Brief Definitive Report

$C\bar{1}$ FIXATION AND CLASSICAL COMPLEMENT PATHWAY ACTIVATION BY A FRAGMENT OF THE $C\mu4$ DOMAIN OF IgM*

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An insight into the structural features of human Waldenström IgM proteins which are responsible for their capacity to bind and activate the first component of complement (C) has been obtained. The basic approach has involved enzymatic and chemical fragmentation of the IgM molecule followed by examination of the resulting fragments for C-fixing activity. A 6,800 mol wt fragment obtained from the terminal $C\mu4$ domain of a Waldenström IgM molecule has been shown to bind active C1 ($C\bar{1}$) (1). This subfragment of IgM, which was designated the C_H4 fragment, was found to be composed of the $C\mu4$ domain of the $Fc\mu$ minus two tryptic peptides in the center of the loop. The first set of experiments described in this communication were designed to evaluate the dependence of $C\bar{1}$-binding ability upon the presence of the intrachain disulfide bond in the C_H4 fragment. In addition, the C_H4 fragment before and after reduction, as well as each of its component chains, were examined for their ability to trigger the activation of the classical C pathway. Based upon these studies we are able to present evidence suggesting that the C1-activating function remains intact in these three fragments.

Materials and Methods

Preparation of IgM and Its C_H4 Fragment. The methods used to isolate IgM and to obtain the C_H4 fragment have been previously reported (1, 2).

Isolation of the Two Peptide Chains Comprising the C_H4 Fragment. C_H4 fragments (5 mg/ml in 5 M guanidine-HCl adjusted to pH 7.5 by Tris base) were reduced by the addition of 2-mercaptoethanol to a final concentration of 0.1 M. After 3 h of incubation at room temperature, a 10% excess of iodoacetamide was added and the pH was maintained at 7.5 by the addition of Tris base until alkylation was complete. The reduction mixture was desalted by passage through a Bio-Gel P-2 column (Bio-Rad Laboratories, Richmond, Calif.) eluted with 1% NH_4HCO_3 and lyophilized. Fractionation of the two peptides comprising the C_H4 fragment was achieved by chromatography through a column of Whatman DE-52 cellulose (H. Reeve Angel & Co., Inc., Clifton, N. J.) equilibrated with 0.025 M NH_4HCO_3, pH 8.0. After application of the sample, the column was washed with one to two total column vol of 0.025 M NH_4HCO_3, pH 8.0, and a linear gradient (0.025 M NH_4HCO_3–1 M NH_4HCO_3) was begun. Pooled fractions from each of the two peaks eluted from this column were lyophilized and redissolved in dextrose gelatin veronal buffer containing

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optimum amounts of Ca^{++} and Mg^{++} (3).

C Assays. A description of the modified $C\bar{1}$-fixation assay has been previously reported (1). The ability to activate C1 was examined by incubating the fragments with normal human serum (NHS), followed by C4 titration. Titration of C4 was carried out by the procedure described by Gaither et al. (4) using C4-deficient guinea pig serum (5).

Amino Acid Analyses. Acid hydrolysis was performed in 6 N HCl at 108°C for 20 h before analysis on a Durrum amino acid analyzer (Durrum Instrument Corp., Palo Alto, Calif.) using the single column high pressure system.

Sequence Determination. The amino-terminal residues of each of the two disulfide-linked peptides comprising the C_H4 fragment were first determined by the Edman-dansyl chloride technique (6, 7). The intact C_H4 fragment was sequenced automatically on a Beckman Model 890 sequencer (Beckman Instruments, Inc., Fullerton, Calif.). The identification of released phenylthiohydantoin amino acids was performed by gas chromatography.

