Localizations of the Serine Protease-binding Sites in the
Collagen-like Domain of Mannose-binding Protein

INDIRECT EFFECTS OF NATURALLY OCCURRING MUTATIONS ON PROTEASE BINDING AND ACTIVATION*

Received for publication, January 8, 2004

Published, JBC Papers in Press, January 14, 2004, DOI 10.1074/jbc.M400171200

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Mutations in the collagen-like domain of serum man- nose-binding protein (MBP) interfere with the ability of the protein to initiate complement fixation through the
MBP-associated serine proteases (MASPs). The result- ing deficiency in the innate immune response leads to susceptibility to infections. Studies have been under- taken to define the region of MBP that interacts with
MASPs and to determine how the naturally occurring mutations affect this interaction. Truncated and modified
MBPs and synthetic peptides that represent seg- ments of the collagen-like domain of MBP have been
used to demonstrate that MASPs bind on the C-terminal
side of the hinge region formed by an interruption in the
Gly-X-Y repeat pattern of the collagen-like domain. The
binding sites for MASP-2 and for MASP-1 and -3 overlap
but are not identical. The two most common naturally
occurring mutations in MBP result in substitution of
acidic amino acids for glycine residues in Gly-X-Y tripl-
ets on the N-terminal side of the hinge. Circular dichro-
ism analysis and differential scanning calorimetry dem-
onstrate that the triple helical structure of the collagen-
like domain is largely intact in the mutant proteins, but
it is more easily unfolded than in wild-type MBP. Thus,
the effect of the mutations is to destabilize the collagen-
like domain, indirectly disrupting the binding sites for
MASPs. In addition, at least one of the mutations has a
further effect on the ability of MBP to activate MASPs.

Serum mannose-binding protein (MBP) interacts with carbohydrate surfaces of microorganisms and thus forms
the pathogen recognition component of the lectin pathway of
complement activation (1, 2). MBP, which is also referred to as
mannose-binding lectin, binds to surface arrays containing re-
peated mannose or N-acetylglucosamine residues. It circulates
as a complex with one or more MBP-associated serine proteases
(MASPs) that autoactivate when the complex binds to an ap-
propriate surface.

The surface recognition function of MBP is mediated by
clusters of three C-type carbohydrate-recognition domains
(CRDs) held together by coiled-coils of α-helices (Fig. 1A). The
N-terminal portion of the MBP polypeptide consists of a colla-
gen-like domain composed of Gly-X-Y triplets with a single
interruption that forms a bend in the domain (3). A short
N-terminal domain contains several cysteine residues that
form interchain disulfide bonds. Serum MBPs assemble into
larger forms containing 2–4 trimeric subunits in rodents and
as many as six subunits in humans. All three oligomeric forms
of rat serum MBP, designated MBP-A, can fix complement,
although the larger oligomers have higher specific activity.
Many species express a second form of MBP. In rats, the second
form, MBP-C, is found in the liver. MBP-C does not form higher
oligomers beyond the simple subunit that contains three
polypeptides. Analysis of chimeras between rat MBP-A and
MBP-C suggests that the collagen-like domains contain the
MASP-binding sites (4). Ficolins contain N-terminal collagen-
like domains and C-terminal fibrinogen-like domains that bind
carbohydrate and also activate complement through MASPs
(5). Ficolins are believed to play a role in innate immunity,
although their physiological ligands are unknown.

Three MASPs in humans, rats, and other mammals share a
common domain organization (6–8). Dimerization and binding
to MBP is mediated by an N-terminal segment consisting of
two CUB domains separated by an epidermal growth factor-
like (EGF) domain (9). MBP dimers form 1:1 complexes with
MASPs, whereas MBP trimers and tetramers bind up to two
MASP dimers (10). The crystal structure of the N-terminal
portion of MASP-2 reveals an elongated molecule in which
protomers interact in an antiparallel arrangement through
interactions involving the first CUB domain and the EGF do-
main (11). The remainder of each MASP polypeptide comprises
two complement-consensus repeat modules and a serine prote-
ase domain. MBP-MASP-2 complexes are sufficient to trigger
complement activation by cleaving C4 and C2 to form C3 con-
vertase, which leads to release of the anaphylatoxin C3a and
deposition of C3b on the cell surface (10, 12). C3b targets the
cell for phagocytosis or lysis by the later complement compo-
nents. MASP-1 and MASP-3 are alternatively spliced products
from a common gene (6). They are identical apart from the
protease domain and a short segment that links it to the
complement consensus repeat-2 module. MASP-1 may form

