Truncated Forms of Mannose-binding Protein Multimerize and Bind to Mannose-rich Salmonella montevideo but Fail to Activate Complement in Vitro*

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Human serum (MBP) and human recombinant (rMBP) mannose-binding protein bind to mannose-rich, serum-resistant Salmonella montevideo (SH5770), enhance C3 deposition, and render the organisms serum-sensitive. We investigated structural features of MBP necessary for this effect. MBP has a cysteine-rich amino-terminal region, a collagen-like region, and a carboxyl-terminal carbohydrate-recognition domain. We prepared carbohydrate-recognition domains lacking the other two domains either by deletion mutagenesis (ΔMBP, 16 kDa) or by collagenase digestion of whole rMBP (edMBP, 16–18 kDa). Whole and truncated MBP were detected on immunoblot by specific monoclonal antibodies that recognize both bound and free MBP. rMBP enhanced C3 deposition on SH5770 8-fold, while edMBP and ΔMBP did not increase C3 deposition over control levels. All forms of MBP bound to SH5770 by enzyme-linked immunosorbent assay and by measuring binding of radiolabeled whole and truncated MBP. Binding by 125I-ΔMBP was inhibited by mannose and by MBP. Thus failure of truncated MBP to enhance C3 deposition was not due to failure to specifically bind carbohydrate residues.

To determine the molecular form of truncated MBP in nondenaturing conditions, 125I-ΔMBP was centrifuged through a 5–20% sucrose-density gradient. The peak of 125I-ΔMBP sedimented to estimated S_{av} 2.01, but larger multimers also were present. Multimers bound SH5770 with higher affinity than monomers.

We conclude that carbohydrate-recognition regions of MBP produced by collagenase digestion or by deletion mutagenesis are sufficient for ligand binding. However, the collagen-like region is necessary for MBP enhancement of C3 deposition on SH5770.

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Materials and Methods

Bacteria—S. montevideo SH5770 (SH5770) was used in all the experiments for several reasons. 1) The lipopolysaccharide is man---

The abbreviations used are: MBP, human mannose-binding protein; rMBP, recombinant human MBP; ΔMBP, cloned truncated MBP; edMBP, truncated collagenase-digested MBP; SH5770, S. montevideo serotype SH5770; PBS, HBS, Hanks’ balanced salt solution; ADS, normal human serum adsorbed with SH5770; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.
MBP Activation of Complement

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**Fig. 1. Mannose-binding protein constructs.** [1], MBP cDNA was stably transfected into SP2 cells, and the recombinant protein harvested from the supernatant had very similar physical chemical properties to serum MBP detailed in Ref. 14. [2], a truncated MBP that encodes for the stalk and carbohydrate recognition domain (CRD) was prepared as described under "Materials and Methods" and stably transfected into SP2 cells. The resultant protein is characterized as shown in Figs. 2 and 6. [3], truncated MBP was derived by collagenase digestion (19) as shown in Fig. 2. Igh SP, immunoglobulin heavy chain signal peptide. collagen-like tail was produced by digestion using methods described by Dinamani et al. (19). Briefly, 250 µl of MBP (300 µg/ml) were mixed with 250 µl of collagenase (43 µg/ml, 2.6 units/ml) from Clostridium histolyticum (Calbiochem, Behring Diagnostics, La Jolla, CA). Both enzyme and substrate were in 200 mM triethyrammonium buffer (pH 7.8, containing 0.05% Triton X-100). The mixture was incubated with agitation for 24 h at 37 °C. Nondigestible and polymeric forms of cdMBP along with collagenase (110 kDa, molecular mass) were separated from monomeric cdMBP (16-18 kDa products) by centrifugation in a Centricon 30 (Amicon Division, W. R. Grace & Co., Danvers, MA) concentration system, repeatedly diluting the retentate with buffer to obtain as much cdMBP as possible in the filtrate. The filtrate was subsequently concentrated in a Centricon 10 (Amicon) concentration tube to 100 µg/ml.

