The Disulphide Bridges of a Mouse Immunoglobulin G1 Protein

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[³⁵S]Cystine-labelled immunoglobulin MOPC21 (IgG1) was prepared from myeloma cells in tissue culture. Carrier myeloma protein was added and the protein was digested with pepsin. The digest was fractionated on Sephadex G-50 into two fractions, further digested with trypsin and again fractionated on Sephadex. Disulphide-bridge peptides were purified by electrophoresis and chromatography and identified by radioautography. A peptide of 96 residues was isolated, which contains both the heavy–light interchain disulphide bridge and all the inter-heavy-chain disulphide bridges. Other peptides were isolated, accounting for all the intrachain disulphide bridges (which could be placed by homology with proteins of other species), except for the variable section of the light chain. Sequences describing this missing disulphide bridge were obtained from totally reduced and alkylated light chains. Peptides related to the interchain disulphide-bridge peptide were isolated from partially reduced and alkylated myeloma protein and from totally reduced heavy chain. The interchain disulphide-bridge peptide was placed at the C-terminal position of the F(ab')₂ fragment, prepared by digestion of the protein with pepsin at pH 4.0. Sequences from the heavy-chain intrachain disulphide bridges of MOPC 21 immunoglobulin are compared with homologous sequences from mouse myeloma proteins of other subclasses and proteins of other species.

The mouse IgG* class has been divided into three subclasses, IgG1 (IgGF), IgG2a (IgGG) and IgG2b (IgGH) (Fahey *et al.*, 1964*a,b*; Potter *et al.*, 1965), and subsequently a fourth subclass, IgG3, has been described (Grey *et al.*, 1971). As in the human subclasses of IgG, the differences in the heavy chains characterized the different subclasses of mouse IgG (Potter *et al.*, 1965, 1966). The location of the heavy–light interchain disulphide bridge and the number and sequences of the heavy–heavy interchain bridges vary considerably in both species (Frangione *et al.*, 1969*a*; de Préval *et al.*, 1970; Svasti & Milstein, 1970). However, in humans there is considerable homology in the sequences defining the intrachain disulphide bridges (Pink *et al.*, 1970).

In the present paper, we describe the characterization of the interchain and intrachain disulphide bridges of a mouse IgG1 (MOPC 21) myeloma protein. Sequences defining the intra-heavy-chain disulphide bridges of MOPC 21 protein are compared with homologous sequences from other species and from mouse myeloma proteins of different subclasses, where available.

A noteworthy innovation over procedures previously used on this type of study was the use of labelled protein obtained by incorporation of [³⁵S]-cystine. Best incorporation was obtained by using cells grown in tissue culture. The labelled protein greatly facilitated the fractionation and purification of peptides.

* Abbreviations: IgG, immunoglobulin G; Ccm, carboxymethylcysteine.

Preliminary reports of this work have been published (Svasti & Milstein, 1970; Milstein & Svasti, 1971).

Materials and Methods

Materials

The plasmacytoma, MOPC 21, originated by Dr. M. Potter, was serially transplanted into Balb/c mice. The myeloma protein was purified from sera kindly supplied by Dr. A. J. Munro and Mr. P. Wright.

P3K (MOPC 21) myeloma cells were kindly given by Dr. K. Horibata (Horibata & Harris, 1970). These cells were grown in spinner flasks in Dulbecco's minimal essential medium (Dulbecco & Freeman, 1959; Smith *et al.*, 1960) fortified with 10% (v/v) heat-inactivated horse serum. The cultures were maintained under an atmosphere of N₂+O₂+CO₂ (83:7:10). Culture media were obtained from Flow Laboratories, Irvine, Ayrshire, U.K.

Iodo[2-¹⁴C]acetic acid and [³⁵S]cystine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Trypsin, pepsin and carboxypeptidase A were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Thermolysin was a gift from Dr. R. P. Ambler.