* This work was supported in part by Wellcome Trust Grant 041384.
CD spectra were obtained in the Oxford Centre for Molecular Sciences,
which is supported by the Biotechnology and Biological Sciences Re-
search Council, and the microcalorimetry facility is supported by the
Wellcome Trust. The costs of publication of this article were defrayed in
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1734 solely to indicate this fact.
‡ Supported by a studentship from the Biotechnology and Biological
Sciences Research Council.
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The abbreviations used are: MBP, mannose-binding protein; MASP,
MBP-associated serine protease; CRD, carbohydrate-recognition do-
main; CUB domain, domain found in complement subcomponents C1r/
C1s, Uegf, and bone morphogenetic protein-1; EGF, epidermal growth
factor; Hyp, hydroxyproline.
Collagen-like Domain of Mannose-binding Protein

part of the complement cascade by cleaving components C2 and C3 upon activation (13). The function of MASP-3 is not known. Genes encoding three different variant forms of human MBP are found at frequencies of up to 30% in some human populations (14). Individuals who are either heterozygous or homozygous for these variant alleles are immunocompromised. The immunodeficiency associated with MBP is particularly evident in children between the ages of 6 months and 2 years, because these individuals are susceptible to recurrent, severe infections. The Arg392 → Cys substitution results in almost complete failure to form higher oligomers. Inefficient complement fixation follows from the inability of single MBP subunits to bind and activate MASPs (3). Proteins containing the Gly34 → Asp and Gly37 → Glu mutations form higher oligomers that fail to fix complement effectively. In previous studies, substitutions equivalent to the human mutations have been introduced into rat MBP-A, and truncated forms of MBPs have been shown to bind less efficiently to these variant proteins (9, 15).

In the work reported here, recombinant fragments of MBP and synthetic peptides have been used to localize the binding site for MASPs within the C-terminal portion of the collagen-like domain of MBP-A. The binding motif is found in other MBPs as well as in ficolins, suggesting a general binding mechanism for the activating components of the lectin pathway of complement activation. Substitution of glycine residues on the N-terminal side of the hinge with aspartate or glutamate destabilizes the collagen-like domain and thus indirectly decreases the binding of MASPs. However, these studies and binding experiments with full-length MASPs indicate that at least the Gly37 → Glu form of MBP is specifically defective in the ability to activate MASPs.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression Systems—Wild-type and mutant rat MBP-A as well as fragments of MASP-1 and MASP-2, comprising the N-terminal CUB and EGF domains with a C-terminal histidine tag, and catalytically inactive rat MASPs were expressed in Chinese hamster ovary cells as previously described (4, 9, 10, 15). The cDNA for MBP-C was modified to insert a factor Xa site by replacement of restriction sites for MASP-1/-3 were incubated with increasing concentrations of competing ligands in the presence of [35S]MBP-A reporter ligand. Levels of complement (4). Lysis was measured by following release of hemoglobin monitored by absorbance at 541 nm. Solid-phase competition assays were performed essentially as described (9), in which polystyrene plates, coated with the N-terminal three-domain fragment of MASP-2 or MASP-1/3 were incubated with increasing concentrations of competing ligands in the presence of [35S]MBP-A reporter ligand. Levels of radioactivity were determined using a PhosphorImager SI (Amersham Biosciences). Data were fitted to multiple ligand binding curves by nonlinear regression using Microcal Origin. The amounts of [35S]MBP-A were below the concentrations required for half-maximal binding in all binding assays. All data represent the mean ± S.E. from at least two separate experiments.

Circular Dichroism—Circular dichroism was measured on a Jasco J600 instrument in a 200-μl quartz cell with 1-mm path length. The temperature was allowed to equilibrate for 10 min between spectra. Spectra were averages of 10 scans obtained at 20 nm/minute with a data collection rate of one point every 0.5 nm, a bandwidth of 1 nm, and a time constant of 2 s.

