–0.43 $\mu$M (47).

According to the above figures, MASP-2 is expected to be about 20–25 times more efficient than C1s with respect to C4 cleavage. As a consequence, despite the low amounts of MASP-2 in serum, a complex utilizing MBL as a recognition unit and MASP-2 as a catalytic unit is probably as active as C1 in terms of complement activation. These considerations provide strong support to the biological relevance of the lectin pathway of complement activation as a major innate defense mechanism. Together with experiments suggesting that MASP-2 has the ability to self-activate (Ref. 45 and this study), and binds individually to MBL with high affinity (27), the above results strongly suggest that the entity responsible for triggering the MBL-mediated lectin pathway of complement is an MBL-MASP-2 complex, in which MASP-2 combines the ability to self-activate as well as to cleave C4 and C2, and hence fulfills the roles of both C1r and C1s in C1. In keeping with previous data (21), our data also demonstrate that the proteolytic activity of MASP-2 is specifically titrated by C1-inhibitor, with a 1:1 C1 inhibitor-MASP-2 stoichiometry. As well documented in the case of the C1 enzymes (41), it may be anticipated therefore that the proteolytic activity of MASP-2 in serum is tightly and specifically controlled by C1 inhibitor.

This work also provides the first analysis of the esterolytic activities and specificities of MASP-1 and MASP-2. In keeping with the fact that MASP-2 and C1s specifically cleave the same protein substrates, they exhibit similar esterolytic activities on the various synthetic substrates tested in this study, which they cleave with comparable $k_{cat}$ and $K_m$ values. However, as measured by the $k_{cat}/K_m$ ratio, the efficiency of MASP-2 was found consistently lower than that of C1s, with the exception of the thioester Z-Gly-Arg-S-Bzl, which, because of a remarkably low $K_m$ value, is the best known substrate of MASP-2. Thus, the shared ability of MASP-2 and C1s to cleave C4 and C2 likely arises in part from common structural features at or in the vicinity of their active sites. On the other hand, the fact that MASP-2 has a much lower $K_m$ value for C4 is probably related to structural determinants located outside the active site area, very likely in the CCP modules, as previously demonstrated in the case of C1s (17). MASP-1, in contrast, exhibits a lower esterolytic activity, that is restricted to two of the substrates tested in this study, Bz-Arg-OEt and Z-Gly-Arg-S-Bzl. Nevertheless, it is noteworthy that MASP-1 cleaves Bz-Arg-OEt with an efficiency comparable with that of C1s, because of a rather low $K_m$ value (see Table II). Also, the fact that MASP-1 does not hydrolyze Ac-Gly-Lys-OMe suggests that its
Specificities of MASP-1 and -2

activity may be restricted to arginyl bonds only. As judged from its rather low and narrow esterolytic activity, MASP-1 appears to display broader activities and are more efficient enzymes.

In keeping with data obtained by Matsushita et al. (21) using material purified from human serum, both recombinant full-length MASP-1 and its catalytic CCP1/2-SP fragment showed detectable C2 and C3 cleaving activities. However, these reactions were quite inefficient, since in both cases only partial cleavage could be obtained after overnight incubation at elevated enzyme:substrate ratios. In this respect, it should be stressed that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that of recombinant MASP-2 (−20,000-fold less). In the same way, the C3 cleaving activity of MASP-1 was consistently found to be less than that of MASP-2, which itself is extremely low compared the MASP-2 activities.

With respect to C2 cleavage by MASP-1, it is stressed that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that of recombinant MASP-2. It appears very unlikely, therefore, that the physiological substrate(s) of MASP-1 in serum, and its rather low and narrow esterolytic activity, MASP-1 appears to be restricted to arginyl bonds only. As judged from the data obtained by Matsushita et al. (21) using material purified from human serum, both recombinant full-length MASP-1 and its catalytic CCP1/2-SP fragment showed detectable C2 and C3 cleaving activities. However, these reactions were quite inefficient, since in both cases only partial cleavage could be obtained after overnight incubation at elevated enzyme:substrate ratios. In this respect, it should be stressed that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that of recombinant MASP-2 (−20,000-fold less). In the same way, the C3 cleaving activity of MASP-1 was consistently found to be less than that of MASP-2, which itself is extremely low compared the MASP-2 activities.

In summary, although our data certainly validate previous findings by Matsushita et al. (21) that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that obtained with our recombinant material, since only partial cleavage was obtained under the conditions used. In this respect, it should also be mentioned that other studies have reported no detectable C3 cleavage by the total MASP fraction (22, 24). In summary, although our data certainly validate previous findings by Matsushita et al. (21) that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that obtained with our recombinant material, since only partial cleavage was obtained under the conditions used. In this respect, it should also be mentioned that other studies have reported no detectable C3 cleavage by the total MASP fraction (22, 24). In summary, although our data certainly validate previous findings by Matsushita et al. (21) that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that obtained with our recombinant material, since only partial cleavage was obtained under the conditions used. In this respect, it should also be mentioned that other studies have reported no detectable C3 cleavage by the total MASP fraction (22, 24). In summary, although our data certainly validate previous findings by Matsushita et al. (21) that the C2 cleaving activity of recombinant MASP-1 is quite negligible compared with that obtained with our recombinant material, since only partial cleavage was obtained under the conditions used. In this respect, it should also be mentioned that other studies have reported no detectable C3 cleavage by the total MASP fraction (22, 24).
Substrate Specificities of Recombinant Mannan-binding Lectin-associated Serine Proteases-1 and -2
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