Stoichiometry of Complexes between Mannose-binding Protein and Its Associated Serine Proteases

DEFINING FUNCTIONAL UNITS FOR COMPLEMENT ACTIVATION *

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Serum mannose-binding protein (MBP) 1 initiates the lectin branch of the complement cascade by binding to sugars on the surfaces of microorganisms and activating two MBP-associated serine proteases (MASP-1 and MASP-2). Rat serum MBP consists of oligomers containing up to four copies of a subunit that is composed of three identical polypeptide chains. Biophysical analysis of intact and truncated MASPs indicates that each MASP is a homodimer that is stabilized through interactions involving an N-terminal CUB domain. The binding sites for MBP are formed from the three N-terminal MASP domains, in which two CUB modules interact with MBP. Each MASP dimer contains binding sites for two MBP subunits. Both sites must be occupied by subunits from a single MBP oligomer to form a stable complex. Thus, the smallest functional unit for complement activation consists of MBP dimers bound to MASP-1 or MASP-2 homodimers. Trimers and tetramers of MBP form complexes containing up to two MASPs. The results reveal how MASP-1 and MASP-2 can function independently to activate the complement cascade.

Mannose-binding protein (MBP) 1 is a key component of the innate immune response. It binds to sugars on the surfaces of pathogenic microorganisms and activates complement by an antibody-independent mechanism (1, 2). Complement fixation by MBP occurs through two associated serine proteases, MASP-1 and MASP-2, that are homologs of C1r and C1s of the classical pathway. Upon activation, MASPs cleave downstream complement components, triggering a cascade of reactions that leads to neutralization of invading cells. Foreign cells are assembled from terminal complement components or are recognized as foreign by MBP. Each MASP dimer contains binding sites for two MBP subunits. Both sites must be occupied by subunits from a single MBP oligomer to form a stable complex. Thus, the smallest functional unit for complement activation consists of MBP dimers bound to MASP-1 or MASP-2 homodimers. Trimmers and tetramers of MBP form complexes containing up to two MASPs. The results reveal how MASP-1 and MASP-2 can function independently to activate the complement cascade.

α-helical coiled coil, and a C-terminal carbohydrate recognition domain. The MASP-binding site on MBP is located within the first part of the collagen-like domain (7). This region forms a central core that links individual MBP subunits, composed of three identical polypeptide chains, to form larger oligomers that resemble bouquet-like structures. Rat serum MBP (MBP-A) forms a heterogeneous mixture of oligomers ranging from monomers to tetramers of subunits (8). The dimers, trimers, and tetramers all fix complement, although trimers and tetramers have higher specific activities than dimers (7). A second MBP, found in the liver, has been identified in rats and other mammals. This protein is designated MBP-C and consists of a single trimeric subunit that does not form larger oligomers. MBP-C has a low complement-fixing activity compared with MBP-A.

MASP-1 and MASP-2 are ~40% identical in sequence (9–12). Each protein consists of two N-terminal CUB domains (domains found in complement subcomponents C1r/C1s, Uegf, and bone morphogenetic protein-1) separated by an EGF-like domain and followed by two complement control protein modules and a C-terminal serine protease domain. MASPs bind Ca 2+ through interactions that probably involve the EGF-like domains (13). They are synthesized as inactive proenzymes. Upon activation, each protein is cleaved, generating an active protease that remains covalently attached to the rest of the molecule by a disulfide bond. Studies using truncated proteins have shown that MASP-1 and MASP-2 bind to MBP in a Ca 2+-dependent manner through interactions involving the CUB and EGF-like domains (13, 14). Fragments encompassing these regions are Ca 2+-independent homodimers that are stabilized by interactions involving the two N-terminal domains. The composition of MBP-MASP complexes that activate the complement cascade is not known.

In the experiments reported here, MBP-MASP complexes have been recreated from recombinant proteins using intact MASPs as well as truncated forms. Analysis of the stoichiometry of these complexes reveals that the three N-terminal domains of each MASP are necessary and sufficient to reproduce the MASP-binding properties of the full-length proteins. A complex between two MBP subunits and either a MASP-1 or MASP-2 dimer is sufficient to form a functional unit that is capable of becoming activated.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and peptide: N-glycosidase F were purchased from New England Biolabs Inc. All tissue culture media were from Life Technologies, Inc. Promix cell labeling mixture (70%; [35S]methionine and 30% [35S]cysteine) was from Amersham Pharma- cia Biotech. Nitrilotriacetic acid-agarose, Sepharose 6B, and protein molecular mass markers were from Sigma. Affi-Gel 10 matrix was from Bio-Rad. Immonul 4 microtiter wells were from Dynex Technologies. Nitrocellulose membranes were from Schleicher & Schuell.