Differential Scanning Calorimetry—Samples were dialyzed extensively against 0.15 M NaCl, 25 mM Na-HEPES, pH 7.8, 1 mM CaCl2. Aliquots (1 ml) of sample and dialysis buffer were degassed for 15 min prior to loading the sample and reference cells of a Calorimetry Systems Nano III calorimeter. All scans were preceded by 10-min equilibration at the starting temperature. In order to ensure the absence of gas in the samples, initial scans from 5 to 20 °C were repeated until a reproducible baseline was achieved. Scans were then performed from 5 to 90 °C. Base lines were calculated using a fourth order polynomial equation to fit the data between 10 and 20 °C and between 80 and 90 °C as well as points at the minimum near 55 °C.

Analytical Procedures—Polyacrylamide gel electrophoresis was performed by the method of Laemmli (19). Gels were stained with Coomassie blue. In the case of the collagen-like tail, destaining was performed against 50 mM Tris-Cl, pH 8.2, and between 80 and 90 °C as well as points at the minimum near 55 °C.

RESULTS

Interaction of MASPs with Truncated MBP-A—In a first approach to defining the region of MBP-A that binds to the MASPs, the interaction of MASP-2 with a series of C-terminal fragments of MBP-A was assessed. Truncated forms of MBP-A lacking the short N-terminal domain, the collagen-like domain up to the interruption, or the entire collagen-like domain were produced (Fig. 1A). For comparison, MBP-A Cys6 → Ser, which cannot form intertrimer disulfide bonds, was used as a source of full-length trimers that do not associate into higher oligomers (4). Proteins that contain part or all of the collagen-like domain were produced in a mammalian expression system to ensure proper modification of proline and lysine residues. The shortest fragment, comprising the neck and CRDs alone, does not contain any of these modifications and was produced in Escherichia coli. All of the expressed proteins were purified by affinity chromatography on mannose-Sepharose and characterized by SDS-polyacrylamide gel electrophoresis and equilibrium ultracentrifugation. Each fragment forms stable homotrimers (data not shown).

Separation of Oligomeric Forms of MBP-A—Conditions for separation of oligomers were similar to those used previously for the wild-type protein (4). Wild-type and mutant MBP-A preparations were dialyzed against 50 mM Tris-Cl, pH 8.2, containing 10 mM EDTA and separated at 25 °C on a Mono-Q HR-5/5 column (Amersham Biosciences) with a gradient of NaCl in this buffer, running from 0 to 0.075 M NaCl in 2 min and from 0.075 to 0.3 M NaCl over 45 min. Gel filtration chromatography was performed in a buffer containing 50 mM Tris-Cl, pH 8.2, on a BioSep S3000 column (300 × 7.8 mm) from Phenomenex (Cheshire, UK) at a flow rate of 0.5 ml/min. Elution positions of different oligomeric forms were deduced from the behavior of the wild-type protein on this column (4).

Peptides—Peptides produced by Bachem UK Ltd. (Merseyside, UK) were adjudged to be at least 95% pure by reverse-phase high performance liquid chromatography, and their masses were verified by mass spectrometry.

COMPLEMENT FIXATION AND BINDING ASSAYS—Complement-fixing activities of wild-type and mutant MBPs were measured using a hemolytic assay in which mannan-coated sheep erythrocytes, preincubated with MBP, were incubated with guinea pig serum as the source of complement (4). Lysis was measured by following release of hemoglobin monitored by absorbance at 541 nm. Solid-phase competition assays were performed essentially as described (9), in which polystyrene plates, coated with the N-terminal three-domain fragment of MASP-2 or MASP-1/3 were incubated with increasing concentrations of competing ligands in the presence of [35S]MBP-A reporter ligand. Levels of radioactivity were determined using a PhosphorImager SI (Amersham Biosciences). Data were fitted to multiple ligand binding curves by nonlinear regression using Microcal Origin. The amounts of [35S]MBP-A were below the concentrations required for half-maximal binding in all binding assays. All data represent the mean ± S.E. from at least two separate experiments.

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Formation of MBP-A-MASP-2 complexes was quantified in solid-phase competition assays, in which MBP-A fragments compete with radiolabeled wild-type MBP-A for binding to the N-terminal portion of MASP-2 (Fig. 2). As expected from previous studies, the affinity of full-length, monomeric MBP-A for MASP-2 is substantially reduced compared with the native proteins that consist largely of higher oligomers. However, removal of the N-terminal domain and the N-terminal portion of the collagen-like domain did not further decrease the affinity of the proteins for MASP-2 (Table I). The smallest fragment, comprising the neck and CRDs alone, did not interact with MASP-2. Taken together, these results indicate that MASP-2 must bind to the C-terminal portion of the collagen-like domain, within the 12 Gly-X-Y triplets that lie between the hinge and the neck.

