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Immunoglobulin M: Pentameric Fcμ Fragments Released by Trypsin at Higher Temperatures

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Abstract. Immunoglobulin M (IgM, 19*S* macroglobulin) is a high molecular weight antibody molecule currently thought to be composed of five identical 7*S* subunits linked by disulfide bonds, perhaps in a cyclic conformation. Reported molecular weights of IgM vary from 8×10^5 to 10^6. Proteolytic cleavage of this molecule under standard conditions with several enzymes results in the formation of ten antigen-binding (Fab) fragments per mole, each Fab having a molecular weight of 40,000. The remainder of the molecule, designated Fc by analogy with the structure of immunoglobulin G (IgG), is highly susceptible to further enzyme cleavage and breaks down to dialyzable peptides. However, under carefully controlled conditions, an intact Fc-like fragment in low yield can be isolated from short-term papain digests of IgM at 37°C. Availability of the Fc portion of the molecule is important not only for structural studies but because it may determine certain important biological properties of the molecule such as complement fixation and placental permeability.

We here report that trypsin cleavage of IgM at temperatures exceeding 50°C results in the production of excellent yields of an intact Fc fragment, whereas no detectable Fc fragment is produced by trypsin cleavage at 37°C. Fab fragments are obtained under both temperature conditions, and an Fab dimer has been identified in high temperature digests. This unusual difference in the products of enzyme digestion with temperature is unexplained but may be related to steric changes induced in the IgM molecule by heat. Fc fragment isolated from high temperature digests contains two-thirds of the carbohydrate of the intact molecule, and has a molecular weight of 342,000. The molecular weight falls to 67,300 after treatment with disulfide reducing agents, thus supporting the concept of a pentameric structure for IgM.

Introduction. Digestion of 19*S* immunoglobulin M (IgM) or its 7*S* reductive subunit with trypsin or chymotrypsin at 37°C results in the formation of antigen-binding Fabμ and $F(ab)_2\mu$ fragments and low molecular weight peptides.[1–3] The latter are nonreactive antigenically and probably are derived from the degraded Fcμ portion of the molecule. Quantitative studies of yields of the fragments and the number and disposition of interchain disulfide bonds[4] suggest that 19*S* IgM is composed of five 7*S* subunits each containing two Fabμ and one Fcμ fragment and that the 7*S* subunits are linked by *S*-*S* bonds between their Fcμ fragments. Some evidence suggests that the 7*S* subunits are linked in a circular

pentamer.[5] Short-term papain cleavage of IgM at 37°C results, in addition to Fabμ, in a fragment with only Fcμ determinants.[2,3,6] However, the production of an Fcμ fragment with papain is erratic and, at best, low yields are obtained. A method of obtaining Fcμ fragments in sizeable yields would be of importance in studies of the structure of IgM and the role of Fcμ in its function.

We here report that tryptic digestion at higher temperatures apparently results in a unique cleavage of the IgM molecule liberating a polymeric Fcμ fragment in good yield.

Materials and Methods. Purified 19*S* human IgM (Type L) from a patient (DiS) with Waldenström's macroglobulinemia was used in all studies. Results were confirmed with four other pathologic macroglobulins and with normal human IgM. Twice-crystallized bovine trypsin (Worthington) was used in all studies but similar results were obtained with trypsin from two other commercial sources. Kunitz pancreatic trypsin inhibitor (Worthington) was employed to stop digestions.

Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 52,640 rpm at 20°C. Infinite dilution values were obtained from plots of *S* vs. concentrations. The concentrations of IgM and IgM subunits were determined by the optical density at 280 using $E_{280}^{1\%}$ of 11.8.[4]

The molecular weight of IgM and its fragments were measured by the meniscus depletion method of Yphantis employing absorption optics.[7] The partial specific volume ($\bar{V}$) of IgM and its fragments were calculated from their amino acid composition and carbohydrate data as previously described.[3] A hexose to total carbohydrate ratio of 1:1.6 was assumed.[8] Amino acid composition was measured using a Beckman model 120C analyzer following hydrolysis for 22 hr at 110°C in 6 *N* HCl as described by Crestfield, Moore, and Stein.[9]

The techniques used to isolate the proteolytic fragments of IgM, to measure hexose content, and the immunological methods have been previously described in detail.[3]