Analytical Methods—SDS-polyacrylamide gel electrophoresis was...
performed by the method of Laemmli (15). MALDI-MS was carried out on a Finnigan MAT Lasermass spectrometer using sinapinic acid as the sample matrix (16). Bovine serum albumin ([M + H]\(^{+}\) = 66,431 Da) and bovine serum albumin dimer ([M + H]\(^{+}\) = 132,860 Da) were used as the calibration standards.

Protein Purification of MBP-A and MBP-C—Recombinant MBP-A and MBP-C were purified by affinity chromatography on mannose-Septose-rose columns (7, 16). Oligomers of MBP-A were separated by anion-exchange chromatography on a MonoQ column (HR 5/5, Amersham Pharmacia Biotech) as described (7). Radiolabeled MBP-A was produced by incubating confluent cells in methionine-free medium supplemented with 10% dialyzed fetal calf serum, 0.5 mM methionine, and 0.1 mM methotrexate, and 0.1 mM [\(^{35}\)S]methionine for 16 h. Radiolabeled protein was purified as described for unlabeled proteins.

Overproduction and Purification of Full-length and Truncated MASPs—Catalytically inactive MASP-1 and MASP-2 were created by modification of the active-site serine residues (Ser\(^{262}\) in MASP-1 and Ser\(^{261}\) in MASP-2) (13). The cDNAs were altered by substitution of double-stranded oligonucleotides for restriction fragments. The modified cDNAs were ligated into the polylinker of plasmid pED (17), and the resulting plasmids were used to transfect adherent DXB11 Chinese hamster ovary cells (18). MASPs were produced in serum-free medium to ensure that preparations were not contaminated with other serum proteins using a protocol that has been described previously for truncated MASPs (19).

MASPs were purified on MBP-A affinity columns prepared as described (7). Culture medium (100–200 ml) diluted with an equal volume of loading buffer (50 mM Tris-Cl, pH 7.5, containing 1.25 M NaCl and 10 mM EDTA) was loaded onto the column equilibrated in the same buffer. Following washing, bound MASPs were eluted with a 0.05–1M NaCl gradient over 20 ml. Yields of MASP-1 and MASP-2—determined by the second moment method and were corrected for the fraction of competent components. Initial studies were undertaken with full-length MASPs, proteins were mixed at 1:1, 1:2, and 2:1 molar ratios to give an average molar dissociation constant of 1.5 × 10\(^{-7}\) M and the fraction of competent components was determined using the program DCDT (23). The molecular mass values ± S.E. from two independent experiments were converted to molar dissociation constants as described (22). Values are means ± S.E. from two independent experiments. It was assumed that no changes in the partial specific volume occur upon complex formation and that the molecular mass of a complex is the sum of that of the components.

Sedimentation velocity experiments were carried out at 40,000 rpm and 20 °C in Epon aluminum-filled centerpieces as described (16). Scans were collected at 2–4 min intervals at 230 nm. Data were analyzed using the program CDCT (23). The apparent sedimentation coefficient derived from the light scattering data when fitted to a single species model at three different loading concentrations. The apparent dissociation constants derived from the best fits were converted to molar dissociation constants as described (22).

To quantify the stoichiometry of MBP/MASP complexes, releasing and scavenger proteins were produced and purified as described for unlabeled three-domain proteins.

RESULTS

Defining Minimum MBP-binding Units of MASP-1 and MASP-2—The first step toward dissecting the MBP/MASP interaction was to reconstitute the MBP-MASP complex from purified components. Initial studies were undertaken with full-length MASPs. Using the results as a basis, subfragments of MASPs with affinities for MBP equivalent to those of the full-length proteins were defined.