The same strategy was used to investigate the interactions of MBP-A with the N-terminal fragment that is common to MASP-1 and MASP-3. Comparable results were obtained in binding assays using truncated MBP-As (Fig. 2 and Table I), indicating that MASP-1 and 3 also interact with the C-terminal portion of the collagen-like domain of MBP-A. Taken together, these results indicate that MASP-2 must bind to the C-terminal portion of the collagen-like domain, within the 12 Gly-X-Y triplets that lie between the hinge and the neck.

The sequences of multiple MBPs were compared in order to identify a potentially conserved MASP-binding site within the collagen-like region. The first five Gly-X-Y triplets on the C-terminal side of the hinge (triplets 6–10) are largely conserved in mammalian MBPs, whereas triplets further toward the C terminus are more variable (Fig. 1B) (21–25). In addition, the regions N-terminal to the hinge and toward the C-terminal end of the collagen-like domain both contain multiple potential sites of glycosylation on hydroxylysine residues. Glycosylation of these sites has been experimentally documented in human MBP and rat MBP-A (4, 26). The bulk of the multiple attached glycans might prevent association of proteins with this portion of the collagen-like domain. Consequently, the conserved region immediately to the C-terminal side of the hinge represents a potentially accessible, conserved target for MASP binding.

Synthetic peptides covering portions of the collagen-like domain were used to map the MASP binding site in greater detail. Overlapping peptides were synthesized across triplets 1–12 (Fig. 1A). To enhance formation of triple helices, the MBP sequences were flanked by two Gly-Pro-Hyp triplets at each end (27). Two of the four peptides tested bind to MASP-2 (Fig. 3). Peptide P3 binds with an affinity only 22-fold lower than full-length MBP-A subunits, indicating that the MASP-2 binding site must be located mainly within the region covered by this peptide, which represents triplets 6–10 of the collagen-like domain. Triplets 6 and 7 are also present in peptide P2, which shows no binding to MASP-2, but peptide P4, which contains triplets 9 and 10, does bind weakly. Thus, the binding site...
within triplets 6–10 appears to involve triplets 9 and 10. MASPs probably only bind to triple helical collagen, so it was important to determine whether the synthetic peptides form stable trimers. Equilibrium analytical ultracentrifugation revealed that peptide P3 is largely monomeric at 4°C, with only a small proportion of trimers (data not shown). Because only a fraction of peptides are in the correct conformation for binding to the MASPs, the measured binding affinity is likely to be lower than if they were all trimeric. Thus, the peptide-binding assays probably underestimate the affinities of MASPs for binding segments of MBP-A.

The roles of individual Gly-X-Y triplets were tested using additional synthetic peptides lacking potentially important residues in triplets 6–10. The strategy was to replace triplets in peptide P3 with the sequence Gly-Pro-Hyp, because these substitutions stabilize the collagen triple helix. Thus, decreased affinity resulting from these replacements reflects a change in the interaction with MASPs and is not an indirect effect of decreased stability of the collagen helix. Triplets 8 and 10 already have the sequence Gly-Pro-Hyp, so three additional peptides were synthesized, in which triplet 6, 7, or 9 was modified. Changing triplet 9 completely abolishes binding, indicating that Lys46 and/or Leu47 make key contacts with MASPs (Fig. 3 and Table II). Mutations in triplet 6 decreased the binding affinity by only 2-fold, whereas mutations in triplet 7 had no effect on binding. These results are consistent with the results comparing peptides P2 and P4, which indicated that the primary binding site probably lies in the C-terminal portion of peptide P3. However, although the effect of changing triplet 6 is small, the result suggests that the binding site might extend back to the hinge.

Comparable binding analysis with the N-terminal portion of MASPs using [35S]MBP-A or MBP-A fragments were incubated with the immobilized N-terminal fragment of MASP-2 (A) or MASP-1/3 (B) using [35S]MBP-A as the reporter ligand. Competing ligands were wild-type MBP-A, MBP-A in which residue Cys6 is changed to serine, truncation 1, truncation 2, and truncation 3.