Reagents were generally of analytical grade. Reagents used for the Edman degradation cycle were redistilled as described by Edman & Begg (1967). HCl for hydrolysis of peptides was obtained as

34.0–36.0% solution (Aristar grade; BDH Chemicals Ltd., Poole, Dorset, U.K.) and was diluted with an equal volume of water; phenol (1 mg/ml) was added to prevent destruction of tyrosine (Sanger & Thompson, 1963).

Methods

Purification of myeloma protein. The protein was prepared from serum by precipitation with 40% satd. $(\text{NH}_4)_2\text{SO}_4$ and chromatography on a column (2 cm $\times$ 20 cm) of DEAE-cellulose (Whatman DE 52) with a linear sodium phosphate gradient (0.01–0.2 M; pH 7.0). Purity was checked by electrophoresis on cellulose acetate strips in 0.05 M-veronal buffer, pH 8.6. Such preparations were about 95% pure. In some cases, further purification was made either by precipitation with an equal volume of 50% (v/v) ethanol at 0°C or chromatography on a column (2 cm $\times$ 10 cm) of CM-cellulose (Whatman CM 52) with a linear sodium phosphate gradient (0.005–0.2 M; pH 6.0). Typical yields were 15–25 mg of myeloma protein/ml of serum.

Preparation of [^{35}S]cystine labelled myeloma protein. The best preparations were obtained as follows. Approx. 2×10^7 MOPC 21 (P3K) cells were washed twice and resuspended in 100 ml of Dulbecco's medium without cystine containing 10% (v/v) of horse serum. After addition of 500 μCi of [^{35}S]cystine the cells were incubated for 3 days at 37°C; then the supernatant fluid was separated from the cells by centrifugation at 2000g. The cells were resuspended in 5 ml of 0.01 M-tris-HCl buffer–0.01 M-KCl–0.001 M-MgCl₂, pH 7.5, disrupted by freezing and thawing and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 40000g. MOPC 21 serum (0.25 ml) was added to both the spent medium and the supernatant from the homogenate. Each fraction was then purified separately on DEAE-cellulose as described above. Electrophoresis and radioautography of the fractions on cellulose acetate strips showed that both the intracellular and extracellular myeloma protein was radiochemically pure, but that the extracellular fraction was contaminated with horse γ -globulins. The total yield from the extracellular fraction was significantly greater than that in the intracellular fraction (2 μCi compared with 0.4 μCi). Both fractions were mixed and 75 mg of MOPC 21 protein was added as carrier before further manipulations.

Reduction of disulphide bridges. Selective reduction of interchain disulphide bridges and carboxymethylation with iodo[2- ^{14}C]acetic acid was carried out as described by Frangione *et al.* (1969b) except that the time of reduction was 45 min instead of 1.5 h. Heavy and light chains were separated by chromatography through a column (3 cm $\times$ 80 cm) of Sephadex G-100 in 5% (v/v) formic acid. Purity of the isolated frac-

tions was established by electrophoresis in sodium dodecyl sulphate–polyacrylamide gels (Shapiro *et al.*, 1967; Weber & Osborn, 1969). Total reduction and alkylation was performed in 6.6 M-guanidine as described by Pink *et al.* (1970).

Peptic digestion of whole myeloma protein and fractionation of the digests. MOPC 21 myeloma protein (75 mg) was dissolved in 5 ml of 5% (v/v) formic acid and digested with pepsin (enzyme/substrate ratio 1:40 by wt.) for 16 h at 37°C. The digest was stopped by adding 2 ml of 1.25 M-pyridine–acetate buffer, pH 6.5, and then applied to a Sephadex G-50 column (2.5 cm $\times$ 90 cm) equilibrated with 5% (v/v) formic acid. Samples from alternate tubes were dried, dissolved in 1 drop of formic acid and 2 drops of performic acid, left at room temperature for 30 min, dried again and subjected to acid hydrolysis. The hydrolysates were subjected to electrophoresis at pH 2.1 for identification of cysteic acid.

The [^{35}S]cystine-labelled myeloma protein was treated in the same manner but the [^{35}S]cystine peptides were followed automatically in a Nuclear-Chicago flow scintillation counter. Cystine peptides from both experiments were eluted in the same pattern and the column profile for the radioactivity experiment is shown in Fig. 1(a).