Production and Characterization of Full-length Homodimeric MASPs—In an initial attempt to isolate large amounts of MASP-1 and MASP-2 for biophysical analysis, the cDNAs were expressed in Chinese hamster ovary cells containing Chinese hamster ovary cells producing other serum proteins using the same expression system (7, 16), suggesting that production of wild-type proteins is cytotoxic. Of 12 cell lines that were tested, only one line produced even trace amounts of MASP-1 that could be detected on immunoblots probed with antibody specific for the N termin
The apparent molecular mass of 65 kDa was consistent with the calculated mass of the N-terminal fragment of processed MASP-1 polypeptide, indicating that the protein is probably activated during biosynthesis. No protein was isolated from the medium of any cell lines transfected with MASP-2 cDNA, implying that the encoded protease is even more cytotoxic than MASP-1.

To avoid the cytotoxicity associated with protease activation during or after secretion, a catalytically inactive form of each MASP was created by replacing the serine residue that forms part of the catalytic triad in the protease domain with an alanine residue (Fig. 1). This substitution was designed to inactivate the protease while retaining its MBP-binding properties. Inactive MASPs, designated MASP-1* and MASP-2*, were purified by affinity chromatography on MBP-A-agarose columns. This strategy enabled rapid purification without the use of extraneous tags. As expected, each MASP* migrated as a single band on SDS-polyacrylamide gels (Fig. 1), demonstrating that neither protein is proteolytically processed during biosynthesis. Weight-averaged molecular masses for MASP-1* and MASP-2* determined by equilibrium ultracentrifugation were in agreement with the calculated values for homodimers of the glycosylated polypeptides (Fig. 2, A and B).

FIG. 1. SDS-polyacrylamide gel electrophoresis of intact and truncated MASP-1 and MASP-2. Upper, schematic representation of the domain organization of full-length and truncated MASPs. The positions of the serine residues that were changed to alanine residues are indicated. Balls on sticks represent potential N-linked glycosylation sites of MASP-1 (above) and MASP-2 (below). The regions that mediate dimerization and MBP binding are indicated. CCP, complement control protein. Lower, purified MASPs* and the MASP-2 fragment encompassing the EGF-like and CUB-2 domains were separated on 10 and 17.5% gels under reducing conditions. Apparent molecular masses were 79 and 73 kDa for MASP-1* and MASP-2*, respectively, and 19–24 kDa for the MASP-2 EGF/CUB-2 fragment. Proteins were detected by staining with Coomassie Blue.

Equilibrium analytical ultracentrifugation was also used to examine whether MASP-1 and MASP-2 interact with each other to form hetero-oligomers. Apparent molecular masses of mixtures of MASP-1* and MASP-2* present in equal molar amounts corresponded to the average molecular mass of the two components over a wide range of protein concentrations (Fig. 2C). Thus, MASP-1 and MASP-2 behave independently and remain dimeric even at protein concentrations in excess of those present in serum.

**MBP Binding by Full-length MASPs**—The interactions of MBP with MASP-1 and MASP-2 were compared using solid-phase competition assays in which increasing concentrations of MASPs* were incubated in wells coated with MBP-A. 35S-Labeled truncated MASPs encompassing the three N-terminal domains (Fig. 1) were used as the reporter ligands. The binding interaction was quantified by determining the concentration of MASP necessary to displace half of the reporter ligand from MBP-A (K<sub>i</sub>). Analysis revealed that MASP-1* and MASP-2* interacted independently with MBP-A with comparable binding affinities to form stable complexes (Fig. 3 and Table I).

**Equivalent Interactions of MBP with Intact and Truncated MASPs Comprising the Three N-terminal Domains**—Purified MASPs* were relatively unstable. Their tendency to aggregate upon storage prevented a more detailed characterization of the
complexes formed with MBP. To identify a stable MASP fragment that could be used in further analysis, the interactions of truncated MASPs with MBP were examined. Initial studies were undertaken using fragments comprising the three N-terminal domains of MASP-1 and MASP-2 (13). Competition assays revealed that three-domain MASPs bound to MBP with affinities similar to those of their full-length counterparts (Fig. 3 and Table I). Comparable $K_I$ values were also obtained when binding assays were performed using immobilized MASPs and soluble MBP-A with labeled MASP-A as the reporter ligand (Table I). Thus, three-domain MASPs are sufficient to reproduce the binding properties of the intact proteins.