Abolishes binding, but in this case a similar loss of binding is also observed when triplet 7 is changed. Surprisingly, changing triplet 6 to Gly-Pro-Hyp actually strengthens the interaction with the MASP-1/3 fragment. This effect is probably due to the increased stability of the triple helical form of this peptide due to the presence of the additional helix-stabilizing residues. This finding serves to emphasize that loss of binding activity resulting from changing the other triplets is probably partially compensated by increased stability of the triple helix, so the intrinsic effects of these changes are likely to be greater than what is measured in the assay. In any case, the results clearly indicate that Lys46 and Leu47 form part of the binding sites for all three MASPs, and Leu40 and Gln41 probably provide important additional contacts for MASP-1 and -3.

**Complement-fixing Activity by MBP-A Containing Mutations within the MASP-binding Site**—In order to confirm the peptide binding results, it was of interest to assess the effect of disrupting the MASP-binding sites on complement-fixing activity of intact MBP-A. Recombinant MBP-A containing the changes Lys46 → Pro and Leu47 → Hyp was produced and purified in parallel with wild-type MBP-A, and binding to the MASPs was tested in the solid phase competition assay. No binding of the mutant protein to the N-terminal fragment of either MASP-2 or MASP-1/3 was detected, demonstrating that the affinity in each case is reduced by more than 100-fold (Fig. 4). Complement-fixing activity was tested using MBP-dependent hemolysis of mannan-coated sheep erythrocytes. No activity was detected at the highest concentration of mutant MBP-A tested, which was more than 500-fold greater than levels of wild-type MBP-A needed to cause 50% lysis of the target cells (Fig. 5). Because the modified MBP-A still binds to mannos- Sepharose in a Ca2+-dependent manner, loss of activity is not due to a failure to bind to terminal-mannose moieties on the sheep erythrocytes. Furthermore, analysis by gel filtration and SDS-polyacrylamide gel electrophoresis showed that the mutant protein resembles wild-type MBP-A both in terms of its oligomeric and its covalent structure (data not shown). A small increase in the proportion of single MBP-A subunits in the mutant MBP-A is not sufficient to account for the decrease in activity observed. Thus, mutation of Lys46 and Leu47 within the collagen-like domain of MBP-A abolishes all detectable complement-fixing activity as a consequence of disruption of the interactions with MASPs.

**Effect of Naturally Occurring Mutations on the Binding of MASPs**—Mutant forms of rat MBP-A have been created to mimic each of the naturally occurring human mutations (15). In previous studies, the ability of these MBPs to interact with N-terminal fragments of MASP-1 and -2 has been investigated (9). Although the mutations lie outside the proposed MASP-binding site, they decrease the affinity of MBP-A for MASPs. Full-length MASPs that are catalytically inactive have recently been created by replacement of the active site serine residue with an alanine residue (10). The availability of these proteins makes it possible to examine the interaction of wild-type and mutant forms of MBP-A with the full-length MASPs to confirm the effects of the mutations. A competition binding assay, in which binding of soluble MBP-A to immobilized MASPs is inhibited by unlabeled wild-type and mutant MBP-A, was utilized to quantify MASP interactions with MBP-A (Fig. 6). Measured in this way, the affinity of each mutant MBP-A for each MASP is reduced compared with wild type, although the effect on affinity is greater in the case of MASP-1.

In order to confirm that the reduced binding of the Arg23 → Cys mutation can be accounted for by the decreased affinity of monomeric MBP-A for the MASPs, the binding competition experiment was repeated using wild-type MBP-A that had been...
The mutations fall outside the portion of the collagen-like domain of this form of MBP-A to form the higher oligomers. Thus, using the full-length proteins, it is possible to explain how, for example, the triple helix of MBP-C can form either covalent trimers or covalent dimers that associate with monomers to form trimers (17). Therefore, in order to have a homogeneous preparation, it was necessary to isolate the covalent trimers by reverse phase chromatography. At low temperature, the circular dichroism spectrum of the isolated domain reveals the characteristic features of a collagen triple helix (Fig. 8A). The mean residue ellipticity at 222 nm is comparable with values obtained for collagen-like peptides (31), suggesting that the isolated fragment is largely triple helical. A thermal melting curve based on changes in ellipticity at 222 nm was used to establish a midpoint for denaturation of the helix of 22 °C (Fig. 8B). The fact that this temperature is well below physiological indicates that the isolated domain is probably substantially less stable than it must be in the intact molecule.

