Molecular Determinants of Oligomer Formation and Complement Fixation in Mannose-binding Proteins*

(Received for publication, September 9, 1998, and in revised form, November 25, 1998)

Russell Wallis‡ and Kurt Drickamer§

From the Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

Rat serum mannose-binding protein (MBP-A) functions as part of the innate immune system by targeting complement toward potentially pathogenic microorganisms. In order to examine the molecular basis for complement activation, rat MBP-A has been overproduced in Chinese hamster ovary cells. Recombinant protein is post-translationally modified in the same way as the native lectin. Hydrodynamic studies indicate that MBP-A consists predominantly of covalent oligomers containing one to four copies of a subunit that comprises a trimer of polypeptides. These oligomers are non-interconverting and do not assemble into higher order structures at concentrations in excess of those normally found in serum. Disulfide bonds formed between cysteine residues at the N-terminal end of the collagen-like domain link polypeptides to form covalent oligomers. Analysis of wild-type MBP-A and MBP-A containing the substitution Cys⁶ → Ser suggests that polypeptides within each trimeric structural unit are mostly linked by disulfide bonds between cysteine residues at positions 13 and 18 arranged in an asymmetrical configuration. Disulfide bonds involving Cys⁶ connect polypeptides within separate trimers. Analysis of chimeras between MBP-A and rat liver MBP (MBP-C) indicates that residues within the N-terminal region of the collagenous domain and the cysteine-rich domain of MBP-A enable assembly of trimers into higher order oligomers. The activity of MBP-A in a hemolytic complement fixation assay using mannan-coated sheep erythrocytes was approximately 20-fold greater than the activity of MBP-C. Analysis of the MBP chimeras and isolated oligomers of MBP-A reveals that the larger oligomers are more efficient at complement activation. These data indicate that the overall complement fixing activity of MBP-A is a function of the individual molecular activities of oligomers and their relative abundance within the serum.

Serum mannose-binding protein (MBP) is an important component in the mammalian innate immune system that binds carbohydrates on the surfaces of pathogenic microorganisms and activates complement in an antibody-independent manner (1, 2). It also functions directly as an opsonin by binding to specific receptors on the surfaces of phagocytic cells (3). Serum MBP is a member of the collectin family of animal lectins. Each collectin polypeptide contains an N-terminal cysteine-rich domain, a collagen-like domain, an α-helical coiled-coil neck region and a C-terminal carbohydrate-recognition domain (CRD) (4).

Complement fixation by serum MBP occurs by a mechanism similar to the classical pathway of complement activation. MBP interacts with two MBP-associated serine proteases (MASP-1 and MASP-2) (5–7) that are homologs of complement components C1r and C1s. On activation, MASP-2 cleaves both C4 and C2 to form a C4b2a complex with C3 convertase activity (8). Human MBP deficiency is a relatively common disorder caused by point mutations in the MBP gene (9, 10). This condition is associated with a high susceptibility to bacterial and viral infections particularly during the first few years of life.

Two distinct rat mannose-binding proteins, MBP-A and MBP-C, have been cloned and sequenced (11). These proteins are found predominantly in the serum and liver, respectively. The two forms adopt distinct higher order structures (4). MBP-C consists of a single trimer of polypeptides whereas MBP-A assembles into higher oligomers. Rotary shadowing electron microscopy and sucrose gradient ultracentrifugation of human MBP suggest the presence of oligomers ranging from dimers to hexamers of trimeric subunits (12, 13). The trimeric building blocks from which MBPs are assembled will subsequently be referred to as subunits. The MBP oligomers adopt a bouquet-like structure, similar to complement component C1q (12). Assembly of each subunit in MBP-C occurs in a C- to N-terminal direction (14), so that association of the CRDs and trimerization of the α-helical coiled-coil region enables formation of the collagen triple helix. The collagen-like domain contains 4-hydroxyproline and 5-hydroxylysine residues, which are further derivatized to form glucosylgalactosyl-5-hydroxylysine (11, 15). MBP-A and MBP-C each contain a single break in the Gly-Xaa-Yaa repeat pattern within the collagenous domain (11). This interruption is thought to introduce a kink or region of flexibility into the protein, which in MBP-A enables the trimeric stems to angle away from the central core of the bouquet-like structure. The polypeptides are linked by cysteine residues within the N-terminal domain which form disulfide bonds within and between subunits in the larger MBP-A structures (11).

In the crystal structure of a fragment of the trimeric MBP-A subunit containing the CRD and neck region, the CRDs of each trimer are clustered such that the binding sites are oriented in a configuration to enable binding to bacterial and fungal cell surfaces (16). In this study, we have used the relatively simple structure of MBP-C to examine the organization of its more complex serum homolog. Biophysical analysis of chimeras of MBP-A and MBP-C indicate that oligomers of MBP-A assemble.

* This work was supported by a grant from the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Glycobiology Institute, Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX1 3QU, United Kingdom. Tel.: 44-1865-275339; Fax: 44-1865-275339; E-mail: rwallis@glycob.ox.ac.uk.

§ Wellcome Principal Research Fellow.

The abbreviations used are: MBP, mannose-binding protein; CRD, carbohydrate-recognition domain; MASP, MBP-associated serine protease; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry.
as a result of the interactions of residues in the cysteine-rich domain and N-terminal part of the collagen-like domain. The presence of these regions of MBP-A also correlates with the level of complement fixing activity, indicating that correct assembly of MBP-A is essential for its biological roles.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from New England BioLabs. All tissue culture medium was from Life Technologies. Sheep erythrocytes and guinea pig serum were purchased from Serotech. Yeast mannan, phenylthiohydantoin-derivatives, and protein molecular weight markers for gel-filtration, SDS-polyacrylamide gel electrophoresis, and MALDI-MS were purchased from Sigma. Proteinase Arg-C, thermolysin, and subtilisin were obtained from Boehringer-Mannheim. Tosylphenylalanil chloromethyl ketone-treated trypsin and chymotrypsin were purchased from Worthington Biochemicals. Reagents for amino acid sequencing were from Beckman Instruments. Iodo[2-14C]acetic acid and Amplify fluorography reagent were obtained from Amersham.