Results

The C_H4 fragment isolated from the Waldenström IgM, Dau, after limited tryptic cleavage of the $(Fc)_5\mu$ fragment was found to consist of 24 residues on the amino-terminal side of the $C\mu4$ domain linked by a disulfide bond to 32 residues on the carboxyl side of the loop [i.e. according to the residue numbering of the IgM, Ou, by Putnam et al. (8) the C_H4 fragment consists of residues 468-491 joined to residues 515-546, with 23 residues having been cleaved out of the center of the disulfide loop (1, 2)].

Separation of the two peptide chains comprising the C_H4 fragment was achieved by chromatography of the totally reduced sample through a DE-52 cellulose column. Amino acid compositional and sequence analysis of the first major peak eluted by the NH_4HCO_3 gradient indicated it consisted of the amino-terminal chain of the $C\mu4$ domain (peptide A), while the second major peak was found to consist of the carboxy-terminal chain of the disulfide loop (peptide B). The sequence of this region as published by Putnam et al. (8) for the IgM, Ou, and by Watanabe et al. (9) for the IgM, Gal, has been given in Fig. 1. In a comparative study of the Fc sequence of three additional Waldenström IgM proteins, including the Dau IgM used for the studies discussed herein, Florent et al. (10) confirmed the sequence of residues 470-472, 515-517, and 519-526 for Dau IgM and concluded that the Fc sequence is closely similar if not identical for most and possibly all human pathological μ-heavy chains. A spot check via double sequencing in our automated sequenator of the intact Dau C_H4 fragment confirmed the residues indicated by arrows in the sequence given in Fig. 1.

The $C\bar{1}$-fixing ability of the Dau C_H4 fragment before and after reduction, as well as of each of its component chains (peptides A and B), was measured by the $C\bar{1}$-fixation assay. As seen in Fig. 2, there was a marked reduction in $C\bar{1}$-binding ability after cleavage of the disulfide bond. However, peptides A and B possessed similar $C\bar{1}$-fixing abilities, each being more efficient than the totally reduced mixture of the two fragments. Table I presents the $C\bar{1}$-fixation molar ratios obtained from the data depicted in Fig. 2. If the number of micromoles of intact C_H4 fragment required to fix one-half of the available $C\bar{1}$ is taken as 1, then peptide B is 76 times less efficient, peptide A is 121 times less efficient, and the reduced C_H4 fragment (peptides A plus B) is 330 times less efficient than the parent fragment.

We next examined the ability of the C_H4 fragment and of its respective purified peptide chains to consume hemolytic C4 on incubation with fresh NHS.

FIG. 1. The amino acid sequence of the C_H4 fragment as published by Putnam et al. (8) and by Watanabe et al. (9) is given in this figure. The residues indicated by arrows are those obtained by sequencing the Dau C_H4 fragment.

FIG. 2. Fixation of human C1 by Dau C_H4 fragments before and after reduction, and by each of its respective purified peptide chains, designated peptides A and B. An amount of $C\bar{1}$ sufficient to produce 63% lysis of the cells in the absence of test material was used. After incubation the amount of unbound $C\bar{1}$ was determined and expressed as a percentage of that found when no test material was added during the incubation.

As seen in Table I, the results indicated that all three fragments were equivalent in their ability to bring about C4 consumption. The negative controls for the C4-consumption assay were the 17 residue cyanogen bromide fragment which is derived from the center of the $C\mu4$ domain (11) (i.e., the portion of the $C\mu4$ domain that is cleaved out to produce the C_H4 fragment) and the pFc′ fragment (i.e., $C\gamma3$ domain) of human IgG1 (12).

