

THE STRUCTURAL BASIS FOR BINDING OF COMPLEMENT BY IMMUNOGLOBULIN M*

By MARY M. HURST, JOHN E. VOLANAKIS, RAYMOND B. HESTER, ROBERT M.
STROUD, AND J. CLAUDE BENNETT

(From the Division of Clinical Immunology and Rheumatology and the Department of Microbiology,
University of Alabama Medical Center, Birmingham, Alabama 35294)

Knowledge of antibody structure is now sufficient to allow investigation of problems of biological function relative to structural parameters. One such structure-function relationship that can be approached is the molecular basis for complement (C) activation by immunoglobulins. On the basis of structural studies it has been proposed that the homology regions of immunoglobulins are arranged in a series of compact domains, with each domain mediating a biological function (1). In line with this hypothesis it has been suggested that the C γ 2 domain may play a role in C fixation by IgG (2, 3). In 1971 Augener et al. by use of active C1 (C $\bar{1}$) fixation assay showed that the 7S subunit of IgM could bind C $\bar{1}$ (4). Plaut et al. subsequently demonstrated that the (Fc) $_{s\mu}$ and Fc μ fragments of IgM were capable of fixing C (5). We have investigated the relative capacity of small molecular weight fragments of a human Waldenström IgM protein (Dau) to bind the first component of C and are thus able to present evidence for the localization of this function to a 24 amino acid sequence.

Materials and Methods

Preparation of IgM and Its Subfragments. IgM was isolated from the plasma of a patient (Dau) with Waldenström's macroglobulinemia as previously described (6). (Fc) $_{s\mu}$ and Fab μ fragments were prepared using trypsin digestion at 60°C (6). After separation of (Fc) $_{s\mu}$ and Fab μ on Sephadex G-200 in 1% NH $_4$ HCO $_3$, pH 8, the (Fc) $_{s\mu}$ was further purified on Bio-Gel A-15 (Bio-Rad Laboratories, Richmond, Calif.). Cyanogen bromide (CNBr) cleavage of (Fc) $_{s\mu}$ and Fab μ was performed as described by Zikan and Bennett (7). Dau Fc $_{s\mu}$ is composed of four CNBr fragments (7), designated CNBr 5 through CNBr 8 (Fig. 1). After CNBr cleavage the smaller fragments, CNBr 6 and CNBr 8, were removed by gel filtration (7). The disulfide bonds in the pentameric CNBr 5 plus 7 fragment were then gently cleaved (either by partial oxidative sulfitolysis [8] or using 0.01 M dithiothreitol followed by alkylation with iodoacetamide) and the sample was applied to a Sephadex G-150 column in 5 M guanidine-HCl to obtain CNBr 5 dimers and monomers which were free of CNBr 7, as shown by polyacrylamide disc electrophoresis in sodium dodecyl sulfate.

In addition, 33,000 mol wt Fc μ fragments were prepared by reducing Fc $_{s\mu}$ with 0.05 M cysteine. The limited tryptic cleavage method of Hester and Schrohenloher (9) was used to release from the Fc μ fragments a homogeneous 6,800 mol wt fragment. This fragment was designated the C $_{\mu 4}$ fragment, since a comparison of its amino acid composition and partial sequence as determined in the sequencer¹ with that published by Putnam et al. (10) for the IgM, Ou, revealed that it was composed of the C $\mu 4$ domain of the Fc μ minus two tryptic peptides in the center of the loop (residues 468-491 and 515-550 according to the numbering of Ou). Relative to Dau CNBr fragments, C $_{\mu 4}$ consisted of the carboxy-terminal 22 residues of CNBr 5 and the first two residues of CNBr 6 disulfide bonded to 36 residues of CNBr 7.

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¹ Hester, R. B., J. E. Mole, and R. E. Schrohenloher. Manuscript submitted for publication.

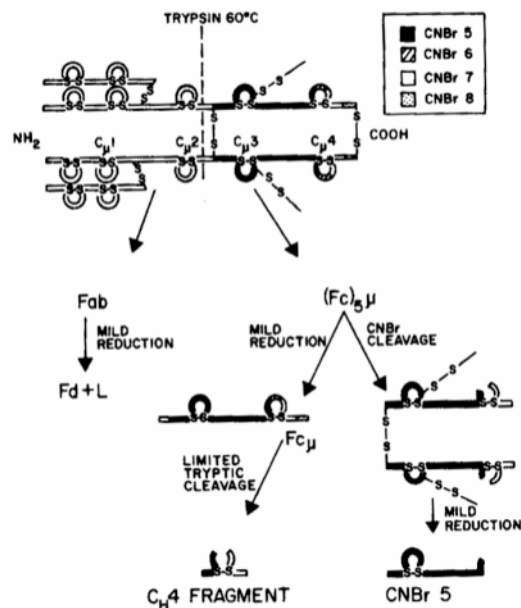


FIG. 1. Fragmentation of human IgM. Flow sheet summarizing the scheme used for isolation of the fragments tested for C \bar{I} -fixing ability. The site of specific cleavage into Fab and (Fc) $_{5\mu}$ fragments by trypsin digestion at 60°C is shown, and the four homology regions of the μ -chain (C μ 1-C μ 4) are identified. Cyanogen bromide cleaves the (Fc) $_{5\mu}$ into four fragments, designated CNBr 5 through CNBr 8. Limited tryptic cleavage of Fc μ produces the C $_{H4}$ fragment which consists of the COOH-terminal segment of CNBr 5 disulfide bonded to a fragment from the center of CNBr 7.