**Analytical Methods**—Amino acid sequencing was carried out on a Beckman LF3000 protein sequencer. Amino acid analysis was performed by the method of Heinrikson and Meredith (17). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18). Standard molecular biology techniques were carried out as described previously (19). The presence of free thiol groups was determined as described previously (14).

**Production of MBP-A—**MBP-A was produced in Chinese hamster ovary cells using a strategy similar to that previously described for MBP-C (14). The cDNA encoding MBP-A (11) was modified to introduce an XhoI restriction site adjacent to the EcoRI site at the 5′ end and a SalI site near the BstXI site (base 835) in the 3′-untranslated region. This fragment was ligated into the unique SaI site restriction site upstream of the dihydrofolate reductase gene in the vector pED2 (20). Chimeric constructs were made using restriction sites that are common to the MBP-A and MBP-C cDNAs or by inserting synthetic oligonucleotide linkers between appropriate restriction sites. Chinese hamster ovary cell transfectants were generated and selected as described previously (14). Cells were grown to confluence in 225-cm2 tissue culture flasks containing 70 ml of medium supplemented with 0.5 mM methotrexate. This medium was harvested daily from 4 days after confluence and MBP-A was purified as described above.

MBP-A was purified from the culture medium of confluent cells, since this procedure yielded protein which resembles preparations of the native lectin (21). The larger oligomers of MBP-A are deficient in protein preparations from the medium of subconfluent cells. Protein isolated from the medium of subconfluent cells was also used to have a reduced apparent molecular mass, which may reflect changes in post-translational modification.

**Ion-exchange Chromatography—**Oligomers of MBP-A and MBP-A Cys6—Ser were resolved by ion-exchange chromatography on a Mono-Q HR 5/5 column (Pharmacia) equilibrated in 50 mM Tris, pH 8.2, containing 10 mM EDTA at a flow rate of 1 ml/min at 25 °C. Oligomers were eluted from the column using a 500 mM NaCl gradient in the same buffer over 45 min. The absorbance was monitored at 280 nm and the medium was stored at −80 °C. MBP was isolated following the method previously used for MBP-C (14). For complement assays, protein was isolated from serum-free medium. Two days after cell confluence was reached, the medium from each tissue culture flask was replaced with 20 ml of CHO-S-SFM II without nucleosides supplemented with 50 μM HEPES, pH 7.5, and 0.5 mM methotrexate. This medium was harvested daily from 4 days after confluence and MBP-A was purified as described above.

**Hydrodynamic Studies—**Gel-filtration chromatography was carried out on a Biosep S-S3000 column (300 × 7.8 mm, Phenomenex) equilibrated in 50 mM Tris, pH 8.2, containing 10 mM EDTA at a flow rate of 1 ml/min at 25 °C. Oligomers were eluted from the column using a 500 mM NaCl gradient in the same buffer over 45 min. The absorbance was monitored at 280 nm and 0.5-ml fractions were collected.

**Proteolysis—**For thermolysin and chymotrypsin digestion, MBP-A was incubated with 10–20% (w/v) of enzyme in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 25 mM CaCl2 at 37 °C for 1 h. Samples were separated on a 17.5% polyacrylamide gel under reducing conditions and blotted onto polyvinylidene difluoride membranes for sequence analysis (23). Trypsin digestion of MBP-A was carried out in 50 mM Tris, pH 7.5, containing 50 mM NaCl, 25 mM CaCl2, and 2.5 mM dithiothreitol. MBP-A (1 mg) was incubated with trypsin (5% w/v) at 37 °C with mixing for 18 h. The reaction mixture was passed through a mannose-Sepharose column equilibrated in reaction buffer without dithiothreitol to remove the CRDs. The peptide mixture was lyophilized to dryness, resuspended in 20% acetic acid (200 μl), and applied to a C3 reverse-phase column. Subtilisin digestion of N-terminal tryptic fragments, reverse phase high performance liquid chromatography, and MALDI-MS was performed as described previously (14).

**Complement Fixation Activity—**Complement fixation activity was determined by measuring MBP-dependent hemolysis of mannan-coated erythrocytes, using a slightly modified version of the assay described by Ikeda et al. (1). For coating of sheep erythrocytes with mannan by the chromium chloride method (24), 4 × 106 cells were washed three times by centrifugation (2000 × g for 2 min) and resuspended in 10 ml of Veronal-buffered saline, pH 7.3, (4.4 mM barbituric acid, 1.8 mM sodium barbitone, 145 mM NaCl). Aliquots of mannan (5 ml at 100 μg/ml) were mixed with 5 ml of erythrocytes (1 × 1010 cells) and 5 ml of CrCl3-H2O (0.5 mg/ml) in Veronal-buffered saline and incubated with mixing for 5 min at 25 °C. The reaction was stopped by the addition of 20 ml of dry-ice cold gelatin-Veronal buffer (Veronal buffer containing 0.1% gelatin, 0.37 M NaCl, and 5 mM CaCl2). The mannan-coated erythrocytes were washed three times in the same buffer and resuspended at 1 × 109 cells/ml. MBP-depleted serum was prepared by passing guinea pig serum (15 ml) through two mannose-Sepharose columns (1 ml), equilibrated in Veronal-buffered saline containing 10 mM CaCl2 at 4 °C. In addition, prior to setting up each complement assay, serum (1 ml) was incubated with mannan-coated erythrocytes (2 × 1010 cells) for 1 h at 37 °C to remove any remaining endogenous lectin. Complement fixation assays were performed in duplicate. Aliquots (0.4 ml) of MBP were incubated with 0.1 ml of cells (1 × 1010 cells) for 1 h in gelatin-Veronal buffer at 25 °C. The cells were washed with 0.5 ml of buffer and incubated with MBP-depleted guinea pig serum in a total volume of 0.5 ml for 1 h with mixing at 37 °C. Sufficient guinea pig serum was used to completely lyse the cells incubated with 1 mg of MBP-A. Gelatin-Veronal buffer (700 μl) was added to each sample and the cells were pelleted by centrifugation. The absorbance was measured at 541 nm and expressed as a percentage of the absorbance of an equivalent volume of cells totally lysed in water, correcting for lysis observed in the absence of MBP. The data were fitted to a sigmoidal curve using Microcal Origin. Relative complement fixing activities were calculated from the concentrations of MBP required for 50% hemolysis compared with MBP-A in assays performed using the same batch of mannan-coated erythrocytes. Values reported are the mean ± S.E. for at least three independent assays.