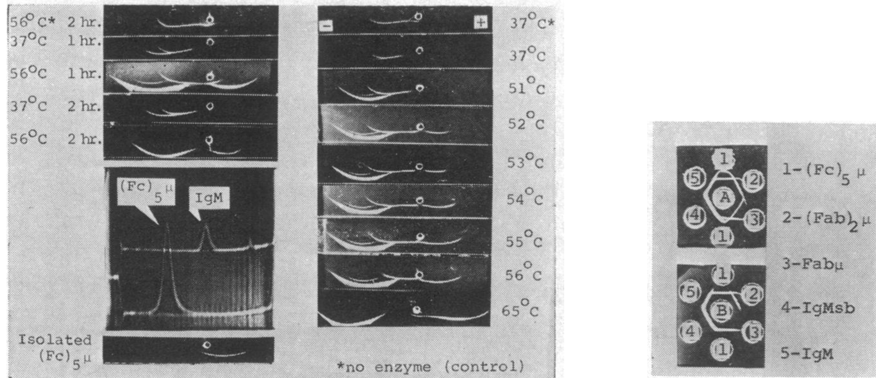
Results and Discussion. Trypsin cleavage of DiS IgM at 37°C for periods ranging from 5 minutes to 48 hours produced only Fabμ and F(ab)$_2\mu$ fragments as previously described.[1,3] IgM showed no apparent change at 56°C in the absence of enzyme. Immunoelectrophoretic analysis of IgM in the course of trypsin cleavage at 56°C (Tris-HCl buffer 0.05 *M*, pH 8.1, with 0.0115 *M* $CaCl_2$; enzyme: protein 1:25, w/w) showed several resultant fragments (Table 1 and Fig. 1).

(*a*) An electrophoretically fast (anodal) band detected by two different anti-human IgM (μ chain specific) antisera, but not by anti-light chain antisera of both κ and λ specificity, or an antiserum showing strong idiotypic specificity for the DiS IgM. This fragment, isolated by starch-block electrophoresis followed by

TABLE 1. *Characteristics of DiS IgM and fragments isolated from 56°C trypsin digests.*

Component	Sedimentation coefficient ($s_{20,w}^{0}$)	Molecular weight	Hexose content† (gm/100 gm protein)
IgM	18.5*S*	845,000	4.8
Fabμ	3.7*S*	41,000	1.9
F(ab)$_2\mu$	6.0*S**	95,000	2.8
(Fc)$_5\mu$	10.8*S*	342,000	12.0
Fcμ [(Fc)$_5\mu$ + DTT 0.01 *M*]	3.4*S*	67,300	...

* At protein concentration 0.8 mg/ml.
† Orcinol method.



(*Left*) FIG. 1.—Immunoelectrophoresis of IgM digests all developed with rabbit anti-DiS IgM (unabsorbed). Digests on right are for 30 min. $(Fc)_5\mu$ is absent at 37°C and becomes detectable at 52°C with progressive increase in concentration to 65°C, when only Fabμ and $(Fc)_5\mu$ are present. The ultracentrifuge schlieren photo was taken of a double-sector cell 32 min after reaching full speed. The upper frame shows the DiS 19*S* IgM; the lower shows the isolated $(Fc)_5\mu$ fragment.

(*Right*) FIG. 2.—Ouchterlony analysis of IgM fragments. Center well A— rabbit anti-DiS IgM (unabsorbed). B— goat antihuman IgM absorbed (μ chain specific). $(Fc)_5\mu$ is antigenically deficient to 19*S* IgM but shows a reaction of immunologic identity with its reductive 7*S* subunit (IgMsb). The $(Fc)_5\mu$ shows a reactions of nonidentity with Fabμ and $F(ab)_2\mu$. The presence of μ-chain determinants on the $F(ab)_2\mu$ lacking on Fabμ are demonstrated.

Sephadex G-200 chromatography (0.14 *M* NaCl) had a $s°_{20,w}$ of 10.8*S*, and a molecular weight by the meniscus depletion method of 342,000 using a partial specific volume ($\bar{V}$) of 0.697. It was reduced by dithiothreitol (DTT: 0.01 *M* in Tris-HCl buffer 0.5 *M*, pH 8.5) to a 3.4*S* fragment with a molecular weight following alkylation with iodoacetamide of 67,300. The 10.8*S* fragment was antigenically deficient to IgM and its 7*S* reductive subunits using anti-IgM antisera (Fig. 2). It was progressively degraded to nonreactive peptides by continued digestion with trypsin at 56°C for 24 hours. However, when digested with trypsin at 37°C it was relatively resistant, significant amounts remaining after 24 hours of incubation. This fragment is referred to as $(Fc)_5\mu$ and its reductive subunit as Fcμ.

(*b*) An electrophoretically slow (cathodal) band detected only by antisera with anti-light chain or idiotypic specificity. This fragment is similar in size, molecular weight, and antigenicity to Fabμ derived from trypsin or papain cleavage of IgM at 37°C and is referred to as Fabμ.

