Characterization of upFc, a Fragment of Human Immunoglobulin G1 Produced by Pepsin in Urea

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The digestion of human IgG1/ κ myeloma proteins with pepsin in the presence of 8M-urea produces fragments that differ from those produced by aqueous peptic digestion, and from other characteristic immunoglobulin fragments. Fb'₂, the larger urea/pepsin fragment, was previously shown to consist of the constant regions of the light chains, and the C_H1 domains and hinge regions of the heavy chains. The smaller fragment, upFc, has now been characterized. After reduction, three peptides were released from fragment upFc. Amino acid sequencing, N- and C-terminal determinations and amino acid compositions have enabled these peptides to be identified as residues Ile-253 to Leu-306, residues Thr-307 to Asp-376 and residues Thr-411 to Gly-446 of the heavy chain. Fragment upFc therefore contains the entire Fc region, beginning at residue Ile-253, except for a 34-residue section from within the C_H3-domain disulphide loop. Peptic digestion of IgG1/ κ proteins in 8M-urea therefore provides a method for isolating from γ 1 heavy chains five homogeneous peptides in good yield, which account for almost the entire constant region. Characterization of fragments Fb'₂ and upFc has shown that the action of pepsin in urea is entirely different from that of aqueous pepsin. Two γ 1 heavy chains have been shown to differ in sequence at three positions from the sequence reported for protein Eu.

Peptic digestion of immunoglobulins in aqueous buffer results in the formation of a large fragment, F(ab')₂, consisting of both light chains and the Fd regions of both heavy chains, with interchain disulphide bonds intact (Nisonoff *et al.*, 1960; Utsumi & Karush, 1965; Heimer *et al.*, 1967), and a small fragment, pFc', from the C-terminal end of the molecule (Heimer *et al.*, 1967; Bennich & Turner, 1969). The remainder of the Fc region appears to be extremely susceptible to proteolytic attack, and is lost as small peptides.

In a previous paper (Parr *et al.*, 1976) we showed that digestion of IgG1/ κ * myeloma proteins with pepsin in the presence of 8M-urea yielded fragments that were entirely different from fragments F(ab')₂ and pFc'. Fragment Fb'₂, one of the two main products of urea/pepsin digestion, was shown to consist of the constant regions of the light chains, the C_H1 domains of the γ chains, and the entire hinge region. Fragment Fb'₂ could also be prepared by urea/pepsin digestion of F(ab')₂ fragments. The second fragment, now designated upFc (urea, pepsin Fc), was not found in urea/pepsin digests of F(ab')₂; it was inferred therefore that this fragment was derived from the Fc region of the molecule.

* Abbreviations: IgG1/ κ , immunoglobulin G with heavy chains of the γ 1 subclass, and κ light chains; fragment Fd, the N-terminal half of the heavy chain; fragment Fc, the C-terminal half of the heavy chain; C_H1, C_H2 and C_H3, constant homology regions of the heavy chain; IgG, immunoglobulin G.

Our characterization has now confirmed this point and has shown that some parts of the Fc region, which are labile in aqueous pepsin, are conserved under the conditions of the urea/pepsin digestion.

Materials and Methods

Immunoglobulins used as substrates were obtained from multiple myeloma patients Tem and McC, and were isolated from serum by electrophoresis in an LKB 5800 Porath column (Porath, 1954).

The peptic digestion of immunoglobulins in urea, reduction and alkylation of fragments, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and N- and C-terminal determinations were carried out as described previously (Parr *et al.*, 1976).

Separation of peptides

Peptides produced by reduction and alkylation of fragment upFc were separated on a column (1.8cm $\times$ 210cm or 2.7cm $\times$ 210cm) of Sephadex G-50 [Pharmacia (Canada), Dorval, Que., Canada], eluted with 0.1M-sodium acetate buffer, pH 5.0, containing 6M-urea. Further purification of one of the peaks obtained by gel filtration was performed by ion-exchange chromatography on a column (1.6cm $\times$ 25cm) of CM-cellulose (Whatman CM32), by using 6M-urea/0.1M-acetate buffer, pH 5.0, for equilibration, with a gradient to the same buffer containing 0.5M-NaCl. A closed 500ml mixing chamber was used. The positions of protein peaks in the eluates

were determined by measuring the u.v. absorbance of the fractions at 280 nm. Pooled fractions were concentrated by ultrafiltration in Diaflo cells (Amicon Corp., Lexington, MA, U.S.A.). Radioactivity in column eluates was determined by counting 50 μ l portions of alternate fractions in 10 ml of scintillation fluid (Bray, 1960) in a Nuclear-Chicago liquid-scintillation counter.