**RESULTS**

**Production of MBP-A—**Mannose-binding protein has generally been purified from mammalian sera by affinity chromatography on immobilized mannan or invertase, or on columns of mannosie linked to agarose (25–27). The purified protein consists of multiple covalent species detected by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Rat MBP-A comprises three major covalent forms which have been interpreted as dimers, trimers, and tetramers of the trimeric polypeptide (21). Quantities of MBP-A sufficient to enable a detailed study of its structural organization were produced in Chinese hamster ovary cells. Typical yields of 1.5–2.5 mg from 500 ml of serum-supplemented medium and 0.5–1 mg from 500 ml of serum-free medium were obtained. In contrast, yields reported from serum preparations range from 0.02 to 0.5 mg for human MBP (12, 26) and 0.2 mg for rat MBP-A (27) from an equivalent volume of serum. MBP-A purified from the expression system is not contaminated with other serum lectins as has been reported for serum preparations (27).

**Post-translational Modifications—**Amino acid sequencing of MBP-A revealed that the signal sequence is processed correctly (11). The MALDI-MS profile of reduced MBP-A is consistent with a single species of mass 24,730 Da. This value is considerably greater than the calculated mass based on the amino acid sequence (23,526 Da), indicating that the polypeptide...
has undergone extensive post-translational modification (11). Amino acid analysis revealed 4-hydroxyproline and 5-hydroxylysine contents of 1.6 and 1.8 mol \%, respectively. These data are consistent with 3.5 residues of 4-hydroxyproline and 4.0 residues of 5-hydroxylysine for each polypeptide chain. Examination of the amino acid sequence of MBP-A indicates that most of the potential sites for hydroxylation must be fully derivatized since there are four sites for both proline and lysine hydroxylsine, within the consensus sequences Gly-X-Pro and Gly-X-Lys. These results are consistent with previous studies in which both 4-hydroxyproline and 5-hydroxylysine have been detected in preparations of the native lectin (11).

N-terminal sequencing of the intact protein and of chymotrypsin and thermolysin fragments indicate that Pro\(^{145}\), Pro\(^{190}\), and Pro\(^{395}\) are fully derivatized, since only phenylthiohydantoic acid-4-hydroxyproline was detected at these positions (Fig. 1). However, Pro\(^{33}\) was found to be unmodified, since only phenylthiohydantoin-proline is detected in the 33rd sequence cycle of Edman degradation of the intact polypeptide. These results were confirmed by MALDI-MS and N-terminal sequencing of tryptic peptides of MBP-A separated by reverse-phase chromatography. Peptide Gly\(^{236}\)-Lys\(^{246}\) elutes in the unbound fraction from the reverse-phase column and peptide Leu\(^{47}\)-Arg\(^{60}\) elutes as a single peak from the column. The masses of these peptides are consistent with complete hydroxylation of Pro\(^{44}\), Pro\(^{50}\), and Pro\(^{56}\). The peptide Asp\(^{24}\)-Arg\(^{38}\) also elutes as a single peak from the reverse-phase column. However, the mass of this peptide indicates that Pro\(^{33}\) is unmodified, confirming the sequencing data. This residue occurs immediately before the break in the collagen consensus sequence. Previous studies have shown that the proline residue at the corresponding position in MBP-C is only partially hydroxylated (14).

MALDI-MS analysis of peptides purified from trypsin digests of MBP-A indicates that the 4 lysine residues within the consensus sequence for hydroxylation are at least partially modified. In each case, the mass of the peptide is consistent with further derivitization of the hydroxylysine residue by the addition of 2 hexose units. In addition, no phenylthiohydantoin-derivative could be detected at these positions by N-terminal sequencing suggesting glycosylation (28). Glucosylgalactosyl-5-hydroxylysine has previously been identified in preparations of MBP (15). Additional minor peaks were observed in the spectra of glycosylated peptides observed by MALDI-MS analysis. The masses were consistent with the loss of single hexose units (\(\sim 162.1\) Da). However, it is not clear whether these peaks reflect heterogeneity of the sample or occur as a result of the ionization/desorption process.

Altogether, the post-translational modifications of MBP-A produced in Chinese hamster ovary cells account for the increased mass of the MBP-A polypeptide determined by MALDI-MS. These modifications, which are characteristic of those found in vertebrate collagens, are consistent with those previously reported for protein purified from rat serum.

**Oligomeric Structure of MBP-A**—Recombinant MBP-A, when analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, migrates as four major bands with apparent molecular masses of 85, 185, 280, and 355 kDa (Fig. 2B). Similar bands were detected in preparations of protein purified by affinity chromatography and in radiolabeled material immunoprecipitated using an antibody specific for the CRD of MBP-A.\(^2\) These findings indicate that multiple covalent forms of MBP-A are secreted from the cells and are not created by rearrangement of disulfide bonds during the purification procedure.

Under reducing conditions, MBP-A migrates as a single band with an apparent molecular mass of 29 kDa. Thus, the bands observed under nonreducing conditions are compatible with the presence of disulfide-linked monomers, dimers, trimers, and tetramers of subunits, with dimers and trimers being the most abundant covalent forms. The composition and ratio of multimers is consistent with previous studies of MBP-A purified from rat serum (21). Although the apparent molecular masses of the trimer and tetramer are beyond the range of the molecular weight markers, the identities of the these species were confirmed by analytical ultracentrifugation (see below). Minor peaks could be observed on SDS-polyacrylamide gels overloaded with MBP-A under nonreducing conditions. The apparent molecular masses of 26, 45, 125, 255, 310, and 340 kDa are consistent with the presence of low levels of intermediate covalent forms comprising 1, 2, 3, 4, 5, 7, 8, 10, and 11 polypeptide chains, respectively.

