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Interchain and Intrachain Disulfide Bridges of a Human Immunoglobulin M: Detection of a Unique Fragment

B. FRANGIONE, F. PRELLI, C. MIHAESCO*, AND E. C. FRANKLIN

Department of Medicine, New York University School of Medicine, New York, N.Y. 10016

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ABSTRACT **Studies of the amino acid sequences around half-cystine residues in an immunoglobulin M have revealed an unexpectedly high number. At least 14 different sequences were found in the μ chain (V_{HIII} subclass). Four of these were involved in interchain disulfide bridges and at least 10 in intrachain bridges. Five came from the κ chain (κ_{III} subclass, Inv b). Several others, although having a high degree of homology, were not identical with those of either the μ or κ chains. These results support the concept of an additional fragment in γM molecules, although its function and localization remain to be determined.**

Although the overall structural features of all five classes of immunoglobulins are strikingly similar, there are differences in the size of the heavy chains and their assembly that distinguish the different classes from each other (1). This is particularly true for the γM fraction, a disulfide-linked pentameric polymer of the basic H_2L_2 subunit whose H (heavy) chains are larger than those of the other classes. Recently, the possibility has been raised that γA and γM contain an additional linking peptide, the size of a light chain, known as the J chain (2, 3), and possibly also another chain related to or identical with light chains (4, 5).

Studies of light chains, and also of the four subclasses of γ chains, have demonstrated that characterization of the interchain disulfide bridges is of particular value in detecting differences between subclasses, while comparison of the intrachain disulfide bonds has revealed striking homologies (6). Experiments were therefore undertaken to examine these features in a macroglobulin (Hel) belonging to a previously defined reactive (minor) μ-chain subclass with κ_{III} light chains. (7).

MATERIALS AND METHODS

Protein Hel ($\gamma M\kappa$) was isolated from the serum of a patient with macroglobulinemia of Waldenström by precipitation of the euglobulin and filtration of the euglobulin solution on Sephadex G-200. The intersubunit bond was selectively reduced with 20 mM cysteine (8). The molecule was partially reduced with 5 mM dithiothreitol (DTT) and completely reduced in 6 M guanidine with 10 mM DTT. The reduced molecules were alkylated with [^{14}C]iodoacetic acid ([^{14}C]IAc) or unlabeled IAc. Heavy and light chains were prepared by filtration of partially reduced γM on Sephadex G-100 in 1 M acetic acid. (Fab″) 2μ fragments were prepared with pepsin (9). Techniques for cleavage of the peptide chains by pepsin and trypsin, separation and characterization of the resulting peptides by high-voltage electrophoresis, radioautography, analysis, and sequencing of amino acids have been described (10).

RESULTS

To label the interchain disulfide bonds, we mildly reduced the protein with 5 mM DTT and alkylated it with [^{14}C]IAc. The labeled heavy chain was digested with trypsin and fractionated in 1 M acetic acid on a Sephadex G-50 column. The precipitate from the trypsin digest was treated with pepsin and fractionated by high-voltage paper electrophoresis at pH 3.5. Fig. 1 shows the elution pattern of the tryptic digest. The material associated with radioactive peaks 2, 3, 4, and 5 was subjected to high-voltage electrophoresis at pH 3.5, 6.5, and 2.1 in order to purify the carboxymethylcysteine peptides. Peak 1 was again digested with pepsin

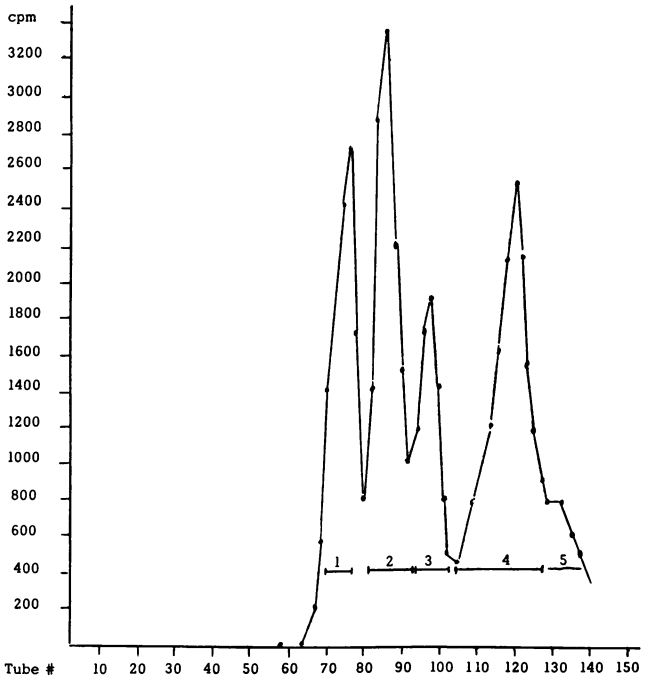

FIG. 1. Chromatographic separation on Sephadex G-50 in 1 M HOAc of a tryptic digest of the heavy chain of γM Hel produced by partial reduction and alkylation with [^{14}C]IAc.

