Intrachain Disulphide Bridges in Immunoglobulin G Heavy Chains

THE F_c FRAGMENT

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The disulphide bridges of the F_c fragment (*C*-terminal half of the heavy chain) have been studied in several human immunoglobulins, containing heavy chains of different antigenic types ($\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$), and in heavy-chain-disease proteins. Two intrachain disulphide bridges were found to be present. The sequences appear to be identical in the F_c fragments of two types of chain studied ($\gamma 1$ and $\gamma 3$), and very similar to corresponding sequences of the F_c fragment in rabbit. These results suggest that the *C*-terminal half of the heavy chains is covalently folded (in a similar fashion to the light chains) with a *C*-terminal loop and an *N*-terminal loop. The similarity is emphasized by comparison of the sequence and location of the disulphide-bridged peptides of the *C*-terminal loop of heavy and light chains. The *N*-terminal loop, on the other hand, appears to be very different in F_c fragments and light chains. The *C*-terminal loop is the only one present in the F_c fragment.

γ G-Immunoglobulin is a highly heterogeneous population of molecules with a remarkable similarity in general structure. Each molecule is composed of two heavy and two light chains held together by disulphide bridges (Porter, 1962). Studies of the disulphide bridges of the two types of human light chains (κ - and λ -chains) have shown that in both cases the molecule is composed of an *N*-terminal and a *C*-terminal loop, which fold the 'variable' and the 'invariable' halves of the molecule respectively (Milstein, 1966*a,b*).

The normal population of human heavy chains contains four types of antigenically distinct polypeptides, $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4^*$ (Grey & Kunkel, 1964; Terry & Fahey, 1964). Peptide 'maps' of the more homogeneous proteins isolated from myeloma patients (monoclonal proteins) showed considerable similarity in the *C*-terminal half of the molecule (F_c fragment) of the different antigenic types (Frangione, Franklin, Fudenberg & Koshland, 1966). The *C*-terminal octadecapeptides of $\gamma 1$, $\gamma 2$ and $\gamma 3$ heavy chains are almost identical (Press, Piggot & Porter, 1966).

It is the purpose of this paper to present studies

of the disulphide bridges of several immunoglobulins and their fragments. These studies define two disulphide bridges located in the F_c fragment. One of the bridges (the *C*-terminal loop) has a striking similarity to the *C*-terminal loop of light chains.

This study has been greatly facilitated by the use of proteins from patients suffering from heavy-chain disease. These proteins are excreted in the urine and appear to be similar to, but not identical with, the artificially produced F_c fragments (Franklin, 1964).

MATERIALS AND METHODS

γ G-Immunoglobulin myeloma proteins and heavy-chain proteins (Cra and Zuc) were isolated from the serum and urine respectively by starch-zone electrophoresis. Papain fragments were prepared and separated as described by Frangione *et al.* (1966). The F_c fragment (Poulik, 1966) was obtained after 18 hr. of papain digestion and isolated on starch-block electrophoresis. Glutamic acid (or glutamine) was the *N*-terminal amino acid of F_c fragment from normal pooled γ G-immunoglobulin and γ G1 (Car) myeloma protein, although, in the case of myeloma protein Car, minor components were also present. Isolated heavy chains were prepared from γ G-immunoglobulin of a normal individual by the method of Fleischman, Pain & Porter (1962).

Disulphide-bridged peptides were isolated by the performic acid-oxidation diagonal technique after peptic digestion [1:40 (w/w) enzyme/substrate ratio in 0.05 N-HCl,

* Abbreviations: $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$, newly accepted designations of the heavy chains of the immunoglobulins γ G1, γ G2, γ G3 and γ G4, formerly known as We or $\gamma 2b$, Ne or $\gamma 2a$, Vi or $\gamma 2c$, and Ge or $\gamma 2d$ respectively (World Health Organisation, 1966); Cy_a and MetSO₂ (in amino acid sequences), cysteic acid and methionine sulphone.