MBP-A analyzed by gel-filtration chromatography elutes at several positions: a well resolved peak with an apparent molecular mass of 250 kDa and three overlapping peaks with apparent molecular masses in the range 450–800 kDa (Fig. 2A). SDS-polyacrylamide gel electrophoresis of fractions collected across the gel-filtration profile indicates that the larger covalent species elute as multiple overlapping peaks from the column (Fig. 2B). The order of elution corresponds to the molecular mass of each covalent oligomer. The elution profile of MBP-A is similar over the entire range of concentrations examined (up to 4 mg/ml). There was no evidence of assembly of the four major oligomers to form more complex, higher order structures.

\(^2\) R. Dodd, E. Leamy, R. Wallis, and K. Drickamer, unpublished observations.
Oligomerization and Complement Fixation by MBPs

Previous studies have revealed that collectins show anomalous behavior on gel-filtration because of the highly extended conformation of the molecules, which in turn is due to the presence of a triple-helical collagen-like domain (13, 14). For this reason, analytical ultracentrifugation was used to determine the accurate mass of oligomers of MBP-A, independent of shape. The equilibrium distribution of purified MBP-A at loading concentrations of up to 1 mg/ml is consistent with a non-interacting heterogeneous mixture (data not shown). The apparent molecular mass as a function of concentration increases throughout the sample cell. However, the data for different loading concentrations, rather than overlapping as would be expected for a self-associating system, show similar apparent molecular mass distributions. Furthermore, the apparent weight-averaged molecular mass is dependent on the rotor speed, since a systematic decrease in apparent molecular mass with increasing rotor speed was observed. This result is also consistent with the presence of heterogeneity.

In order to simplify analysis of MBP-A, the oligomers were separated by ion-exchange chromatography on a Mono-Q column. Four major overlapping peaks were detected. On SDS-polyacrylamide gel electrophoresis these peaks were found to consist predominantly of covalent monomers, dimers, trimers, and tetramers of subunits. Low levels of intermediate oligomers are also detected, suggesting the presence of noncovalent oligomers of subunits. The elution positions of these latter species correspond to their covalent counterparts. Individual oligomers were further purified where necessary by reapplying fractions to the column and eluting using a shallow salt gradient. A sample of each of the purified oligomers is shown in Fig. 2C. Each sample was analyzed by analytical ultracentrifugation to determine the native molecular mass. The equilibrium distributions of apparent molecular mass as a function of concentration of purified dimer and trimer of subunits are shown in the upper and lower panels of Fig. 3. Data from three initial loading concentrations are shown in each case. No self-association can be detected. The weight-averaged molecular mass of each oligomer corresponds closely to the value calculated from the mass of the modified polypeptide (Table I). The sedimentation coefficient of each oligomer is also shown in Table I, together with the Stokes radius and the frictional ratio, calculated from the sedimentation coefficient, and the weight-averaged molecular mass. These values indicate that the oligomers of MBP-A are highly asymmetrical, consistent with their domain organization, and provide an explanation for the anomalous behavior observed on gel-filtration columns. The gel-filtration and analytical ultracentrifugation analysis indicate that MBP-A consists of abundant covalent oligomers with trace amounts of noncovalent oligomers of subunits. These oligomers show no self-association at concentrations in excess of those normally present in serum.

Disulfide-bonding Pattern—Comparison of the sequences of MBP-A and MBP-C reveals that each protein contains 2 cysteine residues at the N-terminal junction of the collagen-like domain (11). Previous studies indicate that protomers of MBP-C are linked by interchain disulfide bonds between these residues arranged in an asymmetrical configuration (14). MBP-A contains an additional cysteine residue at the N terminus of the protein. One possibility is that additional disulfide bonds formed through this extra cysteine residue link polypeptides in separate subunits to form the large covalent structures

gel-filtration chromatography indicates that in some cases the three polypeptides in the trimeric subunits are not covalently linked to each other. For example, small amounts of monomeric and dimeric MBP-A polypeptide elute at the same position as the covalent trimeric subunit, suggesting the presence of some noncovalent monomeric subunits (Fig. 2B). Similar heterogeneity has been observed in preparations of MBP-C, which consists of both covalent and noncovalent trimers of polypeptide chains (14).
were approximately 0.2 mg/ml (trimer of the trimeric unit (5,500 r.p.m.). The loading concentrations are indicated by an arrow. In each case, oligomers were purified by ion-exchange chromatography on a Mono-Q column. Top, dimer of the trimeric unit (7,000 r.p.m.). The loading concentrations were approximately 0.4 mg/ml (C), 0.2 mg/ml (D), and 0.1 mg/ml (E). Bottom, trimer of the trimeric unit (6,500 r.p.m.). The loading concentrations were approximately 0.2 mg/ml (C), 0.1 mg/ml (D), and 0.05 mg/ml (E).

![Equilibrium sedimentation ultracentrifugation of purified oligomers of MBP-A](image)

**FIG. 3.** Equilibrium sedimentation ultracentrifugation of purified oligomers of MBP-A. Apparent weight-averaged molecular mass distribution as a function of concentration for three loading concentrations are shown. The calculated masses of dimers, trimers, and hexamers of subunits, based on the amino acid sequence of MBP-A, are indicated by an arrow. In each case, oligomers were purified by ion-exchange chromatography on a Mono-Q column. Top, dimer of the trimeric unit (7,000 r.p.m.). The loading concentrations were approximately 0.4 mg/ml (C), 0.2 mg/ml (D), and 0.1 mg/ml (E). Bottom, trimer of the trimeric unit (6,500 r.p.m.). The loading concentrations were approximately 0.2 mg/ml (C), 0.1 mg/ml (D), and 0.05 mg/ml (E).