Similar fragments were produced containing the Gly25 → Asp and Gly28 → Glu mutations, but it proved difficult to determine melting temperatures, because even at 1 °C the ellipticity did not reach a value consistent with a completely helical structure (data not shown). Thus, the experiments provided qualitative evidence that the mutations destabilize the helix, but they do not provide a means of quantifying the degree of destabilization.

**Quantitative Comparison of Wild-type and Mutant MBP-As by Differential Scanning Calorimetry**—Differential scanning calorimetry of intact MBP was used as an alternative approach to determining the stability of the collagen-like domain in the context of the full molecule. These experiments were conducted

| Protein          | MASP-2 | MASP-1/3 | Relative $K_I^*$ |
|------------------|--------|----------|-----------------|
| MBP              | 0.056 ± 0.010 | 0.042 ± 0.006 | 34.4 ± 10.8 |
| MBP Cys → Ser    | 1.82 ± 0.26  | 0.93 ± 0.05  | 1.0  |
| Truncation 1     | 2.87 ± 0.11  | 0.12 + 0.24  | <0.2 |
| Truncation 2     | 3.13 ± 0.31  | 2.97 ± 0.60  | 0.79 ± 0.20 |
| Truncation 3     | >8          | >3         | 0.33 ± 0.08 |

$^*$ Expressed as $K_{I,MBP-Cys → Ser}/K_{I,fragment}$. 

**Fig. 3.** Binding of peptides derived from the collagen-like domain of MBP-A to immobilized MASP-2 fragment. Radiolabeled MBP-A was the reporter ligand. A, competing ligands were peptide 1 (▲), peptide 2 (●), peptide 3 (■), and peptide 4 (▲). B, competing ligands were peptide 3 (■), peptide 3Δ6 (▲), peptide 3Δ7 (●), and peptide 3Δ9 (▲).
with rat MBP-A so that comparison could be made directly with the mutant forms that have been extensively characterized (15). Both the wild-type and mutant proteins exist in multiple oligomeric forms. The wild-type protein consists predominantly of dimers and trimers with some tetramers, whereas the oligomer distribution of mutant proteins is shifted more toward monomers and dimers. In order to compare similar oligomeric forms of the wild-type and mutant proteins, dimers of each were isolated by ion exchange chromatography. Separation of the wild-type oligomers has been previously described (4), and a similar protocol was found to separate the mutant oligomers as well (data not shown).

![Figure 4](https://example.com/figure4.png)  
**Fig. 4.** Solid-phase competition assay of MBP-A binding to MASPs. Competing ligands were wild-type MBP-A (■) and MBP-A Lys46 → Pro, Leu47 → Hyp (●).

![Figure 5](https://example.com/figure5.png)  
**Fig. 5.** Complement activation by MBPs. Complement-fixing activity was measured by hemolysis of mannan-coated sheep erythrocytes in the presence of wild-type MBP-A (●) and MBP-A Lys46 → Pro, Leu47 → Hyp (●).

### Table II

**MASP-binding properties of collagen-like peptides**

Inhibition constants ($K_i$) correspond to the concentration of each MBP fragment giving 50% inhibition of $[^{35}S]$MBP binding to the N-terminal fragment of MASP.

| Peptide | $K_i$ (μM) MASP-2 | $K_i$ (μM) MASP-1/3 | Relative $K_i^{rel}$ MASP-2 | Relative $K_i^{rel}$ MASP-1/3 |
|---------|-------------------|---------------------|-----------------------------|-----------------------------|
| Peptide 1 | >700              | >850                | <0.0003                     | <0.0001                     |
| Peptide 2 | >700              | >800                | <0.0003                     | <0.0001                     |
| Peptide 3 | 56 ± 26           | 610 ± 26            | 0.045 ± 0.024               | 0.0017 ± 0.0001             |
| Peptide 3Δ6 | 105 ± 2           | 153 ± 9             | 0.017 ± 0.003               | 0.0065 ± 0.0004             |
| Peptide 3Δ7 | 42 ± 6            | >850                | 0.045 ± 0.013               | <0.0001                     |
| Peptide 3Δ9 | >700              | >850                | <0.0003                     | <0.0001                     |
| Peptide 4 | 500 ± 50          | >850                | 0.004 ± 0.001               | <0.0001                     |