Preparation of peptic F(ab')₂ fragment. The protein

Fig. 1. Sephadex G-50 gel filtration in 5% (v/v) formic acid

(a) Peptic digest of [^{35}S]cystine-labelled MOPC 21 protein. (b) Tryptic digest of [^{35}S]cystine-labelled peptide pool PH. —, Radioactivity (c.p.m.); ---, E_{254} .

(50mg) was digested with 1.25mg of pepsin at 37°C for 16h in 0.1M-sodium acetate buffer, pH4.0. Samples from the digest were subjected to immuno-electrophoresis and sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. The digestion products were partially reduced, blocked with iodo[¹⁴C]acetic acid, totally reduced and alkylated with non-radioactive iodoacetic acid and then extensively dialysed against 1% (w/v) NH₄HCO₃ and digested with trypsin.

Other enzyme digests. Trypsin digests were carried out in 1% (w/v) NH₄HCO₃, pH8.0, for 4h at 37°C (enzyme/substrate ratio 1:100 by wt.). However, to minimize disulphide interchange, digestions of peptides with intact disulphide bridges were carried out in 1% (w/v) ammonium acetate, pH7.5, for 2h at 37°C with an enzyme/substrate ratio of 1:40. Carboxypeptidase A was activated in 2M-NH₄HCO₃ as suggested by Ambler (1967*a,b*) and digests of peptides were made in 0.2M-ammonium acetate, pH7.5, for 10min to 6h at 37°C with 10–20µg of enzyme. The digestion products were identified on an

amino acid analyser and a time-course of release of amino acids was determined. Thermolysin digests of peptides were carried out in 0.1M-ammonium acetate–0.005M-CaCl₂, pH8.5, for 2h at 45°C.

High-voltage paper electrophoresis of peptides. This was carried out as described by Ambler (1963) or Milstein (1966) for 45–90min. Mobilities (*m*) at pH6.5 are expressed as fractions of the distance between the aspartic acid and ε-Dnp-lysine spots (Ambler, 1963; Offord, 1966). Chromatography was performed with a descending butan-1-ol–acetic acid–water–pyridine system (15:3:12:10, by vol.) (solvent BAWP) (Waley & Watson, 1953).

Detection of non-radioactive peptides on paper, acid hydrolysis and amino acid analysis was carried out as described by Milstein (1966). Cystine and cysteic acid peptides from the radioactive experiments were simply followed by radioautography. Peptides were eluted with water or 0.5% acetic acid.

Dansyl–Edman procedure. This was as described by Gray (1967). Dns-amino acids were identified by two-dimensional t.l.c. (Woods & Wang, 1967). A third

Fig. 2. Sequence determination of peptide FRT2 and the identification of carboxymethylcysteine residues by loss of radioactivity

The peptide was subjected to Edman degradation. After each degradation cycle a sample was counted in a scintillation counter and another sample used for determination of the new *N*-terminal residue by the dansyl–Edman procedure.

Table 1. *Amino acid compositions and mobilities of peptides isolated from peptic fraction PH*

The origin of all the peptides is described in Scheme 1.