C \bar{I} -Binding Assay. The C-fixing ability of Dau IgM and its fragments was tested by a modified C \bar{I} fixation assay (4). The buffer utilized throughout the assay was dextrose-gelatin-veronal buffer of relative salt concentration 0.065 containing optimum amounts of Ca $^{++}$ and Mg $^{++}$ (11). An amount of functionally pure human C \bar{I} sufficient to yield 63% lysis was incubated with serial dilutions of the test fragments or with buffer as a control for 10 min at 30°C. EAC4 (sheep erythrocytes [E] sensitized with rabbit antibody [A] to E to which C components have been added) (1.5×10^9 /ml) were then added and incubation was allowed to proceed for an additional 10 min. The resulting EAC $_{1,4}$ cells were washed twice to remove unbound C \bar{I} , and resuspended in the same buffer. An excess of functionally pure C2 (11) was then added, followed by an excess of C3-9 (guinea pig C diluted 1:50 in 0.01 M EDTA buffer which supplies an excess of the late-acting components) and the degree of lysis determined spectrophotometrically at 412 m μ . Appropriate controls were included for each experiment (11). The amount of C \bar{I} fixed by an individual sample was calculated by subtracting the observed z value [$-\ln(1-y)$, where y = percent hemolysis], where z represents the average number of C \bar{I} molecules per cell, from the buffer control z value (11).

Results

A C \bar{I} fixation plot for Dau IgM and its C $_{H4}$ fragment is shown in Fig. 2. 50% of the available C \bar{I} was fixed by 1.5 μ g of intact IgM whereas 46 μ g of the C $_{H4}$ fragment were required to fix an equivalent amount of C \bar{I} . The amounts of the other fragments of IgM which were needed to bind 50% of the available C \bar{I} are summarized in Table I. Whereas only 1.5 μ g of Dau IgM were required to bind half of the available C \bar{I} , to our surprise up to 2,000 μ g of (Fc) $_{5\mu}$, which was the

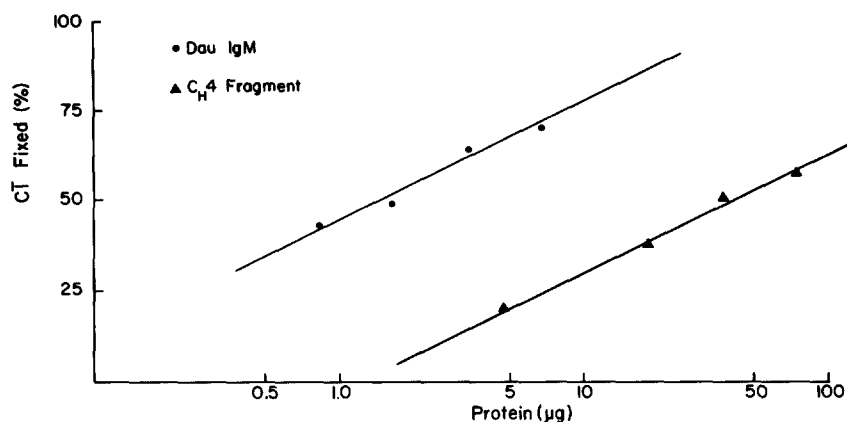


FIG. 2. Fixation of human C1 by Dau IgM and its $\text{C}_{\text{H}4}$ fragment. An amount of $\text{C}\bar{\text{I}}$ sufficient to produce 63% lysis of the cells in the absence of test material was used. After incubation the amount of unbound $\text{C}\bar{\text{I}}$ was determined and expressed as a percentage of that found when no test material was added during the incubation.

TABLE I
C $\bar{\text{I}}$ Fixation by Dau IgM and Its Fragments

Test material	Molecular weight*	Amount‡
	<i>daltons</i>	<i>μg</i>
Dau IgM	840,000	1.5
$\text{Fc}_{\delta\mu}$	340,000	>2,000
$\text{Fc}\mu$	34,000	40
Fab	48,000	>2,750
Reduced Fab	Unfractionated	>1,575
$(\text{CNBr } 5)_2$	40,000	20
Fab_{CNBr}	Unfractionated	>1,125
$\text{C}_{\text{H}4}$ Fragment	6,800	46

* Data on the molecular weights of Dau IgM and its fragments were determined as published [i.e., $\text{Fc}_{\delta\mu}$, $\text{Fc}\mu$, and Fab (6), $(\text{CNBr } 5)_2$ (7), and $\text{C}_{\text{H}4}$ Fragment].¹

‡ Amount required to bind 50% of the available $\text{C}\bar{\text{I}}$.