*Expressed as $K_{L,MBP,Cys6→Ser}/K_i$ fragment.*

Initial experiments indicated that wild-type MBP-A denatures in two steps, suggesting that transition temperatures for the collagen-like domain and the carbohydrate-recognition domain can be resolved (Fig. 9A). It was hypothesized that the transition at 47 °C corresponds to unfolding of the collagen-like domain and the transition at 65 °C results from denaturation of the CRDs. This possibility was supported by further experiments. First, the denaturation temperature for a C-terminal fragment of MBP-A corresponding to the α-helical neck and the CRDs was determined. The denaturation profile matches the second transition temperature for the intact molecule (Fig. 9B).
In both cases, the peak is not a simple transition but appears to be two closely spaced events, and it is not reversible. Such behavior typically represents unfolding followed by precipitation due to insolubility of the denatured protein. Thus, this transition cannot be analyzed as a simple two-state event, and it is not possible to deduce thermodynamic parameters from the data.

In a further set of experiments, the sample was cooled after the first transition but before the second transition, and its stability was assessed by additional cycles of heating and cooling. Under these conditions, the lower temperature transition is at least partially reversible (Fig. 10). In the second and third heating scans, this first transition occurs at a slightly reduced temperature, indicating that the cooled protein has not achieved a fully native conformation. However, the fact that the area under the curve remains about the same suggests that much of the structure has been reformed. Previous studies have documented the ability of molecules containing collagen triple helices as well as collagen-like peptide to reform triple helices (32, 33). Because this transition is largely reversible, an estimate of the enthalpy change of the transition was made based on the area under the curve. The resulting value of 150 kJ/mol for each Gly-Asp and Gly-Glu mutants in the context of the intact protein. Differential scanning calorimetry of the variant forms revealed that the first transition occurs at 39 °C, roughly 8 °C lower than the transition for the wild-type protein (Fig. 11). Experiments with the monomeric forms of the mutants yielded nearly identical thermal denaturation profiles, demonstrating that the transition temperatures are not affected by the degree of oligomerization (data not shown). The relative peak areas of the first transition compared with the second transition are essentially the same for the wild-type and mutant proteins, indicating that they probably contain similar amounts of triple helix at low temperature. This finding rules out one possible effect of these mutations, which would be to prevent helix formation from the site of the mutation on toward the N terminus. Nevertheless, the reduced transition temperatures indicate that the collagen-like domains of both the GlyAsp and the Gly-Glu mutants are significantly less stable than the wild-type domain, and the degree of destabilization is nearly the same for the two mutant forms.

**DISCUSSION**

The data reported here show that MASP-2 binds to the collagen-like domain of MBP-A on the C-terminal side of the interruption in the string of Gly-X-Y triplets. These results are consistent with a recent model of the structural organization of a complex of MBP-A with MASP-2 (11). In this model, a MASP-2 dimer bridges two MBP-A subunits by interacting with the collagen-like domains of MBP-A. Because of the antiparallel arrangement of the MASP dimer, each protomer makes equivalent contacts with a separate MBP-A subunit. The crystal structure of the N-terminal fragment of MASP-2 places the proposed binding sites for MBP-A subunits about 30 Å apart. Assuming a regular triple helical structure, in which each Gly-X-Y triplet is 8.65 Å long (36), two trimeric stalks would need to be separated by -45° at the hinge in order to accommodate the MASP-2 dimer between binding sites on the collagen helices at triplet 9. Such an angle is consistent with images of human MBP determined using rotary-shadowing electron microscopy (37). Interestingly, this angle is also consistent with images of C1q, the first component of the classical complement pathway, which might bind to components C1r and C1s by a comparable mechanism (38).