**Role of the CUB Domains in MBP Binding by MASP-1 and MASP-2**—To establish whether all three of the N-terminal MASPs are necessary for stable binding to MBP, the binding properties of smaller MASPs were analyzed. A fragment of MASP-1 comprising the CUB-1 and EGF modules (13) bound to MBP-A, demonstrating that either or both of these domains form part of the binding site. However, the affinity was reduced compared with intact MASP-1, indicating that the CUB-2 module must also interact with MBP (Fig. 3 and Table I). Comparable results were obtained with the corresponding MASP-2 fragment.

To examine whether the CUB-1 domain is necessary for MBP binding by MASPs, fragments lacking this domain were produced, and their binding properties for MBP-A were analyzed. The MASP-2 fragment comprising the EGF and CUB-2 domains did not interact with MBP-A, so the CUB-1 domain must be essential for stable complex formation. Taken together, these results show that both CUB domains form part of the MBP-binding site on MASPs and that the three N-terminal MASP domains together are thus necessary as well as sufficient for stable binding to MBP.

Because the MASP-2 fragment comprising the EGF and CUB-2 domains did not bind to MBP, it was of interest to determine its oligomeric structure. Characterization of three-domain MASPs and fragments consisting of the two N-terminal domains has shown that one or both of the CUB-1 and EGF modules interact to form homodimers. The molecular mass of the glycosylated MASP-2 fragment comprising the EGF and CUB-2 domains determined by equilibrium ultracentrifugation (21.0 ± 0.8 kDa) is the same as that determined by MALDI-MS (21.5 kDa), so it is monomeric. Because the EGF domains and CUB-2 modules of individual MASP protomers do not bind to each other, the CUB-1 domains must mediate dimerization.

**Stoichiometry of Complement-activating MBP-MASP Complexes**—To define functional units for complement activation by MBP, it was necessary to characterize the interactions of individual MBP-MASP complexes. Because the different MBP-A oligomers activate complement with different specific activities (7), individual MBP-A oligomers were isolated, and their MASP-binding properties were analyzed separately.

**MASP Binding by MBP-A Oligomers with Different Complement-fixing Activities**—The solid-phase competition assay was used to demonstrate that each MBP oligomer interacts with the three-domain MASP-1 and MASP-2 proteins, but with different affinities (Table II). MBP-A oligomers bound to MASP-2 with affinities in the following order: tetramer > trimer > dimer > monomer. However, when the affinities of MBP-A dimers, trimers, and tetramers are rationalized to the number of MBP subunits, they are all equivalent (Table II), suggesting that the higher affinities of the larger oligomers are due to the presence of more MASP-binding sites on such oligomers. Similar treatment of the binding data showed that MASP-1 is different from MASP-2. The affinities of MBP-A trimers and tetramers for three-domain MASP-1 are greater than the affinities of dimers after they are rationalized to the number of subunits in each oligomer (Table II). Thus, MASP-1 binds to MBP-A tetramers and trimers with higher affinities than to MBP-A dimers, even after accounting for the presence of extra MASP-binding sites on the larger MBP oligomers.

The rank order of affinities of MBP-A oligomers for MASPs is the same as their relative complement-fixing activities (Table II), suggesting that the different ability of oligomers to activate complement is dependent on their different affinities for MASP-1 and MASP-2. Thus, the lower specific activity of MBP-A dimers compared with MBP-A trimers or tetramers of subunits may result from the reduced affinity of these oligomers for MASP-1. The absence of detectable activity of single MBP-A subunits and the relatively low activity of MBP-C are probably also due to their low affinities for MASPs.

To establish whether MASP-1 and MASP-2 bind to the same or distinct sites on MBP, assays were carried out using one unlabeled three-domain MASPs to compete with the other radiolabeled three-domain MASPs. Each MASP was able to displace the other reporter ligand, and the rank order of the $K_I$ values was the same whether radiolabeled MASP-1 or MASP-2 fragments were used (Fig. 4). Thus, MASP-1 and MASP-2 must compete for the same binding sites on MBP-A. Slight differences in the $K_I$ values probably reflect the differences in the affinities of MASP-1 and MASP-2 for individual MBP oligomers (Table II).