The proteins were solubilized in sample buffer with or without 2-mercaptoethanol by boiling for 5 min, then analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels (SDS-PAGE). In some cases the proteins were stained with Coomassie Blue. In others, the proteins were immediately transblotted to nitrocellulose then probed with various antibodies specific for MBP.

**Complement Activation—Washed bacteria (300 µl, 2 x 10^9 organisms/ml in HBSS/20 mM CaCl_2) were first mixed with 300 µl of HBSS/20 mM CaCl_2 or 300 µl of HBSS/20 mM CaCl_2 containing 20 µg/ml huMBP, rMBP, cdMBP, or ∆MBP in 1.5-ml microcentrifuge tubes, and incubated at 25 °C for 1 h. The bacteria were then washed and resuspended in HBSS++ at 1 x 10^9 bacteria/ml. Bacteria (250 µl) and 250 µl of 5% serum were mixed (final concentration of bacteria = 5 x 10^7/ml, final concentration serum = 2.5%) and incubated at 37 °C stirring end over end for 30 min. Triplicate 100-µl aliquots of each sample were added to 400 µl of HBSS at 0 °C (ice-water bath) then centrifuged at 12,500 g for 5 min in a microcentrifuge (Microspin 24S, Sorvall Instruments, Du Pont). The supernatant was aspirated and saved, the tip of the tube containing the bacterial pellet was clipped, and each was counted separately in a Cobra Auto-Gamma radiation counter (Packard, Downers Grove, IL). Number of molecules of C3 specifically bound per organism was calculated as described (20).

Other complement activation experiments were performed to determine if any molecular form of ∆MBP was able to activate complement. These studies were executed exactly as described above except

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[1] Igh SP—Collagen Stalk CRD

[2] Truncated MBP cDNA expressed in SP2 cells

[3] MBP monomer forms trimers that multimerize into pentamers/hexamers complexes

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**Fig. 2.** Graph showing serum activation by MBP constructs. Serum from normal volunteers was immediately aspirated and saved, the tip of the tube containing the bacterial pellet was clipped, and each was counted separately in a Cobra Auto-Gamma radiation counter (Packard, Downers Grove, IL). Number of molecules of C3 specifically bound per organism was calculated as described (20).

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**Table 1**

<table>
<thead>
<tr>
<th>MBP Treatment</th>
<th>Mean C3 Activation</th>
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<tr>
<td>Control</td>
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<tr>
<td>rMBP</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>cdMBP</td>
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<tr>
<td>∆MBP</td>
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**Table 2**

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**Table 3**

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**Table 4**

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**Table 5**

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**Table 6**

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**Table 7**

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<td>∆MBP</td>
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that each pool (Fig. 6, pools A–D) of AMBP was recalcified and immediately used individually in place of MBP in the preincubation step of SH5770 prior to incubation in serum and 125I-FC.

**Binding to Bacteria Detected by ELISA—**SH5770 were boiled for 10 min to kill and heat-fix the organisms. After centrifugation, organisms released (2 × 109 organisms/ml) were reconstituted in 0.1 M carbonate buffer, pH 9.55 (15 mM Na2CO3, 30 mM NaHCO3, 3 mM NaN3). Fifty microliters of the bacterial suspension was added to each well of a 96-well microtiter plate (1 × 105 organisms/well), and were allowed to adhere at 37 °C for a 1.5-h shaking. After washing the organisms with HBSS/20 mM CaCl2, 50 µl of MBP (10 pg/ml), cdMBP (5 µg/ml), or ΔMBP (5 µg/ml) in HBSS/20 mM CaCl2 were added and incubated 1 h at 25 °C. The wells were washed with PBS-Tween (PBS containing 0.5 ml/liter polyoxyethylene-sorbitan monolaureate (Tween 20; Sigma), blocked with 5% bovine serum albumin at 4 °C overnight, then washed again with PBS-Tween. Increasing dilutions from 1:3,000 to 1:15,000 of mAb3 (shown by immunoblot to recognize cdMBP and by immunoblot and double immunodiffusion to recognize ΔMBP, data not shown) were added and incubated at 25 °C for 1 h. Wells were again washed with PBS, and horseradish peroxidase–derivated goat anti-mouse IgG (1:250 dilution, Miles Scientific, Naperville, IL) was added and incubated at 25 °C for 1 h. Wells were washed with PBS, and substrate o-phenyldiamine (Bio-Rad) was added and allowed to develop. The reaction was stopped with addition of 4% H2SO4. Plates were read on an Emax precision microplate reader (Molecular Devices Corp., Menlo Park, CA) at 450 nm. The controls for nonspecific binding of mAb3 to SH5770 were wells containing buffer only. The wells for nonspecific binding to MBP were wells containing SH5770 and MBP that were then incubated with murine monoclonal antibody M732 (M732) directed against Group B, type III streptococci. To express specific binding, optical density of the wells containing buffer without MBP were subtracted from sample wells. Optical densities of the wells probed with mAb M732 are graphically depicted in the results.