The weight-averaged molecular mass of each oligomer was determined by equilibrium ultracentrifugation

| Oligomer of subunits | Weight-averaged molecular mass | Frictional ratio | Stokes radius |
|----------------------|-------------------------------|-----------------|---------------|
| Monomer             | 17 ± 4                        | 3.7 ± 0.3       | 1.8 ± 0.3     | 51 ± 8        |
| Dimer               | 147 ± 6                       | 5.5 ± 0.2       | 1.9 ± 0.2     | 66 ± 5        |
| Trimer              | 225 ± 15                      | 6.3 ± 0.2       | 2.2 ± 0.3     | 87 ± 9        |
| Tetramer            | 282 ± 17                      | 7.7 ± 0.1       | 2.1 ± 0.2     | 90 ± 7        |

hydrodynamic properties of purified oligomers of MBP-A

The weight-averaged molecular mass of each oligomer was determined by equilibrium ultracentrifugation

Oligomer | Molecular mass (kDa) | Frictional ratio | Stokes radius |
---|---------------------|-----------------|---------------|
Monomer | 17 ± 4              | 3.7 ± 0.3       | 1.8 ± 0.3     |
Dimer   | 147 ± 6             | 5.5 ± 0.2       | 1.9 ± 0.2     |
Trimer  | 225 ± 15            | 6.3 ± 0.2       | 2.2 ± 0.3     |
Tetramer| 282 ± 17            | 7.7 ± 0.1       | 2.1 ± 0.2     |

results predominately in formation of a single subunit. These data are consistent with the suggestion that Cys⁶ forms disulfide bonds between separate subunits, thus creating the larger covalent forms observed in preparations of wild-type MBP-A.

The single subunit of MBP-A Cys⁶ → Ser was analyzed by analytical ultracentrifugation (data not shown). The apparent molecular mass was 75,500 ± 1,500 Da in two independent experiments. This value is very similar to the calculated value (approximately 74,200 Da) based on the mass of individual MBP-A polypeptides determined by MALDI-MS. No self-association could be detected over the entire concentration range examined (up to 2 mg/ml). These data therefore confirm that the most abundant form of MBP-A Cys⁶ → Ser is a single subunit. This result further supports the conclusion that disulfide bonds between Cys⁶ residues link polypeptides in separate subunits to form larger covalent structures in wild-type MBP-A.

The interchain disulfide bonding pattern in the MBP-A Cys⁶ → Ser trimeric subunit was determined by analysis of an N-terminal fragment generated by digestion with proteinase Arg-C. Peptides were separated by reverse-phase chromatography and identified by MALDI-MS and amino acid sequencing. Under nonreducing conditions, the mass of peptide Ser¹⁴-Arg²⁰ determined by MALDI-MS (6,168.0 Da) corresponds closely to the mass of a disulfide-linked trimer based on the amino acid sequence of MBP-A (6,165.8 Da). Disulfide-linked dimers and monomers of peptide Ser¹⁴-Arg²⁰ were also detected by MALDI-MS. However, the abundance of these species was relatively low. Mass values detected for the monomer and the covalent dimer and trimer were consistent with values calculated from the sequence of MBP-A, indicating that the 2 cysteine residues within these peptides (Cys¹³ and Cys¹⁸) are not modified by linkage to free cysteine or glutathione. Furthermore, the trimeric and dimeric peptides could be reduced to monomers on incubation with dithiothreitol, confirming that the polypeptide chains are linked by disulfide bonds. As in the case of MBP-C (14), two possible isomers can be considered for the covalent trimer, each containing three interchain disulfide bonds. These disulfide bonds can be arranged either in a symmetrical pattern, where each polypeptide is linked by Cys¹³-Cys¹⁸ or in an asymmetrical pattern. Cleavage of the trimeric peptide between Cys¹³ and Ser¹⁴ by subtilisin indicates that the cysteine residues must be linked by disulfide bonds arranged in an asymmetrical pattern. Peaks were detected by MALDI-MS corresponding to all three dimeric peptides: (Ser¹⁴-Cys¹³)₂ (Ser¹⁴-Cys¹³-Cys¹⁸)₂ (1406.6 Da) and Ser¹⁴-Cys¹³/Ser¹⁴-Arg²⁰ (2074.2 Da). A similar strategy was used to determine the disulfide-bonding pattern of the noncovalent trimer of MBP-A Cys⁶ → Ser. Peptides (Ser¹⁴-Cys¹³)₂ and (Ser¹⁴-Arg²⁰)₂ but not peptide Ser¹⁴-Cys¹³/Ser¹⁴-Arg²⁰ could be detected by MALDI-MS following digestion of the dimeric peptide with subtilisin. This result indicates that this peptide is linked by disulfide bonds Cys¹³/Cys¹⁸ and Cys¹³-Cys¹⁸ and suggests that the noncovalent trimer comprises disulfide-linked dimer associated with monomer containing an intrachain disulfide bond.

The disulfide-bonding pattern of the single subunit of MBP-A Cys⁶ → Ser mirrors the bonding pattern described previously for MBP-C (14). Thus, the majority of the polypeptides in individual trimeric subunits of MBP-A are probably linked by disulfide bonds between Cys¹³ and Cys¹⁸ arranged in an asymmetrical pattern. Disulfide bonds between Cys⁶ residues in separate subunits form the larger covalent structures observed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Each subunit contains 3 Cys⁶ residues each of which can potentially form disulfide bonds with polypeptides in separate trimers.
While the data are consistent with these conclusions for most of the oligomers, higher order oligomers are detected in preparations of MBP-A Cys6 → Ser, although with low abundance, indicating that there must be an alternative bonding arrangement in these oligomers. One possible explanation of this observation is that the larger covalent oligomers are formed as a result of the close proximity of N-terminal cysteine residues in noncovalently associated subunits during assembly of the protein in the endoplasmic reticulum. As an alternative proposal, the higher order structures observed in preparations of MBP-A Cys6 → Ser may indicate that a small proportion of oligomers of MBP-A are linked by a different disulfide bonding arrangement, reflecting further heterogeneity in MBP-A.

**Contribution of Multiple Domains in MBP-A to Higher Oligomer Formation**—Measurements of bouquet-like forms of human MBP obtained by electron microscopy are consistent with the central core region consisting of the cysteine-rich domain and the N-terminal part of the collagen-like domain of each protomer, up to the Gly-Gln-Gly interruption (12). Thus, this region is likely to contain residues responsible for assembly of trimeric subunits of serum MBPs into large oligomers. Since MBP-C is a single subunit, while MBP-A forms higher oligomers, analysis of chimeras of the two proteins enables identification of residues that determine the oligomeric state of the molecules.