**Two Independent MBP-binding Sites on MASP Homodimers**—The differences in binding properties of single MBP subunits compared with larger oligomers could be explained if each MASP dimer binds to more than one MBP subunit. Direct evidence for this hypothesis is provided by the stoichiometry of MBP binding to MBP determined by equilibrium ultracentrifugation. MBP-C was used for these studies because it can be purified in large amounts, and it is free of contamination from larger oligomers (16). It is able to fix complement, and its MASP-binding properties are similar to those of single subunits of MBP-A.
Stoichiometry of MBP-MASP Complexes

Table I

| Immobilized protein | Soluble ligand | $K_0$ | Relative $K_0^a$ |
|---------------------|---------------|-------|------------------|
| MASP-1 interactions  |               |       |                  |
| MBP-A               | MASP-1*       | 34 ± 12 | 1.00             |
| MBP-A               | 3-Domain MASP-1 | 15 ± 4  | 2.7 ± 1.5        |
| MASP-1*             | MBP-A         | 23 ± 6  | 1.00             |
| 3-Domain MASP-1     | MASP-1       | 46 ± 9  | 0.64 ± 0.37      |
| MASP-2              | MASP-1 (CUB-1 + EGF) | 1810 ± 200 | 0.013 ± 0.005 |
| MASP-A              | MASP-1 (EGF + CUB-2) | ND     | ND               |

Table II

| MBP oligomer | $K_{\text{MBP oligomer}}$ | $K_{\text{MBP subunit}}$ | Relative $K_0^a$ | Relative complement-fixing activity $b$ |
|--------------|---------------------------|---------------------------|------------------|----------------------------------------|
|              | 3-Domain MASP-1 | 3-Domain MASP-2 | 3-Domain MASP-1 | 3-Domain MASP-2 |                     |
|              | nm | nm | nm | nm |                       |
| MBP-A        | 9 ± 1          | 9 ± 3          | 36 ± 4          | 36 ± 12         | 1.3 ± 0.3          | 1.0 ± 0.5          | 1.0 ± 0.1          |
| MBP-B        | 20 ± 8         | 10 ± 3         | 60 ± 24         | 30 ± 9          | 1.0 ± 0.5          | 1.1 ± 0.5          | 1.2 ± 0.2          |
| MBP-C        | 125 ± 25       | 18 ± 1         | 230 ± 50        | 36 ± 2          | 0.20 ± 0.08        | 0.70 ± 0.21        | 0.24 ± 0.06        |
| MBP-A        | 6500 ± 500     | 1300 ± 300     | 6500 ± 500      | 1300 ± 300      | 0.007 ± 0.002      | 0.024 ± 0.010      | -0.01             |
| MBP-A        | 5100 ± 200     | 3000 ± 1300    | 5100 ± 200      | 3000 ± 1300     | 0.011 ± 0.006      | 0.013 ± 0.007      | 0.06 ± 0.03        |

Equilibrium distributions of mixtures of MBP-C and three-domain MASP-2 fit very well to a model assuming formation of mixed complexes consisting of one and two MBP-C molecules bound to single MASP dimers (Fig. 5), where individual dissociation constants for the first ($K_{D11}$) and second ($K_{D21}$) binding events were 10 ± 5 and 8 ± 1 μM, respectively. These values are in good agreement with the $K_0$ values determined using the solid-phase binding assays, validating the solid-phase assay as a way of measuring binding affinity. Using a similar strategy for MASP-1, $K_{D11}$ and $K_{D21}$ for the interaction with MBP-C were determined as 21 and 14 μM. Thus, the data indicate that there are two independent MBP-binding sites on each MASP homodimer, with each site having comparable affinity for single MBP subunits.

Stoichiometry of Complexes with MBP-A Dimers—The equilibrium data provide an explanation for the observation that MASPs form more stable complexes with MBP-A dimers, trimers, and tetramers than with MBP-A or MBP-C monomers: each larger oligomer contains at least two subunits that can occupy the binding sites of MASP. Initial equilibrium ultracentrifugation experiments confirmed that the larger oligomers bind tightly to MASPs. Indeed, these complexes were too stable to analyze quantitatively using this method because free components were not significantly populated at the lowest concentrations that can be detected using equilibrium methods. For this reason, sedimentation velocity analysis was used to determine the stoichiometry of the complexes.