**Binding of 125I-MBP to SH5770—**In other experiments, fractions of 125I-ΔMBP separated by sucrose-density sedimentation (see below) were combined to form pools A, B, C, and D. One hundred microliters (A or B) or 25 µl (A and B) were mixed with ∼1 × 109 bacteria in PBS containing 20 mM CaCl2 (final volume 300 µl), then incubated for 20 min at 25 °C rotating end over end. Samples were centrifuged at 12,500 × g for 10 min, and the supernatant saved. Tubes of the samples were washed with 1.25 ml buffer, supernatant added to the first, and pellets collected at 12,500 × g. Supernatants and pellets counted. Results were expressed as percentage of total 125I-ΔMBP that bound to the bacterial pellet. In some experiments, 3.3 mg of mannann was added to bacteria simultaneously with each pool to compete with mannose residues on bacteria for specific binding. Mannan, rather than a chelating agent such as EDTA, was used to define background binding because EDTA disrupts the bacterial cell wall (see above). Lipopolysaccharides are released, potentially exposing cell wall constituents not normally available to interact with MBP. Binding to these constituents would result in artificially elevated levels of nonspecific binding. Alternatively, loss of the lipopolysaccharide might result in artificially elevated levels of nonspecific binding. Mannan, rather than a chelating agent such as EDTA, was used to define background binding because EDTA disrupts the bacterial cell wall (see above). Lipopolysaccharides are released, potentially exposing cell wall constituents not normally available to interact with MBP. Binding to these constituents would result in artificially elevated levels of nonspecific binding. Alternatively, loss of the lipopolysaccharide might result in artificially elevated levels of nonspecific binding. Mannan, rather than a chelating agent such as EDTA, was used to define background binding because EDTA disrupts the bacterial cell wall (see above). Lipopolysaccharides are released, potentially exposing cell wall constituents not normally available to interact with MBP. Binding to these constituents would result in artificially elevated levels of nonspecific binding. Alternatively, loss of the lipopolysaccharide might result in artificially elevated levels of nonspecific binding.