	PHTAA*	PHTAAO1	PHTAAO2	PHTBBOb	PHTBBOa1	PHTBBOa2	PHTBBOa3	PHOA
Lys	2.0		1.0	0.9				3.5
Arg								1.0
His				0.8				0.9
Cya	9.6	1.1	3.9	0.8	0.9	1.0	0.9	4.8
Asp	4.0	0.9	1.2	1.2				3.0
Thr	2.0		0.8	2.0			0.8	3.1
Ser	2.2		1.1	2.1				3.2
Glu	4.2	0.8	1.0					1.0
Pro	3.8		2.0	1.2				4.1
Gly	2.1		1.1		1.0	0.9	1.0	1.1
Ala				2.0				2.2
Val	4.0		1.9	1.9			0.9	6.0†
Ile	1.9		0.8					2.1†
Leu					1.0	2.1	2.0	
Phe								
<i>m</i>	0.42	1.05	0.95	-0.18	0.57	0.45	0.35	0.21
<i>N</i> -Terminal	Asx	Asx	Asx	Val	Gly	Leu	Val	Val
	PHOATA	PHOATB1	PHOATB2a	PHOATB2b	PHOAHa1	PHOAHb1	PHOA'	PHOBA4
Lys	0.8	0.9		1.8	0.9	2.0	3.9	
Arg			1.0		0.8		1.0	0.9
His		0.8						
Cya	3.8	1.0			3.8		3.9	1.0
Asp	1.0	1.0		1.2	1.0	1.0	2.2	2.1
Thr	1.0	2.1			0.9		1.2	
Ser	1.2	2.0					1.3	
Glu	1.1				1.0		1.1	0.9
Pro	2.1	1.0	1.0		3.1		2.8	
Gly	1.1				1.0		1.1	
Ala		2.1						
Val	1.9	1.9	1.1†	0.8	2.0†	1.0	4.0†	
Ile	0.9		1.0†		1.8†		2.0†	
Leu								
Phe								0.8
<i>m</i>	0.95	-0.16	-0.38	-0.38	0.65	-0.38	0.25	0.38
<i>N</i> -Terminal	Asx	Val	Ile	Val	Ile	Val	Lys	Phe

* After performic acid oxidation.

† After 96h hydrolysis

solvent (solvent IV of Crowshaw *et al.*, 1967) was used to separate the Dns-derivatives of aspartic acid from glutamic acid, and serine from threonine. Dns-cysteic acid was identified by electrophoresis at pH 4.4 (1.2M-pyridine-acetate buffer). Since Dns-carboxymethylcysteine is sometimes difficult to obtain by direct dansyl-Edman treatment, carboxymethylcysteine was often identified by the loss of radioactivity after each Edman degradation cycle. Samples were taken after each degradation step and

corrected for sampling. A typical result is shown in Fig. 2, which shows the sequence determination of peptide FRT2.

Amide residues were assigned on the basis of electrophoretic mobility at pH 6.5 (Offord, 1966).

The results of sequence determination are shown by arrows under the peptides on which the experiments were performed. Arrows, →, indicate the results of the dansyl-Edman procedure; double arrows, ⇨, indicate that the Dns-amino acid was obtained without

Fig. 3. Derivation of the sequence of the stretch of γ_1 chains containing the interchain disulphide bridges

The peptides described were obtained as performic acid-oxidized derivatives containing cysteic acid (indicated by the presence of an O in the names) or as carboxymethylcysteine derivatives (indicated by the presence of an R in their names). Vertical arrow ↓ indicates site of action of pepsin at pH 4.0 on intact protein [C-terminus of heavy-chain fragment in F(ab')₂].

Fig. 4. Peptides derived from fraction PH but not described in Fig. 3

Related peptides isolated as carboxymethylcysteine derivatives are also shown (RP3, RP4, FRT1).

Fig. 5. Peptic-tryptic peptide PHTAA, defining all the interchain disulphide bridges

It is not known whether all the disulphide bridges on the heavy chain are interchain bridges. Recent experiments show that the light chain is bonded to the sequence Cys-Gly of heavy chains (J. Svasti & C. Milstein, unpublished work).

Table 2. *R_F* values of peptides from peptic pool PH on Sephadex G-50 (5%, v/v, formic acid), expressed relative to Blue Dextran (0) and ϵ -Dnp-lysine (1.0)

The numbers of residues are determined from the sequence of the peptides. * shows the two alternative sizes of the peptides, depending on whether they exist as monomers (no heavy-heavy interchain S-S bridge) or dimers (with at least one heavy-heavy interchain bridge). † oxidized derivatives. The size variation of peptides PH and PHTBB is due to the different alternatives of the components in fraction PHOB as shown in Fig. 4.

Peptide	<i>R_F</i>	No. of residues from sequence
PH	0.14	*45-48 (as monomer H-L) *90-96 (as dimer L-H-H-L)
†PHOA	0.40	36
PHTAA	0.43	*18 (as monomer H-L) *36 (as dimer L-H-H-L)
PHTBB	0.60	18-21
†PHOB	0.82	3-6

hydrolysis, and a broken arrow, $\rightarrow$, indicates a dubious or negative result. Arrows $\leftarrow$ indicate the results of carboxypeptidase A digestion.