highest amount tested, did not bind $\text{C}\bar{\text{I}}$. However, if the assay were run such that whole human serum was incubated with the fragment (i.e., CH_{50} fixation assay), there was C consumption as indicated by a drop from 160 CH_{50} U to 95 CH_{50} U. This is in agreement with the data published by Plaut et al. (5) for the $(\text{Fc})_{\delta\mu}$ of another human monoclonal IgM (Dis). As the latter assay measures total hemolytic activity (11), it might be that $(\text{Fc})_{\delta\mu}$ activates the late-acting C components and very little, if any, $\text{C}\bar{\text{I}}$ is actually fixed. Partial reduction of $(\text{Fc})_{\delta\mu}$ provided $\text{Fc}\mu$ fragments which were effective in binding $\text{C}\bar{\text{I}}$. Controls, consisting of $\text{Fab}\mu$ and partially reduced $\text{Fab}\mu$, did not bind $\text{C}\bar{\text{I}}$ even at high concentrations. The 40,000 mol wt dimer of CNBr 5, which contains the $\text{C}\mu 3$ and a portion of the $\text{C}\mu 4$ domain, was found to bind $\text{C}\bar{\text{I}}$ effectively (only 20 μg being required). In contrast, the control CNBr digest of $\text{Fab}\mu$ was not capable of

binding $\text{C}\bar{\text{I}}$. Finally, 46 μg of the 6,800 mol wt $\text{C}_{\text{H}4}$ fragment were needed to bind 50% of the available $\text{C}\bar{\text{I}}$. Sedimentation velocity analysis of this fragment in the analytical ultracentrifuge in the buffer used for the $\text{C}\bar{\text{I}}$ fixation assay failed to reveal observable aggregation. In view of this, it is remarkable that on a molar basis the 60 residue $\text{C}_{\text{H}4}$ fragment is one-sixth as efficient as the $\text{Fc}\mu$ fragment.

Discussion

The only primary structure common to the two smallest $\text{C}\bar{\text{I}}$ -fixing fragments, $\text{C}_{\text{H}4}$ and CNBr 5, is the 24 residues on the amino-terminal side of the disulfide loop making up the $\text{C}\mu 4$ domain (residues 468–491 in IgM Ou [10]). These data lead us to suggest that this portion of the $\text{Fc}\mu$ is responsible for $\text{C}\bar{\text{I}}$ binding. However, the possibility that the $\text{C}\bar{\text{I}}$ -binding site in the intact molecule may also involve other regions of the $\text{Fc}\mu$ is not necessarily ruled out. In this regard it would be particularly interesting to test the $\text{C}\bar{\text{I}}$ -fixing ability of the $\text{C}\mu 3$ domain when a method for isolating it is found. It should be noted that the 24 residue sequence common to $\text{C}_{\text{H}4}$ and CNBr 5 has the highest degree of homology to human $\beta 2$ -microglobulin of any in the terminal domain, having 8 out of 24 identities and a total of 14 out of 24 conservative replacements (see Putnam et al. [10] and Peterson et al. [12] for comparative sequences). The relevance of this is pointed out by the fact that Painter et al. (13) have shown that $\beta 2$ -microglobulin fixes $\text{C}\bar{\text{I}}$ with high efficiency. On the other hand, a similar sequence comparison to the IgG domains (14) reveals that the 24 residues of $\text{C}_{\text{H}4}$ have a higher degree of homology to $\text{C}\gamma 3$ than to the $\text{C}\gamma 2$ domain which has been suggested to be responsible for C activation on the basis of data accumulated using the CH_{50} assay (2, 3). Specifically, there are 11 out of 24 identities and 4 conservative replacements between these 24 residues of the $\text{C}_{\text{H}4}$ fragment and the analogous 24 residues in $\text{C}\gamma 3$, compared to 6 out of 24 identities and 3 conservative replacements between these 24 residues in $\text{C}_{\text{H}4}$ and $\text{C}\gamma 2$. In view of this and of our data indicating CH_{50} but not $\text{C}\bar{\text{I}}$ fixation by $(\text{Fc})_{5\mu}$ it would be of interest to compare the $\text{C}\bar{\text{I}}$ -fixing ability of $\text{C}\mu 4$ with that of $\text{C}\gamma 2$ and $\text{C}\gamma 3$.

Summary

An insight into the structural features of human IgM that are responsible for its capacity to bind the first component of complement (C) has been obtained by examining the ability of IgM subfragments to bind active C1 ($\text{C}\bar{\text{I}}$). The smallest two fragments found to bind $\text{C}\bar{\text{I}}$ were the major CNBr fragment of the Fc portion of IgM and the $\text{C}_{\text{H}4}$ fragment of the carboxy-terminal domain. The smallest fragment which fixes $\text{C}\bar{\text{I}}$ has a disaggregated mol wt of 6,800, consists of 60 residues, and contains no carbohydrate. Structural considerations and sequence overlaps suggest that the amino-terminal side of the $\text{C}_{\text{H}4}$ domain (24 amino acid residues) might be responsible for fixing $\text{C}\bar{\text{I}}$.

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