**Separation of Molecular Forms of ΔMBP and MBP—**Fifty micrograms of 125I-ΔMBP, 100 µg of nonlabeled ΔMBP, 14.8 µg of 125I-rMBP, or 158 µg of rMBP were diluted in 5 µl sucrose in PBS containing 0.1% gelatin to a final volume of 500 µl, then layered over 4% H2SO4. Plates were read on an Emax precision microplate reader (Molecular Devices Corp., Menlo Park, CA). Tubes were loaded into a chilled Beckman SW 28 rotor, and centrifuged at 4 °C in a Beckman L8-70 Ultracentrifuge at 27,500 rpm for 24 h. Seven-drop fractions were collected from the bottom of the tube. In some cases 125I-MBP fractions were combined to make four pools (Fig. 6, pool A, fractions 40–48; B, fractions 40–60; C, fractions 60–80; and D, fractions 80–100), and each pool individually subjected to a second sucrose density gradient centrifugation. Each centrifuged pool was fractionated and analyzed for radioactivity and/or protein and for binding to SH5770 (see above). Individual fractions of 125I-ΔMBP or 125I-rMBP were collected for polyacrylamide gel electrophoresis (PAGE) on 5–20% polyacrylamide gradient gels that were dried and used for autoradiography. Protein molecular weight standards were sedimented at the same time on identical sucrose gradients. A mixture of bovine thyroglobulin (S20, ρ ~ 6.8), albumin (S20, ρ ~ 4.73), and carbonic anhydrase (S20, ρ ~ 2.8) obtained from Sigma were mixed in 5% sucrose/PBS/gelatin (final volume 500 µl) prior to sedimentation (sedimentation constants are taken from Ref. 22). The standards were treated identically to the MBP samples, except at the end of the experiment, 50 µl of each Bio-Rad protein standard fraction was transferred to a microscope slide well and read on an ELISA reader at absorbance 405 A to detect the myoglobin peak. Because the high sucrose content interfered with our quantitative protein assay, 50 µl of each protein standard fraction was analyzed by SDS-PAGE and stained with Coomassie Blue to ascertain which fraction contained the peak of each protein. Sedimentation constants of the protein standards were used to estimate the sedimentation constants of the MBPs.

**Inhibition of ΔMBP Binding to SH5770 by Mannann—**To determine if ΔMBP was binding specifically to mannose residues on SH5770, 100 µl SH5770 (1.5 × 109 bacteria) were mixed with each of two 100-µl aliquots of each pool (A, B, C, or D) of radiolabeled ΔMBP. 125I-ΔMBP in PBS/20 mM CaCl2 (100 µl) was added simultaneously either with 100 µl PBS/20 mM CaCl2 or 100 µl PBS/20 mM CaCl2 containing mannann (1 mg/ml, final concentration 3.33 mg/ml, Sigma, final total volume 300 µl). The mixtures were then treated exactly as described under "Binding of 125I-MBP."

**Competitive Inhibition of MBP and ΔMBP Binding to SH5770—**To determine relative affinity of MBP and ΔMBP, we performed experiments in which rMBP was used to inhibit binding of 125I-rMBP to SH5770. One hundred microliters of 125I-ΔMBP (4.9 µg in PBS/20 mM CaCl2) was added simultaneously with 100 µl of increasing concentrations of nonradioabeled MBP (0–9.4 pg in PBS/20 mM CaCl2) or ΔMBP (0–9.4 µg/ml in PBS/20 mM CaCl2) to 1.5 × 109 SH5770 in 100 µl PBS/20 mM CaCl2 (total volume mixture 300 µl). The mixture was then incubated at 37 °C for 60 min, washed and assayed in experiments conducted as described in the radiodensity above. Results are presented as percentage inhibition of 125I-ΔMBP binding. Reciprocal experiments of 125I-ΔMBP inhibition of rMBP binding also were performed.

**RESULTS**

**Truncated Forms of MBP—**Non-treated rMBP (maintained at 4 °C), mock-digested rMBP (incubated at 37 °C in the absence of collagenase), ΔMBP, and exhaustively digested cdMBP (all in 50 mM CaCl2) were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Under nonreducing conditions (Fig. 2A), rMBP and mock-digested ΔMBP migrated primarily as doublets of m 82 and 88 kDa as well as two bands of larger multimeric forms and much larger polymers that did not enter the gel (not visible on the gel in Fig. 2, since the stacking gel has been removed). ΔMBP migrated as 16 kDa, and cdMBP as a doublet of 16 and 18 kDa. Upon reduction with 2% 2-mercaptoethanol (Fig. 2B), rMBP and mock-digested ΔMBP migrated as 45 kDa consistent with incompletely reduced trimers. Protein bands at 45 kDa and in some cases at 66 kDa were frequently seen on analysis by SDS-PAGE and by immunoblot of MBP or rMBP reduced with 2-mercaptoethanol (not shown). Complete reduction of full-length MBP may require treatment with 6 M guanidine hydrochloride in 0.1 M Tris, pH 8.5, containing 70 mM 2-mercaptoethanol at 37 °C for 4 h (19) or boiling in 50 mM dithiothreitol for 5 min (14). In contrast, the majority of ΔMBP migrated as 25 kDa and cdMBP as a broad band from 25 to 27 kDa when electrophoresed under reducing conditions. Both truncated MBPs and whole rMBP bound monochromal and polyclonal antibodies as determined by ELISA (see Fig. 4) and immunoblot (data not shown). These results show that truncated forms of MBP produced by collagenase digestion of rMBP or by deletion mutagenesis of the recombinant MBP clone were of similar molecular mass and did not form disulfide-linked multimers.