Fig. 1. 'Map' of the disulphide bridge of: (a) Fc fragment of myeloma protein Car; (b) F'c fragment of heavy-chain-disease protein Cra. Each 'map' was divided into three regions (1, 2 and N), which included the peptides present in a vertical line. The N region contained peptides that were neutral before performic acid oxidation. A peptic digest of each protein was fractionated by electrophoresis at pH 6.5. A marker strip cut along the paper was oxidized with performic acid vapour. The strip was then sewn on to a new sheet of paper, as shown in the Figure, and run again at pH 6.5 at right angles to the original direction. A mixture of amino acids and coloured markers was run at the side of the paper; the arrow indicates the position of the red marker spot, made with a red Pentel pen.

for 16 hr. at 37°. The digest was subjected to electrophoresis on a sheet of Whatman paper in the first dimension at pH 6.5, and a marker strip was then oxidized with performic acid vapour to convert cystine residues into cysteic acid. The strip was sewn on to a new paper and was then run in the second dimension, again at pH 6.5, to complete the diagonal. Peptic peptides were digested with trypsin [1:50 (w/w) enzyme/substrate ratio at pH 8 in 1% (w/v) NH_4HCO_3 buffer, for 6 hr. at 37°]. Peptide 'maps' were stained with cadmium-ninhydrin; this solution contained 14% (v/v) cadmium acetate solution [6g. of cadmium acetate in 30% (v/v) acetic acid] in 1% (w/v) ninhydrin in acetone. For preparative purposes the cysteic acid peptides isolated, after oxidation, by paper electrophoresis at pH 6.5 were further purified by electrophoresis at pH 3.5, except peptides 1c, 3a and 2c, which were purified by electrophoresis at pH 2.1. Amino acid analysis, Edman degradation, N-terminal analysis and electrophoretic conditions were the same as those used by Milstein (1966b).

RESULTS

Fig. 1(a) is a photograph of the disulphide-bridge peptide 'map' of the peptic digest of the Fc fragment from a $\gamma\text{G}1$ myeloma protein (Car). For convenience three regions (1, 2 and N) can be considered in the pattern by dividing it into vertical bands. Each vertical band contains cysteic acid

Fig. 2. Location and nomenclature of the cysteic acid peptides discussed in the text.

peptides departing from the diagonal that derive from a disulphide bridge. These peptides have been numbered as shown in Fig. 2. The presence of these spots in disulphide-bridge 'maps' of several proteins and fragments was investigated and the results are summarized in Table 1.

Table 1. Common intrachain disulphide bridges in γ G-immunoglobulins of different antigenic types

Only the presence of the peptide spots shown in Fig. 2 is recorded in this Table; Nb, Nc and Nd are not included because they are not separated in the 'maps'. * Heavy-chain-disease protein.

Protein	Subclass	Part of molecule	Spot					
			Na	Ne	1a	1c	2a	2c
Car	γ G1	Whole molecule	+	+	+	+	+	+
		Heavy chain	+	+	+	+	+	+
		Light chain	—	—	—	—	—	—
		Fc fragment	+	+	+	+	+	+
		F'c fragment	+	+	—	—	—	—
		Fab fragment	—	—	—	—	—	—
Cra*	γ G1	Whole molecule	+	+	+	+	+	+
		F'c fragment	+	+	—	—	—	—
Zuc*	γ G3	Whole molecule	+	+	+	+	+	+
Bru	γ G3	Whole molecule	+	+	+	+	+	+
Til	γ G2	Whole molecule	+	+	+	+	+	+
Wur	γ G4	Whole molecule	+	+	+	+	+	+
Normal	—	Whole molecule	+	+	+	+	+	+
		Heavy chain	+	+	+	+	+	+

Table 2. Peptic peptides isolated from the heavy-chain-disease protein Cra

			Amino acid composition (moles of amino acid/mole of peptide)					
Peptide	...	...	Na	Ne	1a	1c	2a	2c
Lys						2.7		3.0
Arg							1.2	
Cya			1.0	1.0	0.8	0.9	0.7	Lost (+)
MetSO ₂			0.9					
Asp					1.1	1.0		1.1
Thr				0.8			2.1	
Ser			2.1			1.1	1.0	1.0
Glu						1.2	1.0	1.2
Pro						2.1	0.9	2.0
Ala						1.9		2.0
Val			1.0		(2.7)*	1.2	2.0	1.2
Ile						0.9	1.1	1.0
Leu				1.0		0.9		1.0
Tyr						0.6		0.7
Phe			1.0					
Yield (μ mole)			0.11	0.21	0.006		0.46	0.6
Mobility at pH 6.5 relative to aspartic acid			0.4	0.6	0.83		0.24	—0.15

* Value obtained after 90 hr. hydrolysis; the value was only 1.8 after 20 hr.

