

# Relative Interchain Disulfide Bond Labilities in Human F(ab')<sub>2</sub> $\mu$ Fragments<sup>1</sup> (35989)

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Human IgM comprises five subunits (IgM<sub>s</sub>) each of which consists of two  $\mu$  chains and two light chains. One light chain is covalently attached to one  $\mu$  chain through a cystine residue. Two  $\mu$  chains are covalently joined through a disulfide bond near their C-termini (1) and one near the light- $\mu$  chain linkage (2). In intact IgM, the IgM<sub>s</sub> components also are united through disulfide bridges (3, 4).

The relative susceptibilities of the interchain disulfide bonds in IgM<sub>s</sub> molecules to mercaptoethylamine (MEA) has been studied (5). One light- $\mu$  chain bond and one inter- $\mu$  chain bond had similar labilities to the mercaptan.

F(ab')<sub>2</sub> $\mu$  is a trypsin-produced fragment of IgM (2). The C-terminal half of each  $\mu$  chain is degraded enzymatically leaving a large fragment comprising the two light chains of IgM<sub>s</sub> and the N-terminal halves of the  $\mu$  chains up to the inter- $\mu$  chain disulfide bond.

The purpose of the present study was to investigate the relative labilities to MEA of the light- $\mu$  chain and inter- $\mu$  chain disulfide bonds in F(ab')<sub>2</sub> $\mu$ .

**Materials and Methods.** IgM was purified as previously described (3) from the plasma of a patient afflicted with Waldenström's macroglobulinemia. In brief, lipid-free plasma was dropped into 10 times its volume of water to precipitate the IgM. The crude euglobulin was recovered by centrifugation at 12,000g, dissolved in a solution of 0.32 M

NaCl made to 0.001 M in sodium borate, pH 8 (0.32 SB), and filtered through Bio-Gel P-200 or Sephadex G-200 equilibrated with the same solvent.

F(ab')<sub>2</sub> $\mu$  fragments were prepared essentially according to Miller and Metzger (2). IgM was suspended by dialysis in 0.2 M Tris(hydroxymethyl)aminomethane (Tris) buffer made 0.01 M in calcium chloride, pH 8.0. Trypsin was added to the protein in an enzyme-substrate weight ratio of 1:100. The mixture was incubated for 18 hr at 37°. Tryptic activity was then inhibited by the addition of tosy-L-lysyl-chloromethane to a weight ratio of 1:50. A column (2.5 × 132 cm) was assembled for reverse flow chromatography with a bed of P-200 equilibrated in 0.2 M Tris buffer, pH 8.6. It was charged with 120 mg of the digestion mixture; and protein was eluted at the rate of 4 ml/hour.

Ultracentrifuge analyses were done with a Beckman Model E instrument equipped with electronic speed control, schlieren optics, and an An-H rotor. All runs were made with a rotor temperature of 20° and a speed of 60,000 or 68,000 rpm.

Immuno-electrophoresis was done on glass plates (12 × 5.7 cm) coated with 0.85% agarose in 0.05 M sodium barbital buffer, pH 8.0. Electrophoresis was done at 6 mA/plate for 120 min. The serological reactions were tested with anti-IgM, anti-IgG, anti-kappa, or anti- $\mu$  antisera.

F(ab')<sub>2</sub> $\mu$  fragments were suspended in 0.05 M Tris-0.5 M NaCl solution, pH 8.0. The protein was then reduced with 0.004 M MEA in an atmosphere of N<sub>2</sub> for 30 min at 30°. The reaction was terminated with 0.04 M iodoacetic acid (neutralized). Fragments derived from the reductive cleavage of F(ab')<sub>2</sub> $\mu$  were isolated by filtration of the mixture through a column (1.8 × 60 cm) of

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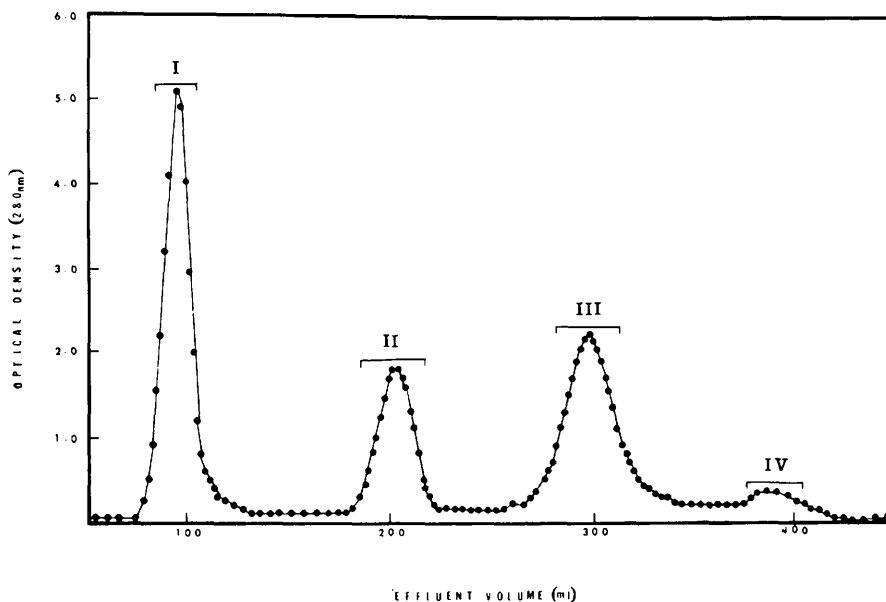