Mixtures of the interacting components were analyzed to determine the composition of MBP-MASP complexes. By changing the molar ratios of the components, it was possible to populate the complexes differentially. Complexes were distinguished by the different rates at which they sedimented using the time derivative method ($s^b$) to display the distribution of apparent sedimentation coefficients ($s^b$) (23). Analysis of mixtures of MBP-A dimers and three-domain MASP-2 showed that (MBP-A)$_2$-MASP-2 complexes were formed. A new peak in the $s^b$ analysis represents a new species that sedimented faster than either of the individual components (Fig. 6, A and B). Only small amounts of unassociated species were detected when proteins were mixed in equal molar amounts, indicating that the $K_0$ is considerably lower than the loading concentration of the components (0.5 μM). In the presence of excess three-domain MASP-2, only a single peak corresponding to the MBP-MASP complex was observed, in which the $s^b$ distribution was similar to that in the 1:1 mixture, demonstrating that larger complexes were not formed. The amount of unbound, three-domain MASP-2 in these experiments corresponded to the molar excess present in the mixture, confirming that the proteins formed only (MBP-A)$_2$-MASP-2 complexes. These complexes are probably more stable than complexes formed between MBP-C and MASP-2 because each subunit of an MBP-A dimer interacts with a subunit of the MASP dimer.

In the presence of excess MBP-A dimers, the $s^b$ distribution of the peak corresponding to the MBP-MASP complex shifted to

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*a* Expressed as $K_{\text{full-length MASP}}$/$K_{\text{truncated MASP}}$.

*b* Not determined.

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In this and subsequent formulas, (MBP-A)$_2$, (MBP-A)$_3$, and (MBP-A)$_4$ represent dimers, trimers, and tetramers of MBP-A subunits, and MASP denotes a dimer of three-domain MASP.
higher values, revealing that additional species were present in the mixture that probably comprised (MBP-A)_2-MASP-2 complexes (Fig. 6B). The average s^n value increased as increasing amounts of MBP-A dimer were added, indicating that components were exchanging between bound and unbound states during the course of the experiment. A large excess of MBP-A dimer was needed to drive formation of (MBP-A)_2-MASP-2 complexes, implying that MBP binding occurs in a stepwise manner, in which the dissociation constant of the second binding event is considerably greater than that for formation of the 1:1 complex.

Analysis of the complexes formed between MBP-A and MASP-1 showed that the MBP-binding properties of the two MASPs are similar (Fig. 7). MBP-A dimers formed predominantly (MBP-A)_2-MASP-1 complexes in the presence of excess three-domain MASP-1, whereas larger species that probably comprised ((MBP-A)_2)_z-MASP-1 complexes were detected in the presence of excess MBP-A dimer. Significant amounts of unbound components were present in the 1:1 mixture, indicating that the affinity of MBP-A dimers for MASP-1 is lower than that for MASp-2, a finding that is consistent with the results obtained in the solid-phase binding assays.

**Stoichiometry of Complexes with MBP-A Trimmers and Tetramers—** Analysis of mixtures of MBP-A trimers or tetramers and three-domain MASP-2 indicated that stable (MBP-A)_3-MASP-2 and (MBP-A)_4-MASP-2 complexes were formed (Fig. 6). Single peaks corresponding to these complexes were detected when the proteins were mixed in equal molar amounts. Only low levels of unbound components were detected, so the K_d values must be significantly less than 0.1 μM. Larger species were formed in the presence of excess three-domain MASP-2 that probably consisted of (MBP-A)_2(MASP-2)_2 and (MBP-A)_2(MASP-2)_2 complexes. The s^n distribution of the most rapidly sedimenting boundary increased with increasing amounts of MASP, and excess MASP was required to populate the ternary complexes, signifying that the affinity of the second MASP-binding site is lower than that of the first. No additional complexes were detected in the presence of excess MBP-A, demonstrating that species comprising multiple MBP-A trimers or tetramers bound to single MASP-2 dimers were not formed.

MBP-A trimers and tetramers formed mainly (MBP-A)_3-MASP-1 and (MBP-A)_4-MASP-1 complexes with the three-domain MASP-1 protein under the conditions examined. There was no evidence of (MBP-A)_3(MASP-1)_2 complexes, even in the presence of a large molar excess of MASP-1 (Fig. 7). In contrast, some (MBP-A)_2(MASP-1)_2 complexes were formed at relatively high MASP-1 concentrations. The s^n of the fastest sedimenting boundary increased under these conditions, and the s^n distribution became asymmetrical, confirming the presence of larger species (Fig. 7F). However, the affinity of the second binding site on MBP-A tetramers for MASP-1 was considerably lower than that of the first.