Abbreviations: DTT, dithiothreitol; IAc, iodoacetic acid.

* Present address: Laboratory of Immunochemistry, Research Institute on Blood Diseases, Hopital Saint-Louis, Paris 10e, France.

TABLE 1. *Sequence and function of carboxymethylcysteine peptides derived from the μ chain of partially reduced γM protein Hel*

Sephadex G-50 Peak	Peptide	Sequence	Function and alternative terminology (12)
1	T1Pb	Ala-Ser-Ile-Cys-Glu-Gln	
	T1Pd	Ile-Cys-Glx-Glx	
	T1Pc	Ile-Cys-Glu	Inter-subunit (μ3, 5)
	T1	(CMCys, Asp_3, $Thr_{1.7}$, $Ser_{2.5}$, $Glu_{2.5}$, Pro, $Gly_{1.5}$, $Ala_{1.6}$, Val, $Ile_{1.4}$, $Phe_{0.6}$, Trp, $His_{1.2}$, Arg, CHO)	
2	T2	CHO Gly-Leu-Thr-Phe-Glx-Glx-Asx-Ala-Ser-Ser-Met-Cys-Val-Pro-Asx-Glx-Asx-Thr-Ala-Ile-Arg T5 (Fab″)2μ TP	Inter-heavy (intra-subunit) (μ2)
3	T3b	CHO Asx-Val-Ser-Leu-Val-Met-Ser-Asx-Thr-Ala-Gly- Thr-Cys-Tyr* T3cP	C-terminal inter-heavy (intra-subunit) (μ4)
4	T4c	Asx(Val,Ser,Leu,Val,Met,Ser,Asx,Thr,Ala,Gly,Thr,CMCys,Tyr)*	"
	T4	Tyr-Val-Thr-Ser-Ala-Pro-Met-Pro-Glx-Pro-Glx-Ala-Pro-Gly-Arg	Alkylated Met
Ppt	TP	Ser-Cys(Glu,Asp,Ser,Thr,Ser,Asp,Ser,Pro)	Heavy-light (μ1)

T, trypsin; P, pepsin; Ppt, precipitate; CMCys, *S*-carboxymethylcysteine.
* The same peptide was obtained without tyrosine.

and treated as above. In each case, the major band containing a radioactive peptide with carboxymethyl cysteine (CMCys) was isolated, analyzed for amino acids, and sequenced. Table 1 lists the partial sequences of the major CMCys-containing peptides. Their function appears to be as follows: Ppt-TP corresponds in composition to the previously identified bridge between heavy and light chains (11–13). It was shown to be in the same region as the C-terminal end of the light chain on a diagonal map of the pepsin–trypsin digest of the intact protein and was therefore presumably bridged to it.

T4 contains methionine and is rich in proline, but is devoid of CMCys. The large amount of proline suggests a relationship to the hinge peptides of γ and α chains.

T3b and T4c, closely related peptides, were devoid of a basic residue and must therefore have come from the carboxy-terminus. T4c differed from T3b in lack of carbohydrate and T3b contained two approximately equal components differing only in the presence or absence of C-terminal tyrosine. This tetradecapeptide (T3b) is somewhat larger than the nonapeptide previously obtained by Wikler *et al.* by cleavage with cyanogen bromide (14). On the basis of prior studies (15) and the evidence presented below, it seems likely that the penultimate half-cystine represents an intra-subunit bond between heavy chains (H-H).

T2 is a large glycopeptide containing CMCys and methionine. Since part of the μ chains was cleaved between Met and Cys, a closely related CMCys-containing decapeptide derived from the C-terminal end of T2 was isolated as T5, as previously noted by Metzger (16). This peptide was identified as the source of the intrasubunit H-H bond located in the (Fab″) 2μ fragment, since a related tryptic peptide (Fab″)-2μ TP could be isolated as the sole radioactive peptide from an (Fab″) 2μ fragment reduced with 10 mM cysteine and alkylated with [^{14}C]IAc so as to cleave only the H-H bond.

T1Pb, T1Pc, and T1Pd were of particular interest, since at least one (T1Pc) appeared to be involved in the intersubunit H-H bridge. To identify the intersubunit bridge, we reduced the protein with 20 mM cysteine and alkylated it with [^{14}C]IAc (8). The labeled subunits were obtained in 50% yield by gel filtration on Sephadex G-200; calculation of the specific activity indicated that two SH residues had been labeled per 180,000 daltons of protein.