However, before concluding that C4 consumption is a reflection of C1 activation it is necessary to prove the following: (*a*) that the immunoglobulin fragments are not activating a serum enzyme other than C1s which can in turn consume C4, and (*b*) that the fragments are not directly interfering with C4 or some other components of the test system. The first point was not examined directly. However, since the fragments have been shown to bind isolated $C\bar{1}$, it seems probable that they, in fact, bind and activate C1 in NHS. In order to determine if the C_H4 fragment could possibly be acting directly on C4 the

TABLE I
Complement Fixation by Dau C_H4 and its Fragments

Test material	C$\bar{1}$ fixation		C4 consumption	
	Amount*	Molar ratio‡	Units C4 fixed per μmol	Molar ratio§
	μg			
Dau C_H4	1.6	1	26,979	1.0
Peptide B	56	76	34,019	1.2
Peptide A	70	121	46,033	1.7
Dau C_H4 reduced	400	330	26,266	1.0

* Amount required to bind 50% of the available C$\bar{1}$.
‡ μmol of fragment required to fix 50% of the available C$\bar{1}$ per μmol of intact C_H4 required to fix 50% of the C$\bar{1}$.
§ C4 site-forming units (SFU) consumed per μmol of fragment divided by C4 SFU consumed per μmol of intact C_H4.

fragments were incubated with functionally purified C4 (Cordis Laboratories, Miami, Fla.) followed by a titration of the remaining C4. When compared with the control, which consisted of buffer plus C4, it was observed that no C4 had been consumed by the C_H4 fragment in the absence of C$\bar{1}$.

Discussion

The ability of the reduced Dau C_H4 fragment and of its individual peptide chains to fix C$\bar{1}$ and to consume C4 in NHS has been examined. The fact that cleavage of the disulfide bond in the C_H4 fragment resulted in a decrease in the efficiency of C$\bar{1}$ fixation was not surprising. However, it was not expected that peptide A and peptide B would fix C$\bar{1}$ and consume C4 in a similar manner. Also of interest was the fact that the individual A and B peptide chains were more efficient at fixing C$\bar{1}$ than the reduced mixture of the two chains. In analyzing these results it is necessary to remember that the C$\bar{1}$-fixation assay measures the competition of immunoglobulin fragments with the EAC4 cells (sheep erythrocytes [E] sensitized with rabbit antibody [A] to E to which C components have been added) for the available C$\bar{1}$, the final result depending on the relative association constants. Perhaps when only one of the peptide chains is present the binding is more avid than when a mixture of the two chains is involved. However, C$\bar{1}$ activation as manifested by C4 consumption occurs to a similar degree with all three fragments. Moreover, the reduced C_H4 fragment appears to consume C4 as effectively as each of its individual chains, and as effectively as the intact C_H4 fragment. This suggests that tight binding of C1 may not be a prerequisite for activation as measured by C4 consumption. Transient binding of C1 by the individual A and B peptide chains may therefore be sufficient to activate C1, which in turn consumes C4.

In conclusion, the observation that C1-activating and weak C$\bar{1}$-fixing ability can be associated with the completely reduced and alkylated peptides A and B indicates that although the intrachain disulfide bond may stabilize the tertiary structure of the area in natural conditions, this bond is not absolutely essential for the expression of these activities.

Summary

A 56 residue fragment derived from a Waldenström IgM protein and consisting of 24 residues of the amino-terminal portion of the $C\mu4$ domain disulfide

bonded to 32 residues of the carboxy-terminal region of the loop has been shown to fix active C1 ($C\bar{1}$) in a $C\bar{1}$-fixation assay. Cleavage of the disulfide bond within the C_H4 fragment resulted in a marked decrease of $C\bar{1}$-fixing ability, although the isolated A and B fragments did retain a limited ability to fix $C\bar{1}$.

Upon incubation with normal human serum the intact C_H4 fragment and equal molar amounts of the isolated A and B peptides consumed C4 suggesting that the C1-activating determinant of IgM remains intact in these three fragments. Furthermore, on a molar basis the intact or the reduced C_H4 fragment consumed C4 as effectively as each of its component chains suggesting that transient binding of C1 by the individual A and B peptide chains is sufficient to activate C1.

On the basis of these observations it is proposed that a classical complement fixation function, i.e. C1 binding and activation, can be localized within a region of the IgM molecule corresponding to the $C\mu4$ domain.

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