**Functional Units for Complement Activation—** MBP-A dimers, trimers, and tetramers all form stable complexes with MASP-1 and MASP-2 and have high complement-fixing activities relative to MBP monomers. To determine the minimum functional unit necessary for complement activation, MBP-A dimers were mixed with both three-domain MASPs simultaneously and analyzed using sedimentation velocity experiments. The data show that each MBP-A dimer could bind to either the MASP-1 or MASP-2 fragment, but that the proteins did not form (MBP-A)_2(MASP-1)-MASP-2 complexes (Fig. 8). Only (MBP-A)_2-MASP-1 or (MBP-A)_2-MASP-2 complexes were formed even in the presence of an excess of both MASPs. Thus, a single binding event between an MBP-A dimer and a MASP dimer must be sufficient to form a functional unit that is
FIG. 6. Sedimentation velocity analysis of mixtures of three-domain MASP-2 and purified MBP-A oligomers. MBP-A dimers (A and B), trimers (C and D) and tetramers (E and F) were mixed with three-domain MASP-2 in the molar ratios indicated. The loading concentrations that corresponded to single molar equivalents of MBP-A dimers, trimers, and tetramers were 0.075, 0.063, and 0.090 mg/ml, respectively. Concentrations were chosen to be at least 10-fold greater than the $K_I$ values determined from the solid-phase assays to ensure that the complexes were significantly populated. Concentrations were calculated based on molecular masses of 150, 225, and 282 kDa for dimers, trimers, and tetramers of MBP-A, respectively, and 74 kDa for three-domain MASP-2 dimers. The sedimentation coefficient ($s_{20,w}$) of the three-domain MASP-2 fragment was determined as $4.5 \pm 0.2$ S.

FIG. 7. Sedimentation velocity analysis of mixtures of three-domain MASP-1 and purified MBP-A oligomers. MBP-A dimers (A and B), trimers (C and D), and tetramers (E and F) were mixed with three-domain MASP-1 in the molar ratios indicated. Concentrations that corresponded to single molar equivalents of dimers, trimers, and tetramers were 0.075, 0.080, and 0.075 mg/ml, respectively. Concentrations were calculated based on a molecular mass of 75 kDa for three-domain MASP-1 dimers. The $s_{20,w}$ of three-domain MASP-1 was determined as $5.0 \pm 0.1$ S.

FIG. 8. Sedimentation velocity analysis of mixtures of MBP-A dimers, three-domain MASP-1, and three-domain MASP-2. MBP-A dimers, three-domain MASP-1, and three-domain MASP-2 were mixed simultaneously in the molar ratios shown. The sedimentation coefficients of three-domain MASP fragments and (MBP-A)$_2$ MASP complexes are indicated.
domains probably interact directly with MBP (Fig. 9A). The EGF-like domain may also form part of the binding site for MBP, or it could orient the CUB domain to enable stable MBP binding. Dimerization of each MASP is mediated through interactions involving the CUB-1 domain. The individual MASP protomers are configured so that both can bind to MBP subunits.

It is interesting to consider the structural implications of the binding data and hydrodynamic analysis on the MBP-MASP complexes. MASPs bind to the N-terminal portion of the collagenous domain of MBP-A (7). This region is assembled from closely associated collagen triple helices and so has a highly extended conformation. Hydrodynamic analysis demonstrates that three-domain MASPs are also asymmetrical. Frictional ratios of 1.58 ± 0.04 and 1.67 ± 0.08 were calculated for the three-domain MASP-1 and MASP-2 proteins based on their sedimentation coefficients and molecular masses. The most likely arrangement is one in which the three N-terminal modules of each MASP protomer extend along the axis of the collagen triple helices (Fig. 9A). Contacts between two MBP subunits and a single MASP dimer stabilize the complexes, in which each MASP protomer probably binds to a single MBP subunit.

The binding properties of MASPs can be explained if there are binding sites for two MBP subunits on each MASP dimer (Fig. 9). Direct evidence for this arrangement is provided by analysis of the interactions of MBP-C with MASP-1 and MASP-2. It is also consistent with the observation that dimers of MBP-A subunits bind to single MASP dimers, whereas trimers and tetramers can bind up to two MASPs. In the presence of excess MASP, MBP-A dimers form complexes with two MASP dimers, indicating that there could be additional binding sites for MBP subunits on each MASP. However, these complexes are formed only in the presence of a large excess of MBP, and it is more likely that one protomer of each MASP dimer interacts with a single MBP-A dimer under these conditions (Fig. 9B).