Sequence comparison shows that the proposed binding sites for MASPs are almost completely conserved in mammalian MBPs (Fig. 1B). Alignment of the collagen-like domains of ficolins with the MBPs reveals that most of the residues that form the core of the MASP-binding sites are also present in ficolins. Thus, MBPs and ficolins probably bind to MASPs by a common mechanism. The motif Hyp-Gly-Lys-X-Gly-Pro, where X is a hydrophobic residue, also occurs in the B chain of C1q, whereas the related sequences Hyp-Gly-Lys-Asn-Gly-Pro and Hyp-Gly-Lys-Val-Gly-Tyr are present in the A and C chains, respectively. These sequences occur 6, 7, and 8 triplets to the C-terminal side of the hinge in C1q and may form binding sites for C1r and C1s that are analogous to the MASP-binding sites in MBPs. The proposal that binding sites for C1r/C1s and MASPs are similar although not identical would explain the observation that C1r/C1s can bind to MBP and activate complement in vitro but not in vivo (37).

The circular dichroism and calorimetry studies indicate that the collagen-like domain in MBP-A has physical properties similar to the triple helical portions of true collagens. The transition temperature of 47 °C is in the range measured for other triple helix-containing molecules and is lower than that of most globular proteins. Comparison of the circular dichroism results with the calorimetry data indicates that the collagen-like domain is stabilized by the adjacent coiled-coil of α helices at the C-terminal end. The reversibility of unfolding of the collagen-like domain in the presence of a folded C-terminal domain corresponds to proposed biosynthetic mechanism in which trimerization is initiated at the C-terminal end of the...
polypeptide (3). Such initiation of trimerization through coiled-coil domains has been proposed to be a common feature of triple helix-containing molecules (39). Poor reversibility of the folding of modified MBP-C in which the junction between the triple helix and the coiled-coil has been altered to introduce a factor Xa site provides further evidence that triple helix formation is strongly influenced by the adjacent sequence.

A key finding in these experiments is that the collagen-like domains of the Gly\textsuperscript{25}Asp and Gly\textsuperscript{28}Glu mutants of MBP-A are largely triple helical, although they are less stable than the wild-type domain. This destabilization probably results from distortions caused by the larger side chains that do not pack into the center of the collagen helix. The finding that the collagen-like domain remains triple helical is consistent with the finding that these mutant proteins assemble into oligomers nearly as efficiently as the wild-type protein (15).

The binding studies with full-length MASP’s suggest that the affinities of the Gly\textsuperscript{28}Glu and Gly\textsuperscript{55}Asp mutants of MBP-A for MASP-1 and -2 are reduced by both mutations. The transition temperatures for these mutant proteins lie just above 37 °C, suggesting that under physiological conditions the equilibrium between helical and unfolded conformations of the collagen-like domain may only slightly favor helix formation, whereas the much higher transition temperature for the wild-type protein would ensure that the protein remains in the helical conformation more of the time. Because MASPs pre-

**Fig. 8.** Determination of collagen-like domain stability by circular dichroism analysis. A, circular dichroism spectra obtained at various temperatures for a 100-μg sample of collagen-like domain dissolved in 200 μl of 25 mM Tris (pH 7.8) containing 5 mM CaCl₂. B, comparison of the ellipticity at 222 nm (average of values from 221 to 223 nm) at various temperatures. The fitted line reveals that the midpoint of the melting curve is 22 °C. The contribution to the absolute ellipticity from the non-collagen-like N-terminal domain is unknown, but the ellipticity of the triple helical region was estimated from the difference between the values at 35 and 1 °C.

**Fig. 9.** Differential scanning calorimetry of intact MBP-A and the N-terminal neck plus CRD fragment.

**Fig. 10.** Reversibility of collagen-like domain denaturation measured by differential scanning calorimetry. The first two scans were stopped at 55 °C, and the sample was cooled to 5 °C before being reheated.
Gly\textsuperscript{28} to the hinge may affect the ability of the collagen-like domain to alter its conformation at the bend formed by the interruption in response to binding of the C-terminal carbohydrate-recognition domains to pathogen surfaces. A change in conformation at this site is proposed to be an essential part of the mechanism by which the two catalytic domains in a MASP dimer are brought together so that they can activate each other (11). The affinities of the mutant proteins for MASP-2 are not reduced as much as the affinities for MASP-1. This result, along with the peptide binding data, indicates that there are subtle differences in the geometry of MASP-2 and MASP-1/3 binding to MBP-A, so that these interactions are differentially sensitive to the effects of the Gly\textsuperscript{28} → Glu mutation on the geometry at the Gly-Gln-Gly bend site.

**Acknowledgment**—We thank Maureen Taylor for helpful comments and critical reading of the manuscript.

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