Table 3. Peptides from trypsin digestion for 6 hr. of peptide 2c of protein Cra, fractionated by paper electrophoresis at pH 6.5 and 2.1

Peptide	Yield (μ mole)	Amino acid composition (moles of amino acid/mole of peptide)	Mobility (pH 6.5)	Sequence ('dansyl'-Edman)
2cT1	0.12	Glu, 1.0; Pro, 2.0; Ala, 2.0; Ile, 1.0; Leu, 1.0	0.32	Ala-Leu-Pro-Ala-Pro-Ile-Glu
2cTN	0.05	Lys, 1.0; Cya, 0.8	0.13	Cya-
2cT2	0.04	Lys, 1.9; Cya, 0.7; Tyr, 0.9	—0.36	Tyr-
2cT3	0.1	Lys, 1.0; Asp, 1.0; Ser, 1.0; Val, 1.0	—0.43	Val-Ser-Asn-Lys
2cT4	0.04	Lys, 1.0; Tyr, 1.0	—0.47	Tyr-

Fig. 3. Proposed sequence of peptide 2c of heavy-chain-disease protein Cra.

Fig. 4. Proposed sequence of cystine peptides of heavy-chain-disease protein Cra.

Table 4. Peptic peptides isolated from the heavy-chain-disease protein Zuc

		Amino acid composition (moles of amino acid/mole of peptide)								
Peptide	...	...	Na	Nc	Nd	Nb	Ne	1a*	2a	2c
Lys										3.1
Arg									1.0	
Cya			1.0	1.3†	1.0	1.4†	1.2	0.8	0.8	0.9
MetSO ₂			0.80	0.9						
Asp								1.2		1.0
Thr						1.0	0.9		1.8	
Ser			2.1	2.0	2.0				1.0	1.0
Glu									1.2	1.0
Pro									1.0	1.8
Ala										1.9
Val			0.9	1.0				3.0	1.9	0.9
Ile									1.01	0.9
Leu						1.8	1.0			1.0
Tyr										0.7
Phe			1.0							
Yield (μmole)			0.10	0.10	0.01	0.05	0.01		0.28	0.04
Mobility at pH 6.5 relative to aspartic acid			0.4	0.49	0.49	0.46	0.6	0.83	0.24	-0.16
N-Terminus			Phe	Ser	Ser	Leu	Thr			

* 90 hr. hydrolysis.

† Erratic base lines in the analyser are the probable cause of some unduly high cysteic acid values.

Heavy-chain-disease protein Cra (γG1 type)

The protein resembles the Fc fragment of a γG1 myeloma protein (Frangione *et al.* 1966). The following cysteic acid peptides were isolated and studied (Table 2).

Peptide Na. The sequence of this peptide, by the 'dansyl'-Edman technique, was Phe-Ser-Cya-Ser-Val-MetSO₂.

Peptide Ne. The sequence of this peptide, by the 'dansyl'-Edman technique, was Thr-Cya-Leu.

Peptide Nb. This peptide was lost during

Fig. 5. Proposed sequence of peptides Na, Nc and Nd of heavy-chain-disease protein Zuc. The *N*-terminal residue of each peptide is shown underlined.

Fig. 6. Proposed sequence of peptides Nb and Ne of heavy-chain-disease protein Zuc. The *N*-terminal residues are underlined.

purification. It was identical in mobility and staining characteristics with peptide Nb from heavy-chain-disease protein Zuc (see below).

Peptide 1a. This peptide was present in minor amounts. Its sequence, by the 'dansyl'-Edman technique, was Cya-Val-Val-(Val,Asp).

Peptide 1c. The mobility, amino acid composition and *N*-terminus of this peptide were identical with those of peptide 2c.

Peptide 2a. This peptide gave a faint ninhydrin stain, which became stronger after 48hr. Its sequence, by the 'dansyl'-Edman technique, was Ile-Ser-Arg-Thr-Pro-Glu-(Thr,Val₂,Cya). Glutamic acid, and not glutamine, is indicated, since the peptide was acidic.

Peptide 2c. The sequence was established as shown in Table 3 and Fig. 3. Peptide 2cT1 was acidic and peptide 2cT3 basic, indicating that glutamic acid was present in peptide 2cT1 and asparagine in peptide 2cT3. The *N*-terminal residue of peptide 2c was tyrosine and, from the specificity of trypsin, peptide 2cT1 should be the *C*-terminus.

The above results indicate the presence of the cystine peptides shown in Fig. 4.

Heavy-chain-disease protein Zuc (γ G3 type)

Purified peptides were analysed (Table 4). Peptides Nd and Nc were separated by electrophoresis at pH3.5. The relationship between peptides Na, Nc and Nd is indicated in Fig. 5. Peptides Nb and Ne appear to be related as shown in Fig. 6.