Three sets of reciprocal chimeras of MBP-A and MBP-C were created (Fig. 5). In each case the site of recombination was chosen to be within a region of high sequence identity, in order to minimize disruption of the protein structure. Chimeras were constructed at the junctions of the collagen-like domain and at the Gly-Gln-Gly interruption of the collagen-consensus sequence. MBP-C contains an insertion for which there are no corresponding residues present in MBP-A, at the start of the collagen-like domain. For this reason, two sets of reciprocal chimeras were created at this site, in which this sequence is either included or omitted. Since MBP-A contains an additional cysteine residue (Cys6) within the cysteine-rich domain, one further construct was made consisting of MBP-C with the N-terminal 4 amino acid residues replaced by the first 6 residues of MBP-A. The resulting chimeric proteins were overproduced and purified as described for MBP-A.

The native structures of the purified chimeric proteins were examined by a combination of gel-filtration chromatography and SDS-polyacrylamide gel electrophoresis. Analysis by gel-filtration chromatography indicates that exchanging the C-terminal half of the collagen-like domain, the neck and CRDs of the MBPs has little effect on assembly of the chimeras (Fig. 6). However, both the cysteine-rich domain and the N-terminal part of the collagen-like domain of MBP-A are required for full assembly into higher order oligomers. The presence of only one of these regions from MBP-A (constructs 2AC, 2CA, 3AC, and 3CA) gives an intermediate phenotype, suggesting that residues in both domains contribute to assembly of MBP-A.

Gel-filtration peaks corresponding to low molecular weight species (elution volume ≥ 8.0 ml) are detected in preparations of MBP-C and chimera 4AC (Fig. 6). These peaks were found to consist of a proteolytic fragment of MBP-C by N-terminal sequencing, comprising the C terminus from residue Val54. This proteolytic fragment has been previously identified in preparations of MBP-C (14).

The conclusion that residues in the cysteine-rich domain and in the N-terminal part of the collagen-like domain of MBPs determine their oligomeric structure is supported by analysis of the chimeras by SDS-polyacrylamide gel electrophoresis under nonnondenaturing conditions (Fig. 7). The apparent molecular masses of the covalent oligomers observed for each chimera is dependent on the origin of both the cysteine-rich domain and the N-terminal part of the collagen-like domain. Thus, the source of these regions within each chimera determines the overall covalent structure. Again, chimeras containing one of these regions from each protein have an intermediate phenotype, indicating that residues from both domains contribute to the assembly of MBPs.

The N-terminal part of the collagen-like domain of MBP-C contains an insertion of 9 amino acid residues for which there are no corresponding residues present in MBP-A (Fig. 5). The presence of this insertion does not exclude the formation of higher order oligomers, since large covalent structures are detected in chimeras containing this region. However, construct 2CA in which the insertion is adjacent to the collagen-like domain of MBP-A comprises a higher proportion of noncovalent oligomers than constructs in which it is not present (Fig. 7). Thus, this region appears to modulate oligomer formation, while not preventing the assembly of higher order structures.

The collagen-like domain of MBP-A up to the Gly-Gln-Gly interruption appears to promote the formation of higher order oligomers, since chimeras containing this region (2CA and 3CA) comprise a higher proportion of the larger covalent structures than those containing the corresponding region of MBP-C (Figs. 6 and 7). This part of the collagen-like domain contains...
only three differences between the MBPs, in which Lys<sup>37</sup>, His<sup>39</sup>, and Ala<sup>33</sup> in MBP-C correspond to Arg<sup>20</sup>, Arg<sup>23</sup>, and Pro<sup>26</sup> in MBP-A, respectively (Fig. 5). Thus, one or more of these residues in MBP-A must contribute to the differences in the phenotypes of the MBPs conferred by the collagen-like domains and are determinants of the oligomeric state of the molecules.

The construct 4AC, containing the first 6 amino acid residues of MBP-A in place of the N-terminal 4 residues of MBP-C, consists mainly of a single trimeric subunit. Despite the additional cysteine residue (Cys<sup>58</sup>), only low levels of higher order structures are detected (Fig. 6). This finding suggests that the contribution of the N-terminal cysteine-rich domain of MBP-A toward the formation of large covalent oligomers is not due simply to the presence of an extra cysteine residue but that additional residues within the C-terminal half of this domain are involved.

*Complement Activation.*—In order to assess the complement fixing activity of the recombinant MBPs, complement activation was measured by hemolysis of erythrocytes coated with yeast mannan (Fig. 8). The complement fixing activity of recombinant MBP-A is consistent with that described previously for preparations of the native lectin, using a similar assay system (1). Interestingly, complement-dependent hemolysis was also detected in the presence of MBP-C, but only at concentrations 20 times higher than for MBP-A. The amount of MBP-C required to cause detectable hemolysis is greater than that tested in previous studies, since more protein was available due to production in the expression system. Thus, these results are consistent with previous studies of MBP-A and suggest that MBP-C can activate complement but with lower activity that its serum homolog.

Chimeras of MBP-A and MBP-C were tested for their ability to activate complement in order to identify regions of MBP-A responsible for its enhanced activity. Specific hemolysis as a function of concentration for samples of recombinant MBP-A, MBP-C, and a selection of chimeras is shown in Fig. 8. As with oligomerization, the complement fixing activities of the chimeras correlate with the presence of the cysteine-rich domain and the N-terminal part of the collagen-like domain of MBP-A. These data suggest that the higher oligomeric forms of MBP-A are more efficient at complement activation in this assay. These findings are consistent with previous studies of partially purified oligomers of native MBP isolated from mammalian sera (12, 21).