In order to obtain the labeled cysteinyl peptide, the same labeled γM subunits were digested with pepsin and trypsin and subjected to high-voltage electrophoresis at pH 3.5; this gave one major and two minor radioactive bands. The sequence of the major peptide from the partially reduced heavy chain was Ile-Cys-Glu, while the minor ones were similar to peptides T1Pd (Ile-Cys-Glx-Glx) and T1Pb (Ala-Ser-Ile-Cys-Glu-Gln). Although the smallest peptide might be related to the two larger ones, its precise origin is uncertain; in a recent report, Miekka and Deutsch (17), using a different method, have localized the intersubunit bridge to a peptide with the sequence: Ala-Ser-Ile-Cys-Glu-Asp, which could also have given rise to Ile-Cys-Glu. Two alternative explanations might therefore be in order. Although a Glu–Asp interchange might have occurred in the proteins studied by Deutsch and by us, it seems likely that two different cysteinyl residues are labeled under the conditions of the experiments, one or both of which might serve as a direct or indirect link between subunits.

In another experiment, the intrachain disulfide bridges

TABLE 2. *Sequence and possible position of peptides containing half-cystine obtained from completely reduced and alkylated μ chains and from a diagonal map of the whole molecule*

Peptide	Sequence	Possible position of disulfide bridges	
A. Peptides containing half-cystine derived from intrachain disulfide bridges of a μ chain			
TP 1	Leu-Ser-Cys-Ala-Ala-Ser-Gly	Fd variable	1*
TP 2	Tyr-Cys-Ala-Lys		
TP 2′	Tyr-Cys-Ala(Asp_2,$Thr_{0.9}$,$Glu_{1.1}$,$Gly_{1.1}$,$Val_{0.7}$,$Met_{0.4}$,His,Lys)		
TP 3	Val-Gly-Cys-Leu	Fd constant	2*
T 4	Asx-Val($Asp_{1.8}$,$Thr_{1.2}$,Glu_3,$Gly_{2.1}$,$Val_{0.6}$,$Met_{0.7}$,$His_{0.9}$)Cys-Lys		
T 5	Leu-Ile-Cys-Gln-Ala-Thr-Gly-Phe-Ser-Pro-Arg	Fc_2-NH_2 terminal (?)	3*
T 6	Phe-Thr-Cys-Thr-Val-Thr(Thr,Pro_2,Asp,Ser,Leu_2,His)Lys		
TP 7	Leu-Thr-Cys	Extra	4*
TP 8	Phe-Thr-Cys-Arg		
TP 9	Ile-Thr-Cys-Leu	Fc-C-terminal	5*
TP 9′	Glu-Ser-Ala(Thr,Ile,Cys)		
TP 10	Tyr-Thr-Cys-Val		
TP 10′	Tyr(Thr,Cys,$Val_{1.2}$,Ala)		
B. Peptides containing half-cystine derived from intrachain disulfide bridges and the C-terminal end of the κ chain			
TP 11	Gly-Glu-Cys	C-terminal	
TP 12	Ser-Cys-Arg	Variable $κ_{III}$†	
TP 13	Tyr-Cys-Gln-Gln-Arg		
TP 14	Val-Cys-Leu	Constant	
TP 15	Val(Tyr,Ala,Cys-Glu)		
C. Additional peptides containing half-cystine obtained from the whole molecule			
TP 16	Ala-Asx-Tyr-Glx-Cys-Lys		
TP 17	[ ] Asp-Thr-Ala(Cys,Ser,Glu,Gly)		
TP 18	Ala(Ser,Ile,Cys,Glu,Asp_2)		

T, trypsin; TP, trypsin–pepsin; [ ] = negative N-terminal.

* *1*, Ref. 18; *2*, ref. 23; *3*, slight homology of no. 6 with γ chains (ref. 6); *4*, marked homology to peptides T5 and T6; *5*, homology to γ chains, Fc-C-terminal loop (ref. 6). † N-terminal light chain — Glu.

in the μ chain were obtained from the heavy chain prepared by mild reduction and alkylation of γM with nonradioactive IAc. This μ chain was then further reduced with 10 mM DTT in 6 M guanidine and alkylated with [^{14}C]IAc. After trypsin digestion, the soluble material was fractionated into five major radioactive peaks by elution from a Dowex 1×2 column with increasing amounts of acetic acid. The precipitate was further treated with pepsin and divided into seven radioactive bands by chromatography in butanol–acetic acid–water–pyridine 15:3:12:10. All peaks and bands were further purified by high-voltage electrophoresis. Table 2*A* shows the partial sequences of the 10 labeled peptides obtained in highest yield. One of these, TP1, identified the μ chain as belonging to the V_{HIII} subclass (18).