Peptide 2a was digested with trypsin and gave the following peptides (the compositions are in moles of amino acid/mole of peptide); peptide

Fig. 7. Proposed sequence of peptide 2a of heavy-chain-disease protein Zuc.

2aT2 (arginine 1.0, serine 1.0, isoleucine 1.0) and peptide 2aT1 (cysteic acid 0.9, threonine 1.9, glutamic acid 1.1, proline 1.1, valine 1.9). The sequence of peptide 2a by 'dansyl'-Edman degradation is given in Fig. 7.

It seems therefore that the above peptides are identical with the peptides obtained from the heavy-chain-disease protein Cra, in spite of the difference in γ -chain antigenic type.

Disulphide-bridge 'maps' of other proteins

Table 1 shows the presence or absence of some spots in disulphide-bridge 'maps' of several proteins and their fragments. 'Maps' of whole myeloma proteins of the same type as the heavy-chain-disease protein Cra show the presence of the spots characteristic of the disulphide bridges described. They appear to be similar, not only in their mobility, but also in their staining characteristics with cadmium-ninhydrin reagent. It is significant that normal pooled γ G-immunoglobulin also gave the characteristic spots, in very good yields, as judged by the ninhydrin intensity. The same spots were also observed in disulphide-bridge 'maps' of pooled isolated heavy chains, indicating that these disulphide bridges are not reduced during partial reduction. However, the F'c fragment gave only the set of spots characteristic of the neutral (N) region (Fig. 1b). The F'c fragment has been reported to be a minor component present in papain digests of whole γ G-immunoglobulin. It appears to include only one of the two loops.

DISCUSSION

The results presented indicated that the Fc fragment (the *C*-terminal half of the heavy chains) of human γ G1- and γ G3-immunoglobulins contains two intrachain disulphide bridges. Disulphide-bridge 'maps' of γ G4-immunoglobulin and of γ G2-immunoglobulin suggest that this arrangement is common to the four types of γ G-immunoglobulin molecule, although minor differences in the sequences around the cysteines are not excluded. These disulphide bridges are considered to be intrachain because they were also present in isolated heavy chains after reduction and carboxymethylation of the interchain bridges. The

Table 5. *C-Terminal loop in light and heavy chains*

The positions of the half-cystines are numbered from the *C*-termini. References: κ -chains (Hilschmann & Craig, 1965; Milstein, 1966a,b; Titani, Whitley & Putnam, 1966); λ -chains (Milstein, 1966a; Milstein, Clegg & Jarvis, 1967; Wikler, Titani, Shinoda & Putnam, 1967); rabbit Fc fragment (Hill *et al.* 1966); human γ 1- and γ 3-chains (the present paper).

Source	Position of half-cystine	Sequence	Position of half-cystine	Sequence
Human κ -chain	20	Tyr-Ala-CyS-Glu-Val-Thr	80	Val-Val-CyS-Leu
Human λ -chain	19	Tyr-Ser-CyS-Gln-Val-Thr	78	Leu-Val-CyS-Leu
Rabbit Fc fragment (pooled)	22	Phe-Thr-CyS-Ser-Val-Met	80	Leu-Thr-CyS-Met
Human γ 1-chain		Phe-Ser-CyS-Ser-Val-Met		(Leu)-Thr-CyS-Leu
Human γ 3-chain		Phe-Ser-(CyS,Ser)-(Val, Met)		Leu-Thr-(CyS, Leu)

Table 6. *N-Terminal loop in light chain and Fc fragment*

References are as given in Table 5. The position of the half-cystine is numbered from the *C*-terminus. The exact position of the second half-cystine in the rabbit Fc fragment is still uncertain. The deletions and insertions suggested by Hill *et al.* (1966) to maximize similarities have been preserved.

Source	Position of half-cystine	Sequence
Human κ -chains	126	Tyr—Cys-Gln-Gln-Tyr-Asp- Phe Leu* Arg Glu
Rabbit Fc fragment (pooled)	126	Thr-Phe-Gly-Gly-Gly—Thr Gln Pro
Human γ 1-chain		Phe-Lys-Cys-Lys-Val-His-Asp-Lys-Ala-Leu-Pro-Ala-Pro-Ile-Glu
Human γ 3-chain		Tyr-Lys-Cys-Lys-Val-Ser-Asn-Lys-Ala-Leu-Pro-Ala-Pro-Ile-Glu (Tyr, Lys, Cys, Lys, Val, Ser, Asx, Lys, Ala, Leu, Pro, Ala, Pro, Ile, Glu)
Human κ -chains	189	Val-Gly-Asp-Arg-Ile-Thr-Ile - Thr-Cys-Gln-Ala Val Ala Leu Ser Arg
Rabbit Fc fragment (pooled)		Ile-Ser-Arg (not known)
Human γ 1-chain		Ile-Ser-Arg-Thr-Pro-Glu-(Val,Thr,Cys)-Val-Val-(Val,Asp)
Human γ 3-chain		Ile-Ser-Arg-Thr-Pro-Glu-Val-Thr-Cys-Val

* Three residues have been deleted in this position.