Results

By using molecular weights of 50000 for heavy chains and 22000 for light chains, the molar specific-radioactivity ratio of light to heavy chains was calculated to be $1:3.9 \pm 0.3$. The myeloma protein was shown to have no free thiol groups since no radioactive carboxymethylcysteine-containing peptides were obtained after carboxymethylation with iodo-[¹⁴C]acetic acid in 6.6M-guanidine buffer, pH8.2, without prior reduction.

The peptic digest of intact MOPC 21 protein was fractionated by Sephadex G-50 ultrafiltration into two peptide pools, PH and PL (Fig. 1a). Further treatment of these two fractions is shown in Fig. 1(b) and Scheme₁ and was as follows.

Peptic pool PH

A tryptic digest of this pool was fractionated on Sephadex G-50 as shown in Fig. 1(b). Two [³⁵S]cystine-containing fractions were taken (PHTA and PHTB) and further fractionated by electrophoresis at pH 6.5. Fraction PHTA yielded only one peptide, PHTAA (Table 1), which seemed to be pure on the basis of amino acid composition. After performic acid oxidation it gave rise to two peptides, PHTAAO1 and PHTAAO2, which were separated by electrophoresis at pH 6.5. Peptide PHTAAO2 contained four cysteic acid residues (Table 1, Fig. 3) and peptide PHTAAO1 (Table 1, Fig. 4) was identical with the C-terminal sequence of other mouse κ -chains (Gray *et al.*, 1967).

Fraction PHTB yielded two cystine peptides, PHTBA (identical with peptide PHTAA) and PHTBB ($m = -0.37$), which yielded four peptides on performic acid oxidation: PHTBBOb (Table 1, Fig. 3) and PHTBBOa1, PHTBBOa2 and PHTBBOa3 (Table 1, Fig. 4).

Peptide pool PH was oxidized with performic acid and the derivatives were fractionated by Sephadex G-50 into fractions PHOA and PHOB. PHOA seemed to be a pure peptide (Table 1); it was digested with trypsin and five derivatives isolated: peptides PHOATA, PHOATB1, PHOATB2a, PHOATB2b (low yield) and free lysine (Table 1, Fig. 3). Confirmation of the sequence and alignment of the tryptic peptides was achieved by isolation of PHOA' (a peptide prepared in the same manner as PHOA but from a different peptic digest of MOPC 21 protein), and the thermolysin derivatives PHOAHa1 and PHOAHb1. Fraction PHOB yielded peptides PHOBA1, PHOBA2, PHOBA3 (Fig. 4; amino acid analyses not shown) and PHOBA4, the light-chain C-terminal peptide (Table 1, Fig. 4).

It was concluded that fraction PH contains all the interchain bridges as well as one of the intraheavy-chain bridges. The intrachain bridge was the one formed by half-cystine residues in the γ 1 chain peptide of Fig. 4 and in the peptide PHOATB1 of Fig. 3. This is (on homology grounds) the first intrachain disulphide bridge of the invariable region (see Fig. 9). The interchain bridges are in the peptic-tryptic peptide PHTAA (Fig. 5) which must contain both the heavy-light bridge and at least one heavy-heavy interchain disulphide bridge. The latter must be so for the following reasons. First, there are four cysteic acid residues on the heavy-chain moiety, none of which existed as a free thiol group as the protein has no free thiol groups. One of the half-cystine residues must be bonded to the light chain. Thus, even if two of the remaining half-cystine residues form an intrachain disulphide bridge, the fourth must form an interchain bridge. Also the apparent molecular weight of peptide PHTAA is significantly greater than that of peptide PHTBB and similar to that of peptide

Table 3. Amino acid composition of peptides from peptic fraction PL after tryptic digestion

The origin of the individual peptides is described in Scheme 1.