**C3 Binding to Bacteria Preincubated in MBP—**We studied binding of C3 to SH5770 in the presence of MBP, ΔMBP, or cdMBP (Fig. 3). Organisms were incubated with MBP, rMBP, ΔMBP, cdMBP, or buffer, then incubated in 2.5% ADS. After
the presence or absence of 10 pg/ml collagenase as a control for presence of 10 pg/ml collagenase. Gelatin (150 μg) was incubated in the presence or absence of 10 μg/ml collagenase as a control for collagenase activity. ΔMBP was made as described under "Materials and Methods." Each was solubilized in sample buffer and electrophoresed on a 12.5% polyacrylamide gel under nonreducing and reducing conditions. Panel A, in the absence of collagenase, rMBP migrated as a doublet at 82 and 88 kDa plus large multimers, with (+/+) or without (−/−) incubation. Incubation in the presence of collagenase (+/+ ) produced a doublet at 16 and 18 kDa. ΔMBP migrated at 16 kDa, exactly opposite the smaller digestion product. Gelatin (−/+ ) migrated as two easily seen high molecular weight bands and a long smear of protein not clearly apparent in this photograph. Digested (+/−) gelatin was not visible, confirming activity of the collagenase. Panel B, under reducing conditions, (−/−)rMBP and (−/−)ΔMBP migrated at 45 kDa, probably representing incompletely reduced trimers. ΔMBP migrated at 25 kDa, at the lower end of the 25–27-kDa broad band of (+/+)rMBP in the adjacent lane. Gelatin lanes again confirm collagenase activity.

FIG. 2. SDS-polyacrylamide gel electrophoresis of different forms of MBP. For 24 h prior to electrophoresis, rMBP was either maintained at 4 ºC, incubated at 37 ºC, or incubated at 37 ºC in the presence of 10 μg/ml collagenase. Gelatin (150 μg) was incubated in the presence or absence of 10 μg/ml collagenase as a control for collagenase activity. ΔMBP was made as described under "Materials and Methods." Each was solubilized in sample buffer and electrophoresed on a 12.5% polyacrylamide gel under nonreducing (panel A) and reducing (panel B) conditions. Panel A, in the absence of collagenase, rMBP migrated as a doublet at 82 and 88 kDa plus large multimers, with (+/+) or without (−/−) incubation. Incubation in the presence of collagenase (+/+ ) produced a doublet at 16 and 18 kDa. ΔMBP migrated at 16 kDa, exactly opposite the smaller digestion product. Gelatin (−/+ ) migrated as two easily seen high molecular weight bands and a long smear of protein not clearly apparent in this photograph. Digested (+/−) gelatin was not visible, confirming activity of the collagenase. Panel B, under reducing conditions, (−/−)rMBP and (−/−)ΔMBP migrated at 45 kDa, probably representing incompletely reduced trimers. ΔMBP migrated at 25 kDa, at the lower end of the 25–27-kDa broad band of (+/+)rMBP in the adjacent lane. Gelatin lanes again confirm collagenase activity.