† Very variable position

peptides described do not account for all the cysteine content of the Fc fragment. However, in the experiments described in this paper no other disulphide bridge could be characterized.

The sequence of human heavy chains is not yet available. However, a partial sequence of the Fc fragment of rabbit γ G-immunoglobulin has been reported and a comparison between the peptides reported in this paper and those obtained by Hill, Delaney, Fellows & Leboritz (1966) in the rabbit protein can be made. From this comparison it is obvious that the two sequences found in the neutral region (peptides of the N series) are very similar to the sequences around the cysteines in positions 22 and 80 (numbering from the *C*-terminus; see Table 5). The cysteic acid of peptides 1c and 2c

can be identified as residue 126, starting from the *C*-terminus (see Table 6). The position of peptides 2a and 1a is less certain because the sequence of rabbit γ G-immunoglobulin is still incomplete. However, a sequence Ile-Ser-Arg is present near the *N*-terminal end of rabbit Fc fragment (see Table 6). This comparison suggests that the Fc fragment is covalently folded, as shown in Fig. 8. This arrangement resembles the ring structure of light chains (Milstein, 1966b) with a *C*-terminal loop and an *N*-terminal loop. The similarity is much emphasized by the sequences and location of the disulphide-bridged peptides of the *C*-terminal loop of human heavy and light chains (Table 5). Three out of six residues and three out of four residues near position 80 are identical in human λ -chain and heavy chain.

Fig. 8. Intrachain disulphide bridges in human light chains and Fc fragment. (a) Light chains (Milstein, 1966b); (b) Fc fragment (the positions of the half-cystine residues are taken by comparison with rabbit Fc fragment) (Hill *et al.* 1966); (c) F'c fragment.

Even more striking is the finding that the homologous cysteine residues are bridged together, suggesting that the tertiary structure of the two loops of the light and heavy chains might also be very similar. The presence of the C-terminal loop in rabbit heavy chains has been observed by R. R. Porter and his co-workers (Prah, 1967). A similarity between light and heavy chains has already been suggested (Hill *et al.* 1966; Singer & Doolittle, 1966).

The N-terminal loop, on the other hand, appears to be very different in light and heavy chains. There are only three to four residues (including the half-cystine) that are identical between κ - or λ -chains and heavy chains of human γ G-immunoglobulin, out of 25 residues depicted in Table 6. This does not mean that they have not departed from a common ancestor, since the evolutionary changes might have been sufficiently large to erase the similarity of origin. It is pertinent in this context that the N-terminal loop of light chains is variable, whereas the N-terminal loop of the Fc fragment does not seem to be so.

The observation that the F'c fragment only contains the C-terminal loop peptides gives a good indication of its origin. However, the results do not show whether the end of the fragment is actually at the C-terminus of the whole heavy chain. Further, the absence of part of the C-terminal loop could escape detection in the disulphide-bridge 'fingerprints'. However, this seems unlikely since the F'c fragment contained essentially a single N-terminal residue (glutamic acid or glutamine).

The sequences reported in this paper appear to be identical in the two antigenic types (γ 1 and γ 3) of human heavy chains studied. The C-terminal octadecapeptides of the two human heavy chains are also identical, except for one or two residues (Press *et al.* 1966; Prah, 1966). The total number of known residues of the Fc fragment is now 55, all of which are identical in the two types of heavy chain, with one or two exceptions. Great similarity between the Fc fragments of different types had been indicated by their 'fingerprint' patterns (Frangione *et al.* 1966). The results suggest that the separation of these different types from a common ancestor is a recent event in evolutionary terms. A comparison with the known residues of the rabbit Fc fragment is illustrative. Out of 25 residues in the three cysteine peptides shown in Tables 5 and 6, five are different between rabbit and man. Adding the 18 residues, with two differences, of the C-terminus (Press *et al.* 1966) gives 84% identical residues, which compares with about 85% identical residues between human and rabbit haemoglobin α -chains.

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