	PLTAB201	PLTCB101	PLTCB102	PLTAB101	PLTCB201	PLTAB102	PLTCB202	PLTCN101	PLTCN102	PLTCN202	PLTCA101	PLTCA102
Lys	1.0					1.0						
Arg						0.8						
His			0.8									0.7
Cys	0.8	0.8	1.0			1.0		1.0		0.8	1.0	1.0
MetSO ₂				1.0	1.0							
Asp	2.0							1.0	1.1			
Thr								1.0				1.1
Ser		2.0			1.1				0.9	1.0		0.9
Glu									0.9	1.0		1.2
Pro												1.1
Gly	1.0	1.2										
Ala	1.9	1.9										
Val				4.0*	2.9*						1.7	
Leu	1.0	1.0						1.1	1.1	1.0		
Tyr			0.7					0.8		1.0		
Phe		0.37	0	0.57	0.44	1.0				0.8	1.0	0.8
m	0			Val	Val	-0.37		Thr		0.35	0.40	
N-Terminal	Lys	Leu	Tyr	Val	Val	Phe	Cys	Thr	Thr	Phe	Val	His

* After 96h HCl.

Fig. 6. Deduction of sequence and the S-S bridge patterns of peptides present in pool PL after tryptic digestion

The dansyl-Edman results were obtained on the two cysteic acid derivatives. For instance, peptide PLTAB2 was oxidized with performic acid and two peptides, PLTAB201 and PLTAB202, were separated by electrophoresis at pH 6.5 (mobilities and compositions of oxidized peptides are shown in Table 3). The probable origin of each bridged peptide is based on its homology with other proteins (see Fig. 9).

PHOA (Table 2), suggesting that it has 36 residues rather than 18. Further, the peptide pool PH has a significantly increased R_F after performic acid oxidation to yield its largest component PHOA. This suggests that it existed in a dimeric form before oxidation and was a peptide of 90-96 residues.

Peptic pool PL

This was digested with trypsin and fractionated on a Sephadex G-25 (fine grade) column in 5% (v/v) formic acid. Three partially resolved fractions were obtained and termed PLTA, PLTB and PLTC. Each was subjected to electrophoresis at pH 6.5 and the radioactive bands obtained were further fractionated

by chromatography in solvent BAWP and a second electrophoresis at pH 6.5 after performic acid oxidation in the vapour phase. The amino acid compositions of the peptides isolated are shown in Table 3. Figs. 6(a), 6(b), 6(c) and 6(d) show the disulphide-bridge peptides obtained from this digest and the derivation of their sequences.

Two peptides, LRTN2 and LRT α HR2 (Table 4 and Fig. 7), were isolated from a separate digest of totally reduced and alkylated light chains, which indicated the presence of two unaccounted half-cystine residues. Since there are no free thiol groups in the protein, these two half-cystine residues are likely to form a disulphide bridge with each other. These peptides define a bridge, which was shown (Milstein

Table 4. *Amino acid compositions of carboxymethylcysteine peptides*

The origin of the peptides is described in the Results section. Peptides RP and HR are described under *Disulphide bonds labile to partial reduction*, except for HRT3 which is described in Fig. 6. FR peptides are described under *F(ab')₂ fragment*. LR peptides are described under *Peptic pool PL*.

	RP1	RP2	RP3	RP4	RPHT1	RPHT2	FRT1	FRT2	HRT4	HRT4C	HRT4PA	HRT4PB	HRT3	LRTN2	LRT α HR2
Lys			0.9	0.7	0.9	1.0		1.1	2.8	0.9	1.0	2.1	0.9		
Arg															
His															
Ccm	0.6	0.6	0.7	0.6	2.2	1.7	0.7	2.5	2.3	2.3	2.6		0.5		1.1
Asp			1.9	2.0	1.0	1.0	1.0	0.9	0.8	0.9	0.9		1.0	0.6	0.5
Thr	1.0	1.0			0.8			0.9	0.8	1.0	1.0		1.0	1.8	1.2
Ser	1.2	1.9						1.0	1.9	2.3	1.8		0.9		
Glu	1.2	1.2	1.2	1.2			1.0	1.0	1.1	1.2	1.0		1.0		1.0
Pro	1.0	0.8						1.8	5.1	1.9	2.1	3.0			
Gly					0.9	0.8		0.9	1.2	1.2	0.9				2.1
Ala					1.3	1.2							2.1	0.8	0.9
Val	1.9	2.5						2.1	3.0	3.0	3.1				
Met													1.0		
Ile					0.7	0.9		0.8	1.9	0.8	0.9	0.9			
Leu														1.1	0.9
Tyr													1.7		0.7
Phe				0.8					1.9	1.0					
m				0.33	0.89	0.80	1.0	0.9	0.34	0.72	0.80	1.6	0.43	0	0.25
N-terminal	0.64	0.58	0.42	Phe	Asx	Asx	Asx	Asx	Asx	Asx	Asx	-0.44	Ser	Val	Leu