FIG. 3. Specific C3 binding to SH5770. SH5770 were preincubated in buffer, cdMBP, ΔMBP, or rMBP, then in 2.5% ADS, exactly as described under "Materials and Methods." MBP-sensitized SH5770 bound more than 3.8% (9,876 molecules C3/organism) total C3 in the assay, while cdMBP (668 molecules C3/organism) and ΔMBP (735 molecules C3/organism) bound no more C3 than the control organism preincubated in buffer (1,237 molecules C3/organism). This experiment is representative of four separate experiments performed in triplicate.

exposure to MBP or rMBP (not shown), 8-fold more C3 (9,876 molecules C3/organism) bound to SH5770 than in control samples (1,237 molecules C3/organism). By contrast, cdMBP (668 molecules C3/organism) and ΔMBP (705 molecules C3/organism) did not increase C3 deposition over control levels. Thus loss of the collagen-like tail from the carbohydrate recognition region removed the ability of MBP to enhance complement activation.

MBP Binding to SH5770—To examine the possibility that failure of ΔMBP to activate complement on SH5770 was due to the inability of MBP to bind in the absence of the collagen-like tail, we compared binding of truncated MBP to binding of equimolar amounts of whole MBP by ELISA. All three forms of MBP, whole rMBP, ΔMBP, and cdMBP, specifically bound (Fig. 4) to SH5770 as detected by mAb3 that recognized bound and free MBP. Low nonspecific binding of negative control mAb M732 is also shown in Fig. 4. Subsequent binding assays with ¹²⁵I-ΔMBP (see below) confirmed that truncated MBP binds to SH5770. Thus failure to enhance complement activation was not due to failure of truncated MBP to bind to the target organism.

Molecular Form of ΔMBP—The inability of bound ΔMBP to activate complement may reflect either the requirement of the collagen-like tail for complement activation or for multimerization of the molecule. Therefore, we examined the molecular form of ΔMBP, and the relationship of form to bacterial binding and complement activation. These initial experiments were designed to determine whether ΔMBP existed as monomers, polymers or both under nondenaturing conditions. We also wanted to compare the sedimentation of whole MBP and truncated MBP in nondenaturing conditions.

In order to compare the size and sedimentation rate of nondenatured molecules, ¹²⁵I-ΔMBP and ¹²⁵I-rMBP were centrifuged through 5–20% sucrose-density step-gradients in the absence of calcium. The peak of ¹²⁵I-rMBP sedimented at a rate of approximately S₂₀,₅₀ = 2.75 (Fig. 5), consistent with the known molecular mass of monomeric MBP of 28–32 kDa (1, 12). The slope of the leading limb of rMBP implied that there were multimeric forms, and their presence was verified by SDS-PAGE and autoradiography (not shown). In fractions 43–47, containing low concentrations of sucrose, there were 29–32-kDa monomers. In addition, there were bands consistent with trimers and larger complexes that barely entered the
gel (pentamers or larger). This observation suggests that upon standing at 4 °C after separation from larger multimers in the gradient, monomeric rMBP associated to form polymers.

In contrast to rMBP, the ΔMBP peak sedimented at a rate approximately $S_{20, w}$ 2.0 (Fig. 6A), consistent with the molecular mass of monomeric ΔMBP of 16 kDa (estimated by SDS-PAGE). The sloping ascending limb of the ΔMBP peak also suggested that multimeric ΔMBP complexes also were present. This was tested by preparing pools A through D as indicated in Fig. 6A, and resedimenting pools individually on 5–20% sucrose gradients (Fig. 6B). Pool A sedimented at 9.9 $S_{20, w}$, pool B at 5.4 $S_{20, w}$, pool C at 3.02 $S_{20, w}$, and pool D at 2.01 $S_{20, w}$. In each case, the peak of the pool sedimented at approximately the mid-point of the fractions it was composed of, confirming that oligomers as well as monomers of ΔMBP were present in the sample. We therefore asked whether all forms of ΔMBP would bind to SH5770.

**Relationship of Molecular Form of MBP to Bacterial Binding—**Binding of monomers and multimers of ΔMBP to mannose-rich bacteria was tested (Fig. 7). After incubation with SH5770, bacteria-associated $^{125}$I-ΔMBP from pooled fractions A, B, C, or D was measured and expressed as percentage of total $^{125}$I-ΔMBP in that pool. In some experiments, mannan was added simultaneously with ΔMBP to SH5770. Specifically bound $^{125}$I-ΔMBP was calculated by subtracting percentage $^{125}$I-ΔMBP bound in the presence of mannan from the percent bound in the absence of mannan. Pool A specifically bound 18% of $^{125}$I-ΔMBP, pool B 11.3%, pool C 3.6%, and pool D 3.7%. These results show that ΔMBP binds specifically to mannosides on SH5770 and also suggest that polymers of ΔMBP bearing multiple carbohydrate-recognition domains bind to the mannoside-rich bacterial surfaces with higher affinity than the monomeric forms.