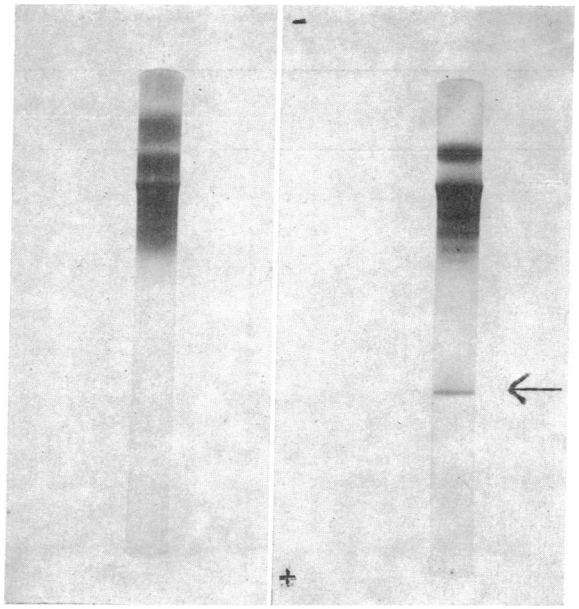

FIG. 2. Acrylamide gel electrophoresis of (*left*) light chains from γG, (*right*) light chains from γM Hel. The most anodal band (→) has a mobility similar to that reported for J chains (2,3).

Peptides containing additional half-cystine residues were isolated from a diagonal map of the pepsin- and trypsin-digested macroglobulin. Five of these (Table 2*B*) were derived from the light chain, and the sequence Ser-Cys-Arg is characteristic of the V_{III} subclass (1). Three additional peptides (Table 2*C*), isolated in relatively poor yield, could not be placed in any of the heavy- or light-chain peptides. Since it seemed possible that they were derived from the J chain, the "light-chain" fraction was examined by polyacrylamide disc gel electrophoresis in 10 M urea, pH 9.4, to determine if J chains were present. A band migrating rapidly to the anode with a mobility similar to that of J chains was seen (Fig. 2*b*) (2, 3). Since a similar band was absent from light chains prepared from γG, it seems likely that J chains were associated with the γM molecule. One of the three peptides (TP18) Ala (Ser-Ile-Cys-Glu-Asp_2) corresponds to part of the intersubunit bridge identified by Miekka and Deutsch (17); it is not known if it is also present in peak T1, Table 1 (see above).

DISCUSSION

Our results confirm observations from several other laboratories (16, 19) that cleavage of all interchain disulfide bonds yields at least four half-cysteinyl residues per μ chain, and provide direct support for some of the previously postulated functions of these residues. Counting from the N-terminus, the first of these four half-cystine residues is involved in the H-L bridge, as shown by our diagonal electrophoresis and previously (11–13). The second half-cystine participates in a μ-μ bridge linking the two halves of the pepsin fragment (Fab″) 2μ, and is probably located in the "hinge" region (12, 16, 20). Unlike the γ chains, the hinge region of the μ chain contains only one half-cystine and a carbohydrate group, and lacks the proline-rich sequence characteristic of γG and γA molecules. A peptide high in proline (T4) but devoid of cysteine was located, but its precise position is unknown. The third half-cystine, linking the 8S (H_2L_2) subunits, is known to be in the Fcμ fragment (21). Its precise position has not been located, nor is it known whether it binds two H chains directly or via the J chain (3). The fourth, the penultimate half-cystine residue in the μ chain, represents another intrasubunit H-H bond (15, 22).

The precise number and location of the intrachain disulfide bridges of the μ chain are difficult to determine because sufficient data are not available on the J chain, the recently discovered extra chain that resembles light chains under the conditions of fractionation used in this study (3). Since the heavy chain was free of light chains on immunoelectrophoresis, and since the J chain is largely dissociated under the conditions of reduction used, it seems likely that all the peptides isolated in good yield from the completely reduced μ chain were derived from the H chain. Since at least 10 cysteinyl-containing peptides were isolated from the completely reduced μ chain, it seems possible that there are at least five intra-H-chain disulfide loops, rather than the usual four. The possible location of each of these, based on homology considerations and on data obtained from the diagonal map, is shown in Table 2. Such a value would be in line with the larger molecular weight of μ chain and might be the result of an additional gene duplication, an evolutionary relationship that is supported by the close degree of homology around the cysteine residues. The homology between peptides 5, 6, 7, and 8 and the peptides making up the amino-terminal loop of the Fc fragment in the γ-chain subclasses (6) suggests that the duplication might have involved the gene coding for this region.

The origin of the additional peptides isolated from the diagonal map presents a special problem, since little information is available about the extra chains reported to be present in γM molecules. Diagonal electrophoresis of a pepsin–trypsin digest of the "light chain" fraction revealed not only the usual four cysteic acid peptides derived from the intrachain disulfide bridges of κ chains (Table 2*B*), but several additional peptides. While these may come from an uncommon κ chain, it seems more likely that they derive from one of the recently-described extra chains. Attempts to isolate such fragments and subject them to similar studies are currently in progress.

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