Fig. 7. Variable S-S bridge of light chains

These were isolated as carboxymethylcysteine derivatives from a tryptic digest of isolated chains. Peptide LRTα4HR2 is a fragment of a tryptic peptide obtained by digestion with thermolysin.

& Svasti, 1971) to be part of the *N*-terminal half of light chains.

Disulphide bonds labile to partial reduction

A peptic digest of partially reduced and [^{14}C]-carboxymethylated protein was fractionated by gel filtration through Sephadex G-50 in 5% (v/v) formic acid, followed by paper electrophoresis at pH 6.5. Seven radioactive peptides were obtained. Peptides RP3 and RP4 (Table 4, Fig. 4) were the κ -chain C-terminal peptides. Peptides RP1 and RP2 were present in lower yields and appeared to include one of the interchain half-cystine residues of the heavy chain (Fig. 3). The other three radioactive peptides were of very high molecular weight and were separately digested with trypsin to give the same pattern of two smaller radioactive peptides, RPHT1 and RPHT2. This indicates that the three larger peptides were the product of incomplete peptic splits around the half-cystine residues from the heavy chains, involved in heavy-chain binding. Peptides RPHT1 and RPHT2 were analysed and the results (Table 4 and Fig. 3) show that they include the same half-cystine residues from the interchain-bridges stretch.

The sequence around this area was extended by isolating peptide HRT4 and a related peptide HRT4C (Table 4) from a tryptic digest of totally reduced heavy chains. Peptic digestion of peptide HRT4 yielded two peptides, HRT4PA (radioactive) and HRT4PB (non-radioactive) (see Table 4 and Fig. 3). The C-terminal sequence of peptide HRT4PA was established as Val-Ser-Ser-Val by a time-course of carboxypeptidase A digestion (at 10 min: Val 0.5, Ser 0.1; at 1 h: Val 0.95, Ser 0.7; at 3 h: Val 1.05, Ser 1.3; at 6 h: Val 1.3, Ser 1.8).

F(ab')₂ fragment

Immunoelectrophoresis showed that the peptic digest at pH 4.0 (see the Materials and Methods section) had Fab determinants. The molecular weight of the fragment was determined as 95 000 by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels. After partial reduction and alkylation with iodo[^{14}C]acetic acid, two fragments were obtained with indicated molecular weights of 22 000 (light chain) and 25 000 (heavy-chain fragment, Fd').

Fig. 8. Site of action of pepsin at pH 4.0 on MOPC 21 protein, and molecular weights of fragments

For experimental details see the text.

Radioautography of the gels showed that both fragments were [^{14}C]carboxymethylated, the Fd' fragment being more strongly labelled. The pH 4.0 peptic digest was partially reduced, carboxymethylated with iodo[^{14}C]acetic acid, totally reduced and carboxymethylated with non-radioactive iodoacetic acid (see the Materials and Methods section), exhaustively dialysed and digested with trypsin. Two radioactive peptides were obtained. Peptide FRT2 (Table 4, Fig. 3) did not contain a basic residue in the C-terminal position. It was in fact part of a larger peptide HRT4 isolated from intact heavy chains containing the half-cystine residues involved in formation of interchain S-S bridges. It appears then that it is the C-terminal peptide of the heavy-chain fragment Fd' indicating that the interchain S-S bridges of MOPC 21 protein are near the C-terminus of the F(ab')_2 fragment (Fig. 8).