MASP-1 and MASP-2 compete for the same binding sites within the collagen-like domains of MBP oligomers. Trimmers and tetramers of MBP-A subunits bind with comparable affinities to both MASPs, but MBP-A dimers bind more tightly to MASP-2 than to MASP-1. Based on these properties, it is likely that in serum, MBP-A dimers are mainly associated with MASP-2, whereas MBP-A trimers and tetramers form complexes with either MASP-1 or MASP-2. Because there are binding sites for two MASP dimers on the larger MBP-A oligomers (Fig. 9, C–F), it is also possible that (MBP-A)₃₋₅MASP-1-MASP-2 complexes are formed, although formation of these complexes is not essential for effective complement activation.

MAP19 is an alternatively spliced version of MASP-2 comprising the two N-terminal domains (26–28). It copurifies in MBP/MASP preparations from rat and human sera. The role of MAP19 is unclear because it lacks the serine protease domain of intact MASPs, and so it is unable to activate complement directly. Based on the properties of the truncated MASPs described here, it can be predicted that MAP19 is probably a homodimer that binds to MBP, but with lower affinity compared with MASP-2. Because the larger MBP-A oligomers can bind up to two MASPs, MAP19 could form a complex with an MBP oligomer and an intact MASP. Thus, it might form part of an MBP-MASP complex that triggers complement activation, although its contribution to the activation mechanism is unclear.

Human serum MBP, like rat MBP-A, consists of multiple oligomeric forms. It comprises mixtures of oligomers ranging from two to eight trimeric subunits (29). Based on their similar structural organizations, it is likely that the mechanism for MBP binding and complement activation by human MBP is the same as that for rat MBP-A. The larger MBP oligomers in human serum could potentially form complexes containing additional MASP molecules. For example, MBP octamers could bind up to four MASP dimers.

MBP-C is able to activate complement, whereas single MBP-A subunits have no detectable activity (7). Comparison of the binding data shows that these proteins appear to have comparable affinities for MASPs. However, single subunits of MBP-A may bind with lower affinities than the data suggest because even trace contamination by larger MBP-A oligomers would increase the $K_c$ values observed. Because MBP-C is monomeric and is not contaminated by larger oligomers, the binding data probably reflect accurately the affinities of the interactions.

A fundamental goal in understanding complement activation by MBP is to define how binding to a pathogen triggers MASP activation. MBP interacts with microorganisms through the C-terminal carbohydrate recognition domains, whereas the binding site for MASPs is located within the collagenous domain, near the N-terminal end of the protein. Because these binding events occur in distinct parts of the MBP molecule, it is likely that activation is mediated through a conformational change. Binding to the surface of a microorganism could induce conformational changes within individual MBP subunits, leading to MASP activation. In this model, a single MBP subunit would be expected to be sufficient to activate MASPs. Evidence against this model comes from crystallographic data that show...
no structural changes occurring to individual carbohydrate recognition domains upon sugar binding (30).

In an alternative model for complement activation, binding to the surface of a microorganism could cause a global change in the structure of MBP, resulting in displacement of the relative positions of subunits. This model would explain why MBP oligomers consisting of at least two trimeric subunits are required to trigger MASP activation effectively. It would also explain why MASPs are dimeric because each protomer of the dimer binds to a separate subunit within an MBP oligomer. This hypothesis is compatible with the structural organization and biophysical properties of MBP oligomers. Potentially flexible regions have been identified between the collagenous domain and the neck as well as immediately adjacent to the C-terminal hinge regions of subunits upon binding to the surface of a microorganism could cause a global change in the overall architecture of the molecule. Because MASPs are located immediately adjacent to the N-terminal hinge regions, they would be sensitive to such conformational changes. Indirect evidence suggests that a global conformational change in C1q leads to activation of C1r and C1s (32). Thus, MBP-MASP complexes may serve as templates for understanding the activation mechanism of the classical pathway as well as the lectin pathway of the complement cascade.

Acknowledgment—We thank Kurt Drickamer for helpful discussions and assistance in preparation of the manuscript.

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