Pentameric/hexameric forms of rMBP, but not smaller multimers, may substitute for C1q to initiate activation of the classical complement pathway (11–13). Removal of the collagen-like tail of C1q by collagenase digestion eliminates its ability to initiate complement activation (23) but does not remove the ability of truncated C1q to multimerize and to bind to antigen-antibody complexes (24). Likewise, a mixture of truncated MBP of all multimeric states was unable to support complement activation despite binding to mannosese-rich substrates.

In order to determine whether multimeric ΔMBP supported...
complement activation while monomers or smaller oligomers could not, each recalcified pool (Fig. 6) from the sucrose density gradient separation of ΔMBP was tested individually. None of the pools supported deposition of C3 on SH5770, the mannosse-rich test organism (data not shown).

Competitive Inhibition of 125I-ΔMBP Binding by MBP or ΔMBP—We performed studies to determine the relative affinities of 125I-ΔMBP and whole rMBP for the mannosse-rich organism. As increasing amounts of rMBP were added to SH5770 simultaneously with a constant amount of 125I-ΔMBP, rMBP inhibited 125I-ΔMBP binding in a dose-response fashion at all concentrations tested (Fig. 8, panel A). Although most of the rMBP used in this assay existed as a monomer (as shown by sucrose density sedimentation, Fig. 5, and autoradiogramy, not shown), multimeric forms also were present. Nonetheless, assuming that ΔMBP and rMBP were each present primarily as monomers, 0.0761 mol of monomeric rMBP was needed to inhibit 50% of the binding of 1 mol of 125I-ΔMBP, demonstrating the greater affinity of whole MBP for the mannosse-rich surface.

When non-radiolabeled ΔMBP was used to compete with 125I-ΔMBP, binding to SH5770 was inhibited in a dose-related manner (Fig. 8, panel B). Binding of 5 μg of 125I-ΔMBP was reduced by 50% in the presence of 5 μg of ΔMBP.

Reciprocal inhibition experiments (ΔMBP inhibition of 125I-rMBP binding, data not shown) did not demonstrate ΔMBP interference with rMBP binding at the concentrations of ΔMBP used. A maximum of 7.3 mol of ΔMBP was added for each mol of 125I-rMBP used in the assay. Concentrations of ΔMBP were never sufficient to inhibit the more avidly bound 125I-rMBP.

DISCUSSION

Whole human MBP, whether recombiant or purified from serum, binds to mannosse-rich Salmonellae (8) and activates the alternative complement pathway (7), a characteristic corroborated by the present investigations. MBP also activates complement on mannan-coated sheep erythrocytes (11) and on zymosan (12) via the classical pathway. The present studies did not clarify whether multiple ligand-binding sites are sufficient or if the collagen-like region also was essential for complement activation to proceed. Our experiments demonstrated that the collagen-like tail is necessary for complement activation, regardless of the multimeric state of the molecule.

Sucrose density sedimentation clearly established that truncated MBP exists as multimers even in the absence of the collagen-like region, consistent with earlier work (25). Predictably, larger multimers with presumably more ligand-