Since the contribution to the overall complement-dependent hemolytic activity of MBP-A is determined both by the relative abundance and the specific activity of each of the component oligomers, the hemolytic activities of these oligomers were determined individually. Based on SDS-polyacrylamide gel electrophoresis and gel-filtration analysis, the most abundant forms of MBP-A are dimers and trimers of subunits. Specific hemolysis is shown as a function of concentration for purified oligomers of MBP-A in Fig. 9. The complement fixing activities of the tetramer, trimer, and dimer of subunits relative to total MBP-A are 0.95 ± 0.05, 1.18 ± 0.23, and 0.24 ± 0.06, respectively. These data suggest that most of the hemolytic activity of MBP-A is due to the trimer of subunits. The tetramer and dimer of subunits also contribute to the complement fixing activity of MBP-A, although to a lesser extent due to lower abundance and lower activity of these oligomers, respectively. As noted above, MBP-C, which consists of a single subunit, also activates complement in this assay. Therefore, these findings suggest that each oligomeric form of MBP can interact with and activate downstream components of the complement cascade.

No complement-dependent hemolytic activity could be detected for the single subunit of MBP-A under the conditions tested. It is possible that the binding affinity of this oligomer for mannan-coated erythrocytes is insufficient to enable complement activation in the hemolytic assay. Binding studies show that the affinities of MBP-A and MBP-C for monosaccharide ligands are very similar. However, isolated CRDs of MBP-A have a much lower affinity than CRDs of MBP-C for multivalent ligands containing clusters of mannose moieties (29). The difference in the affinities of MBP-A and MBP-C for multivalent ligands is likely to be most apparent for smaller oligomers of MBP-A that contain fewer binding sites. This finding emphasizes that the hemolytic assay used to detect complement activation reflects a complex series of molecular interactions, including both ligand binding and binding to and activation of the downstream components of the complement cascade.

**DISCUSSION**

**Heterogeneity in MBPs**—The biochemical and biophysical data reported here indicate that MBP-A produced in Chinese hamster ovary cells faithfully resembles protein isolated from rat serum. MBP-A comprises a heterogeneous mixture of oligomers consisting predominantly of dimers and trimers of trimeric subunits, along with lower levels of tetramers and monomers. Equilibrium ultracentrifugation indicates that these oligomers do not interact even at high protein concentrations, suggesting that multiple forms of MBP-A exist in the serum. Detailed hydrodynamic analysis of serum MBPs has not been...
possible previously due to the relatively low amounts of protein isolated from mammalian sera. However, biophysical analysis suggests that human MBP can form larger structures than its rat counterpart, with oligomers ranging from dimers to octamers of subunits (13), and electron microscopy reveals mixtures of oligomers ranging from trimers to hexamers of subunits (12).

Many purification procedures described for MBPs have incorporated steps such as gel-filtration chromatography in order to separate serum MBP from liver MBP and from other contaminating proteins. A consequence of the heterogeneity of serum MBPs is that these procedures are likely to introduce a bias in the oligomeric composition, by selectively purifying certain oligomeric species. This problem may explain discrepancies in the reported compositions of MBPs in preparations isolated by different purification strategies.

### Assembly of MBP-A—Analysis of chimeras between MBP-A and MBP-C

Assembly of MBP-A indicates that assembly of MBP-A subunits to form larger oligomers is mediated by amino acid residues within the cysteine-rich domain and the N-terminal part of the collagen-like domain. Images of human MBP observed by rotary shadowing electron microscopy are consistent with this region forming a core which links the trimeric stems of separate subunits (12). The mechanisms by which collagen triple helices assemble into larger oligomers is unclear, although electrostatic interactions involving charged residues have been implicated, both in the association of collagen triple helices to form fibrils and in the binding of various molecules to collagen (30–32). The N-terminal region of the collagen-like domain of MBP-A is rich in both acidic and basic residues. This region also contains glu-
bonds between Cys 13 and Cys 18 link MBP-A polypeptides and the peptides link separate subunits. Thus, based on this model, modulation of oligomer formation through interactions involving the relatively large, derivatized side chains.

Mutations within the N-terminal region of the collagen-like domain are associated with MBP deficiency in humans (9, 10). This common genetic defect results in an increased susceptibility to infections particularly during the first few years of life. Protein isolated from patients with this disorder has an altered oligomeric structure consisting predominantly of lower molecular weight forms (13). The mutations known to cause MBP deficiency are all localized within the region of the protein identified as being critical for assembly of MBP-A. Thus, it seems likely that the altered oligomeric composition of MBP from patients with this disorder arises due to defective assembly of the protein.

The covalent structure of MBP-A, consisting of trimeric subunits linked by disulfide bonds, indicates that these subunits must associate at some stage during assembly of the protein. However, analytical ultracentrifugation experiments indicate that no self-association of the secreted oligomeric forms occurs at high protein concentrations. It is possible that the conformation of MBP-A oligomers changes during assembly, so that the secreted forms can no longer self-associate. MBPs are thought to assemble in a C-to-N-terminal direction in which one of the last steps involves formation of disulfide bonds that link the polypeptide chains (14). This step may prevent further self-assembly of subunits. Biochemical analysis indicates that there are no free cysteine residues within any of the oligomeric forms of MBP-A. This finding implies that at least some of the cysteine residues within the cysteine-rich domain must be derivatized, perhaps by linkage to cysteine or glutathione as has been observed for certain secreted proteins (33). For example, in the form of MBP-A consisting of a single subunit of three polypeptide chains, each polypeptide contains three N-terminal cysteine residues. At least one of these residues cannot form an intrachain or interchain disulfide bond and must be derivatized. Analysis of MBP-A Cys<sup>6</sup> $\rightarrow$ Ser suggests that disulfide bonds between Cys<sup>13</sup> and Cys<sup>18</sup> link MBP-A polypeptides within subunits, while bonds between Cys<sup>6</sup> of certain polypeptides link separate subunits. Thus, based on this model, modification is likely to occur to Cys<sup>6</sup> residues which do not form disulfide bonds. These modifications may prevent assembly of MBP-A subunits in the secreted protein.