(*c*) A second slow band detected by antisera with anti-light or μ-chain activity which was antigenically deficient in μ-chain determinants with respect to 19*S* IgM or its 7*S* reductive subunit (Fig. 2). This 6*S* fragment had a molecular weight of 95,000 and was readily degraded by trypsin at 56°C to fragments which lost their μ-chain determinants and were electrophoretically indistinguishable from Fabμ. The 6*S* fragment was not detectable in 24-hour digests and probably represented an $F(ab)_2\mu$ fragment bearing a specific μ-chain determinant not present in the Fabμ or $(Fc)_5\mu$ fragments (Fig. 2 $F(ab)_2\mu$). Its similarity to Fabμ is supported by amino acid composition data showing a nearly identical content of amino acid in the Fabμ and $F(ab)_2\mu$ fragments.

Timed trypsin digests at 56°C at periods of 5 minutes to 24 hours showed maximal $(Fc)_5\mu$ yields in approximately 30 minutes. Preliminary observations indicate that the "residual substrate" at 30 minutes, although indistinguishable from starting material by immunoelectrophoresis , was no longer 19*S* and had experienced a considerable decrease in molecular size. At 65°C cleavage of I_sM was complete within eight minutes, and the mgm. yield of $(Fc)_5\mu$ was approximately 30% of the digested protein. The amount of low molecular weight peptide material produced in the course of IgM digestion at 56°C or higher was very low. This was based on an analysis of the peptide region on Sephadex G-200 chromatography using absorption at 280 mμ and was therefore dependent upon the tryptophan and tyrosine content of the material. Bovine α- or β-chymotrypsin digestion of 19*S* IgM at 56°C also released Fcμ fragments in yields similar to trypsin. Papain at 56°C produced small amounts of Fcμ fragments as determined by immunoelectrophoresis. Pepsin studies were complicated by the apparent instability of IgM at acidic pH at 56°C.

Cleavage of IgM by trypsin at 37°C proceeded normally if either the substrate or the enzyme were initially preheated to 56°C and then cooled to 37°C. The unique cleavage occurred at 56°C if trypsin and IgM were separately preheated at 56°C and then combined without cooling. Cleavage of IgM at temperatures between 37°C and 65°C for 30 minutes showed a gradual increase in yield of $(Fc)_5\mu$ beginning at 52°C (Fig. 1). At temperatures between 41°C and 52°C, $(Fc)_5\mu$ was found if digestions were carried out for longer periods; the length of time required being roughly inversely proportional to the temperature. At 70°C for 30 minutes the IgM was irreversibly denatured.

The formation of an $(Fc)_5\mu$ by trypsin at 56°C as compared to 37°C is unexplained. Preliminary observations using optical rotatory dispersion at various temperatures indicate a conformational change in the IgM molecule at 56°C relative to 37°C. A change in conformation could allow the exposure of a trypsin susceptible peptide bond not readily accessible to the enzyme at 37°C. The observation that the longer digestion times at temperatures lower than 52°C produces an $(Fc)_5\mu$ fragment is consistent with a temperature dependent equilibrium between two structures, only one of which is attacked by the enzyme to effect the unique cleavage. The stability of the $(Fc)_5\mu$ fragment to trypsin when returned to 37°C may also be based on conformational factors induced by recoiling at the lower temperature. We have no evidence to support the possibility that trypsin specificity changes at higher temperatures. The similar effect of chymotrypsin and papain at 56°C would be against such a possibility and would point to the substrate as the variable factor. However, studies are continuing with other substrates in an attempt to clarify this point.

The production of a reductive subunit of the $(Fc)_5\mu$ which is one-fifth its molecular weight supports a pentameric structure for IgM. Investigations of the number and position of the *S-S* bonds involved in the reductive cleavage are in progress. The carbohydrate data suggest that the sugar units are present in two or more regions of the molecule with more than two-thirds being located in the Fc region.

An apparently similar carbohydrate-rich fragment having only μ-chain determinants has been isolated by Mihaesco and Seligmann[2] from papain digests of

human IgM at 37°C. Unlike the $(Fc)_5\mu$ reported here, their fragment had an apparent weight average molecular weight of 150–165,000 and was obtained in low yield. The molecular weight differences may in part be explained by the presence of the reducing agent cysteine in their reaction mixture, leading to partial depolymerization of a polymeric Fc. An important point of similarity between the trypsin and papain Fc's is that both experienced a considerable reduction in molecular size with disulfide reducing agents, indicating the presence of several disulfide linked subunits. Unlike trypsin digestion at 56°C, however, large amounts of dialyzable peptides are produced by papain digestion.

The availability of the $Fc\mu$ fragments in quantity will hopefully facilitate further studies of the structure and function of the IgM molecule. Also, the application of proteolysis with various enzymes at higher temperatures to other systems will be of considerable interest. Further studies on the underlying mechanism of the cleavage at higher temperatures could possibly yield interesting data concerning protein conformation and mechanisms of proteolytic cleavage by enzymes.

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