FIG. 8. Inhibition of 125I-ΔMBP binding to SH5770. SH5770 (1.5 × 10^9) were incubated with 4.9 μg 125I-ΔMBP and increasing amounts of rMBP (0.9-4 μg) or ΔMBP (0-250 μg) and treated exactly as described in Fig. 7. Results are expressed as percent inhibition of 125I-ΔMBP binding. 125I-ΔMBP binding was inhibited in a dose-response fashion by both rMBP (panel A) and ΔMBP (panel B). The binding of 1 mol of 125I-ΔMBP could be diminished by 50% with 0.0761 mol of rMBP (panel A, dashed line), while a molar ratio of 1:1 (ΔMBP:125I-ΔMBP) diminished binding by 50% (panel B, dashed line). In additional studies (not shown) inhibition of 125I-rMBP binding by ΔMBP was not observed at concentrations tested. However, limited availability of ΔMBP prevented testing of inhibition with amounts of ΔMBP sufficient to demonstrate displacement of the high affinity rMBP.
binding sites, bound mannose-rich Salmonellae with greater affinity than smaller multimers and monomers, but higher affinity did not influence the ability of truncated MBP to activate complement. In addition, the overlap in sedimentation rates of MBP and AMBP suggests that smaller AMBP exists as multimers with at least as many binding sites as a substantial portion of whole MBP. Thus if the ligand-binding region were sufficient for complement activation, at least some C3 deposition would have been observed.

The collagen-like region also contributes to the stability of the MBP-ligand interaction. Truncated MBP specifically binds to mannose glycoconjugates and can be successfully competed from its target mannose residues by mannan, just as mannan prevents interaction of whole MBP with the mannose-rich target surface. However, avidity of ΔMBP binding is less than whole MBP since whole MBP successfully competes with ΔMBP for mannose residues, while ΔMBP does not inhibit binding of whole MBP at concentrations tested (data not shown). The explanation for this observation may be that the collagen-like region stabilizes polymerized monomers in the optimal conformation for ligand binding.

The collagen-like tail of human rMBP is cleaved by collagenase to produce inactive carbohydrate-recognition fragments of the carboxyl terminus. Collagenase preferentially cleaves polypeptides after proline in the amino acid sequence Gly-X-Pro-Gly (26). Human MBP has six such repeats in its collagen tail (4, 5) and is susceptible to cleavage. Despite exhaustive digestion, cdMBP remained a doublet of 16 and 18 kDa under denaturing conditions of SDS-PAGE. It is unlikely that one of these two bands represents incomplete digestion since under the same conditions collagen-like regions of rat MBP (19) and Clq (23, 24) are completely removed. Produced in mouse myeloma cells, rMBP may be glycosylated to varying extents, producing two sizes (82 and 88 kDa) of rMBP on SDS-PAGE (see doublets of nondigested rMBP in Fig. 2). The cdMBP doublet under nonreducing conditions probably represents digestion of two rMBPs of different glycosylation states. Less likely, carbohydrate residues may be lost from a portion of the undigested carboxyl terminus during the enzymatic degradation.

The apparent inconsistency in form (multimer versus monomer) of rMBP present on SDS-PAGE and in sucrose density gradient was caused by differences in experimental conditions. Our hypothesis was that presence or absence of calcium was responsible for the apparent differences in multimerization state of rMBP on SDS-PAGE and in the sucrose-density gradient. We confirmed the hypothesis by incubating rMBP in calcium-free PBS at 4 °C, simulating the calcium-free environment of the sucrose gradient experiments, or in triethylammonium buffer containing 50 mM CaCl₂ at 37 °C, simulating the collagenase-digestion experiment. After a 1-h incubation, an aliquot of each sample was electrophoresed, and no difference was detected in multimerization state of rMBP in the presence or absence of calcium as determined by migration on SDS-PAGE. However, after 24 h of incubation, the migration of rMBP confirmed that incubation in the absence of calcium resulted in separation of the multimers of rMBP, causing it to migrate primarily as the 32-kDa monomer, while in the presence of calcium, it migrated primarily as a doublet at ~82 and 88 kDa, plus several much larger bands. The loss of multimeric state occurred even in the absence of dilution in the sucrose gradient.

In conclusion, our results indicate a requirement for the MBP collagen-like region for complement activation. Even without the stabilizing effect of the collagen-like region, globular carbohydrate-recognition “heads” exist as monomers and variously sized, noncovalently linked multimers that maintain their ability to bind specifically to mannose-rich targets.

REFERENCES