Discussion

The use of [^{35}S]cystine-labelled myeloma protein simplified the problem of determination of the disulphide bridges of MOPC 21 protein, so that all but one of the disulphide bridges were obtained in one experiment. There was no need to use ninhydrin, and the yields of the cystine peptides were greatly improved as they could be located by radioautography. In this work, the major use of radioactivity was confined as a tracer used in conjunction with unlabelled protein. Preliminary experiments with a

Table 5. *Interchain bridge region in several γ chains*

References: human: γ 1, Steiner & Porter (1967); Frangione & Milstein (1967); Edelman *et al.* (1969): γ 2, Milstein & Frangione (1971): γ 3, Frangione & Milstein (1969): γ 4, Pink *et al.* (1970): rabbit, O'Donnell *et al.* (1970): mouse: γ 1, Svasti & Milstein (1970): γ 2a and γ 2b, de Prval *et al.* (1970): guinea pig, Oliveira & Lamm (1971); Turner & Cebra (1971).

Species	Subclass	No. of amino acids in bridge region	No. and nature of half cystine residues			
			Intrachain bonds	H-L bonds	H-H bonds	Total
Human	γ 1	18	—	1	2	3
	γ 2	15	—	—	4	4
	γ 3	24	—	—	5	5
	γ 4	15	—	—	2	2
Rabbit		14	1	—	1	2
Mouse	γ 1	16	—	1	3	4
	γ 2a	14	—	—	3	3
	γ 2b	?	—	—	4	4
Guinea pig	γ 2	20	—	—	3	3

protein (γ 1) are compared with the available homologous sequences from MOPC 173 protein (γ 2a), MOPC 141 protein (γ 2b) and rabbit, human and guinea-pig proteins. The comparison suggests that mouse γ 1 protein is more similar to that of rabbit than to that of human, suggesting that the human γ gene diverged before the mouse γ 1/rabbit γ divergence. Unfortunately it is not yet possible to say whether mouse γ 1 protein is more similar to mouse proteins of other subclasses than to proteins of other species, since results for other mouse proteins are incomplete.

The variable sequences of human heavy chains fall into three subgroups (Wang *et al.*, 1970), which are independent of the subclass of the constant-region portion (Wikler *et al.*, 1969; Press & Hogg, 1970; Edelman *et al.*, 1969; Pink *et al.*, 1970; Wang *et al.*, 1970). One mouse myeloma protein (MOPC 173) shows considerable homology with proteins of the same subgroup as Vin (Bourgeois & Fougereau, 1970b). This (Fig. 9a) seems also true for MOPC 21 protein. The results support the idea that the variable and invariable regions of heavy chains have originated before the divergence of mouse and man (Milstein & Pink, 1970). They furthermore give experimental evidence to the suggestion (Bourgeois & Fougereau, 1970b) that there is a pool of variable regions that is common to the heavy chains of different subclasses of mouse IgG.

In contrast with the large homology in the sequences defining the intrachain S-S bridges of heavy chains, there is very little homology in the sequences containing the heavy-chain interchain bridges. This region should probably be referred to as the interchain 'bridge' region or 'bridge' region rather than 'hinge' region (as it is often referred to). The homology between proteins of different sub-

classes and different species is quite clear up to residue 214 (human myeloma protein Eu numbering) after which it becomes of little significance or less general until the glutamic acid residue 233. These numbers are somewhat arbitrary and may require modification as more sequence results are obtained. Table 5 shows the size variation and disulphide-bridge patterns in the 'bridge' region for a number of proteins of different species and different subclasses. The size of this section varies considerably from 14 residues (rabbit IgG) to 24 residues (human IgG3), and the number of heavy-heavy interchain S-S bridges from 1 (rabbit IgG) to 5 (human IgG3). In rabbit IgG, one half-cystine residue in the bridge region forms an intrachain S-S bridge whereas in human and mouse IgG1 one half-cystine residue forms a heavy-to-light interchain S-S bridge.

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