FIG. 1. Elution profile of trypsin-digested IgM: The digest was filtered through Bio-Gel P-200. Brackets designate the protein fractions which were pooled, and Roman numerals denote the pooled protein.

P-200 equilibrated with 0.2 *M* Tris solution, pH 8.6.  $F(ab')_{2\mu}$  and the smaller fragments ( $Fab'$ ) were then dialyzed in 6.7 *M* guanidine hydrochloride (Gd·HCl) solution, pH 7.0, prior to ultracentrifugation.

Proteins were analyzed for carboxymethylcysteine (CM-cysteine) as previously described (3).

Concentration of proteins was carried out in an Amicon ultrafiltration device. A UM-10 membrane was used and the pressure was exerted by compressed  $N_2$ .

**Results.** The IgM was shown by immunoelectrophoresis to be free of contaminating serum proteins. It was digested with trypsin and the resulting mixture was filtered through Bio-Gel P-200. The elution profile is shown in Fig. 1. The protein of the pooled fractions was identified by analytical ultracentrifugation and immunoelectrophoresis. Fraction I consisted of undigested IgM. Fractions II and III reacted with anti- $\kappa$  antiserum but not with anti- $\mu$  antiserum. The sedimentation coefficient of fraction II was 6.4 S and that of fraction III was 3.7 S. The fragments were designated  $F(ab')_{2\mu}$  and  $Fab_{\mu}$ . Fraction IV consisted primarily of peptides but about 17% of it was small frag-

ments of greater than approximately 15,000 mol wt retained by the UM-10 membrane during concentration.

$F(ab')_{2\mu}$  was treated with 0.004 *M* MEA. The schlieren pattern of the reduced material (Fig. 2A) disclosed the presence of a component which sedimented slower than  $F(ab')_{2\mu}$ . The apparent sedimentation coefficient of the small protein was 4.2 S. Filtration through P-200 effected the separation of two different sized molecular entities (Fig. 2). The portions of the eluted proteins pooled to form fractions I and II are indicated in Fig. 2. Following electrophoresis, neither of the isolated components reacted with anti- $\mu$  antiserum, but both did precipitate with anti- $\kappa$  antiserum (Fig. 2B). Fraction I was residual  $F(ab')_{2\mu}$  and fraction II was  $Fab'_{\mu}$ .

Samples of the pooled protein of fractions I and II (Fig. 2) were centrifuged in 6.7 *M* Gd·HCl solution at 68,000 rpm to complete sedimentation. Close examination of the schlieren patterns showed there was no dissociation of the light chains.

Analysis for CM-cysteine indicated the presence of 1.0 mole of CM-cysteine/mole of  $Fab'_{\mu}$ .