Vertebrate collagens are known to interact with many different proteins within the endoplasmic reticulum during their assembly. For example, collagens have been shown to bind to protein disulfide isomerase, which is thought to mediate efficient folding by suppressing aggregation during collagen biosynthesis (34). Thus, it is conceivable that the interaction of MBP polypeptides with certain molecules during folding may be necessary for the correct assembly of MBP oligomers to form the larger structures secreted from the cell. Once the MBP structures have left the endoplasmic reticulum, these molecules will no longer be available to assist in assembly, therefore preventing further MBP oligomer formation.

**Complement Fixation by MBPs**—The role of serum MBPs in the innate immune system is well established (1–3). In contrast, the function of liver MBP is unknown. MBP-C is able to bind to certain mammalian glycoproteins, indicating that it may interact with endogenous glycoproteins within the liver (35, 29). However, the high degree of sequence identity with MBP-A implies that MBP-C may have a role in innate immunity. The results reported here indicate that MBP-C is able to activate complement in an *in vitro* assay, although with lower activity than its serum homolog. While the physiological relevance of this observation is unclear, it seems possible that within the liver MBP-C may have a role similar to that of MBP-A in serum. Since MBP-C is smaller than MBP-A, the enhanced affinity for complex sugar ligands observed for isolated CRDs of MBP-C may reflect an alternative mechanism for achieving high affinity binding to carbohydrate structures on the surfaces of microorganisms.

The formation of large oligomeric structures is critical for efficient complement fixation by MBP-A. However, each oligomeric form appears to be able to interact with downstream components of the complement cascade leading to some degree of complement fixation. Since oligomers do not interact to form higher order structures, the total complement fixation activity is a function of the individual molecular activities of oligomers and their relative abundance within the serum. The mutant MBPs associated with MBP deficiency in humans have an altered composition, consisting predominantly of lower molecular weight forms (13). The decreased activity associated with these structures may provide an explanation for the low complement fixing activity of serum isolated from patients with this disorder. Alternatively, mutations within the collagen-like domain of serum MBP may disrupt the binding site for MASP-1 or MASP-2, thus directly preventing activation of the complement cascade.

**REFERENCES**

1. Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T., and Yamashina, I. (1987) *J. Biol. Chem.* 262, 7451–7454
2. Super, M., Levinsky, R. J., and Turner, M. W. (1990) *Clin. Exp. Immunol.* 79, 144–150
3. Kuhlman, M., Joiner, K., and Ezekowitz, R. A. B. (1989) *J. Exp. Med.* 169, 1733–1745
4. Drickamer, K., and Taylor, M. E. (1993) *Annu. Rev. Cell Biol.* 9, 237–264
5. Matsuhashi, M., and Fujita, T. (1992) *J. Exp. Med.* 176, 1497–1502
6. Takahashi, A., Takayama, Y., Hatsuse, H., and Kawakami, M. (1993) *Biochem. Biophys. Res. Commun.* 196, 681–687
7. Thié, S., Verup-Jensen, T., Stover, C. M., Schwæble, W., Laursen, S. B., Poulsen, K., Willis, A. C., Eggertsen, P., Hansen, S., Holmskov, U., Reid, K. B. M., and Jensenius, J. C. (1997) *Nature* 386, 506–510
8. Ji, Y. H., Fujita, T., Hatsuse, H., Takahashi, A., Matsuhashi, M., and Kawakami, M. (1990) *J. Immunol.* 145, 571–578
9. Super, M., Thié, S., Lu, J., Levinsky, R. J., and Turner, M. W. (1989) *Lancet* ii, 1236–1239
10. Sumiya, M., Super, M., Tabona, P., Levinsky, R. J., Takayaki, A., Turner, M. W., and Summerfield, J. A. (1991) *Lancet* 337, 1569–1570
11. Drickamer, K., Dordal, M. S., and Reynolds, L. (1986) *J. Biol. Chem.* 261, 6878–6887
12. Lu, J., Thié, S., Wiedemann, H., Timpl, R., and Reid, K. B. M. (1990) *J. Immunol.* 144, 2287–2294
13. Lipscombe, R. J., Sumiya, M., Summerfield, J. A., and Turner, M. W. (1995) *Immunol. Rev.* 136, 65–74
Oligomerization and Complement Fixation by MBPs

18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Kaufman, R. J., Davies, M. V., Wasley, L. C., and Michnick, D. (1991) Nucleic Acids Res. 19, 4485–4490
21. Yokota, Y., Arui, T., and Kawasaki, T. (1995) J. Biochem. (Tokyo) 117, 414–419
22. Cohn, E. J., and Edsall, J. T. (1943) in Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, pp. 370–381, Reinhold, New York
23. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
24. Perucca, P. J., Faulk, W. P., and Fudenberg, H. H. (1969) J. Immunol. 102, 812–820
25. Kozutsumi, Y., Kawasaki, T., and Yamashina, I. (1980) Biochem. Biophys. Res. Commun. 95, 658–664
26. Kawasaki, N., Kawasaki, T., and Yamashina, I. (1983) J. Biochem. (Tokyo) 94, 937–947
27. Oka, S., Ikeda, K., Kawasaki, T., and Yamashina, I. (1988) Arch. Biochem. Biophys. 260, 257–266
28. Butler, W. T. (1982) Methods Enzymol. 82, 339–346
29. Quesenberry, M. S., Lee, R. T., and Lee, Y. C. (1997) Biochemistry 36, 2724–2732
30. Holmes, D. J. S., Miller, A., Parry, D. A. D., Piez, K. A., and Woodhead-Galloway, J. (1973) J. Mol. Biol. 79, 137–148
31. Li, S. T., Golub, E., and Katz, E. P. (1975) J. Mol. Biol. 96, 835–839
32. Wallace, D. G. (1990) Biopolymers 29, 1015–1026
33. Reddy, P., Sparvoli, S., Fagioli, C., Fassina, G., and Sitia, R. (1996) EMBO J. 15, 2077–2085
34. Kellokumpu, S., Suokas, M., Risteli, L., and Myllyla, R. (1997) J. Biol. Chem. 272, 2770–2777
35. Childs, R. A., Feizi, T., Yuen, C. T., Drickamer, K., and Quesenberry, M. S. (1990) J. Biol. Chem. 265, 20770–20777