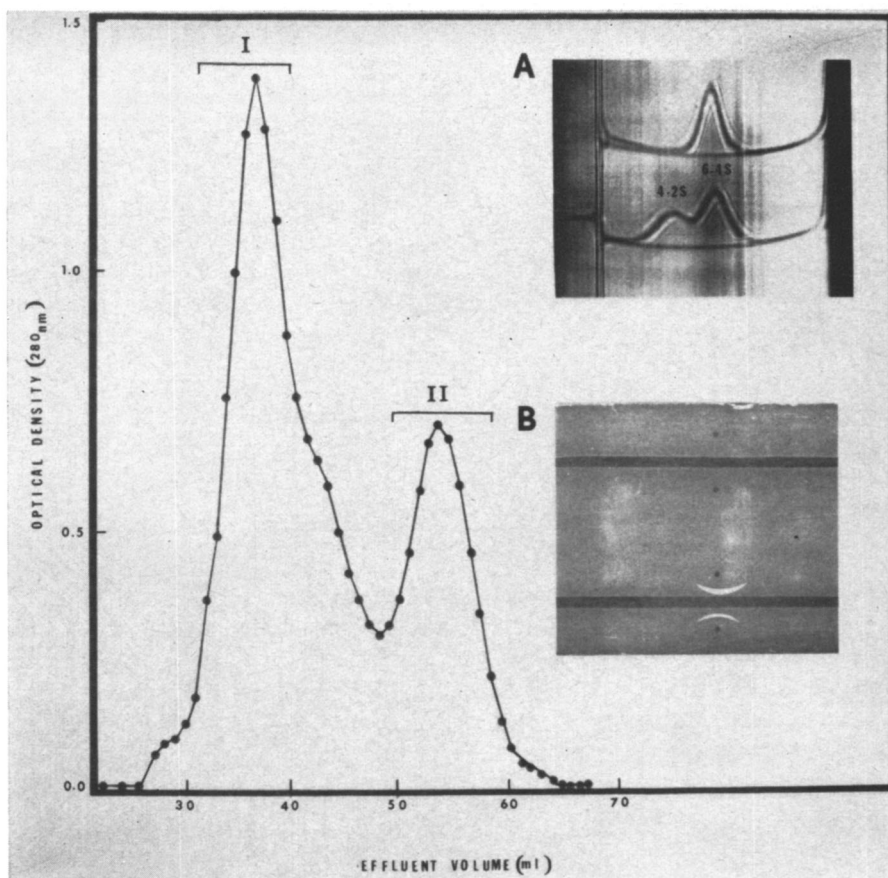


FIG. 2. Elution profile of reduced and alkylated  $F(ab')_{2\mu}$ : After reduction with 0.004  $M$  MEA, the protein was filtered through Bio-Gel P-200. Brackets designate the protein fractions which were pooled, and Roman numerals denote the pooled protein. (A) Schlieren patterns: (top) unreduced  $F(ab')_{2\mu}$  (6 mg/ml); (bottom) reduced and alkylated  $F(ab')_{2\mu}$  (10 mg/ml). Preparations were suspended in 0.2  $M$  Tris buffer, pH 8.6. Photo was taken 58 min after reaching rotor speed of 60,000 rpm. (B) Immunologic analyses of fractions I and II of elution profile: (outside wells) protein of fraction I; (inside wells) protein of fraction II; (top trough) anti- $\mu$  antiserum; (bottom trough) anti-kappa antiserum. The anode is to the right.

*Discussion.* Miller and Metzger (2) have shown that trypsinolysis of IgM results in the formation of  $F(ab')_{2\mu}$  and  $Fab_{\mu}$  fragments. It was noted in the present experiments that for periods exceeding 18 hr the digestion progressively yielded more  $Fab_{\mu}$  than  $F(ab')_{2\mu}$ . Apparently trypsin initially hydrolyzed the  $\mu$  chains at a position on the C-terminus side of the inter- $\mu$  chain disulfide bond located nearest the N-terminus, thus creating the  $F(ab')_{2\mu}$  fragment. The enzyme subsequently hydrolyzed the  $\mu$  chain at a second point on the N-terminus side of the inter-chain disulfide bond liberating the  $Fab_{\mu}$  fragments.

The lability of the interchain disulfide bonds in human IgM molecules has been extensively investigated. The bonds most labile to MEA are those between the subunits (IgM<sub>s</sub>) (3). In IgM<sub>s</sub> molecules the disulfide bonds linking one of the  $\mu$ -light chain pairs, and one of the inter- $\mu$  chain bonds, were the most labile (5). The second  $\mu$ -light chain bond was less susceptible to reduction than the first and the remaining inter-chain bond was rather resistant to cleavage by MEA.

Because of the similarity in labilities of two bonds, one joining the light to the  $\mu$  chain and the other joining two  $\mu$  chains, we

were particularly interested in studying the stability of two similar bonds located in  $F(ab')_{2\mu}$ . It is certain that the  $\mu$ -light chain bond in question resided in  $F(ab')_{2\mu}$ , but it is not known that the fragment contained the proper inter- $\mu$  chain bond. Nevertheless, it was clearly evident that the inter- $\mu$  chain bond was more susceptible to reduction by MEA than either of the  $\mu$ -light chain bonds. The analysis for CM-cysteine showed that each Fab' had 1 residue/mole which could have originated only by reduction of a single inter- $\mu$  chain disulfide bond in  $F(ab')_{2\mu}$ . Additional proof that a  $\mu$ -light chain bond was not broken was given when Fab' fragments failed to dissociate in Gd·HCl solution.

These data do not answer the question of which bond is the more labile to the mercaptan in  $IgM_s$ . They do indicate that the inter- $\mu$  chain bond in  $F(ab')_{2\mu}$  is more susceptible to MEA than the  $\mu$ -light chain bonds. Since a large amount of protein was removed from  $IgM_s$  in the proteolytic production of the  $F(ab')_{2\mu}$  fragment, the inter- $\mu$  chain bond probably was left unprotected and readily

accessible to the mercaptan.

*Summary.*  $IgM$  was prepared from the plasma of a patient suffering with Waldenström's macroglobulinemia. The protein was fragmented with trypsin and  $F(ab')_{2\mu}$  was isolated from the digestion mixture. The fragments readily dissociated into Fab' after treatment with 0.004 *M* mercaptoethylamine. The  $\mu$ -light chain disulfide bonds were not broken. In  $F(ab')_{2\mu}$  fragments, the inter- $\mu$  chain disulfide bond is more susceptible to reduction with mercaptoethylamine than  $\mu$ -light chain bonds.

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