Amino acid analysis

Approx. 20 nmol of peptide was hydrolysed in 200 μ l of constant-boiling HCl for 24 h at 110°C under vacuum. Hydrolysates were evaporated to dryness, and redissolved in 520 μ l of 0.2 M-sodium citrate buffer, pH 2.2, containing 250 nmol each of norleucine and L- α -amino- β -guanidinopropionic acid hydrochloride/ml as standards.

Sequencer analysis

Sequence analyses were carried out in a Beckman Sequencer model 890C by using a programme essentially the same as that described by Hermodson *et al.* (1972) and the volatile *NN*-dimethylbenzylamine buffer of Hermodson *et al.* (1972). Amino acid phenylthiohydantoin derivatives were converted into silylated derivatives (Hermodson *et al.*, 1972) and identified by g.l.c. Half-cystine residues, in the form of *S*-carboxy[methyl- 14 C]cysteine, were confirmed by liquid-scintillation counting. Arginine and histidine residues were identified by recovering the amino acids from their thiazolinones, by using a procedure based on that described by Smithies *et al.* (1971). Thiazolinones were evaporated to dryness, after the addition of 20 nmol of norleucine to each, heated for 20 min at 60°C, and hydrolysed with 200 μ l of 66% (w/v) HI for 24 h at 130°C in evacuated sealed tubes. The hydrolysates were evaporated to dryness and dissolved in 400 μ l of sodium citrate buffer, pH 2.2; samples (50 μ l) were applied to each column of the Beckman 121M amino acid analyser. Confirmation of some residues identified by g.l.c. was also carried out by converting the phenylthiohydantoin derivatives into free amino acids by this method.

Results

Preparation of peptides

The isolation of fragment upFc(Tem) as pool B from the urea/pepsin digestion mixture has been described (Parr *et al.*, 1976). *N*-Terminal determinations had shown that more than one sequence was present in this pool, so fragment upFc(Tem) was totally reduced and alkylated, and passed through a column of Sephadex G-50. The elution profile obtained is shown in Fig. 1; fractions were pooled

as illustrated. When examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, pool A showed two bands and pool B one (peptide B). Pool A was then concentrated, and passed through a column of CM-cellulose. The ion-exchange elution profile is illustrated in Fig. 2. The ratio of absorbance to radioactivity was twice as high in peak Aa as in either peak Ab or peak Ac. Fractions were pooled as shown. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis demonstrated that peak Aa corresponded to the peptide with lower mobility eluted in pool A from the Sephadex G-50 column and that both peaks Ab and Ac corresponded to the electrophoretically faster band. The corresponding peptides from another IgG1/ κ myeloma protein, IgG(McC), were also prepared by the methods described above. At this stage all peptides isolated appeared as single bands when examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3).

N-Terminal determinations

The *N*-terminal residues of peptides in the various pools were determined at each stage. After gel filtration, both pool A (Tem) and pool A (McC) were found to contain peptides with *N*-terminal isoleucine and threonine residues. Peptide B from each Sephadex G-50 column also had an *N*-terminal threonine, together with small amounts of phenylalanine and tyrosine. After the ion-exchange step, peptide Aa in both cases contained only *N*-terminal

Fig. 1. Separation of the products of reduction and alkylation of fragment upFc(Tem) by gel filtration

The column (1.8 cm $\times$ 210 cm) was packed with Sephadex G-50 and eluted with 0.1 M-sodium acetate containing 6 M-urea, pH 5.0. The flow rate was 24 ml/h; 2 ml fractions were collected.

isoleucine, whereas peptides Ab and Ac had threonine in this position. Peptide Ab also showed some *N*-terminal valine and leucine.

Sequence studies

Table 1 shows the results obtained from the sequencer for five of the peptides analysed, and a comparison of these sequences with those of three regions of the heavy chain of IgG(Eu) (Edelman *et al.*, 1969). Peptide Aa from fragment upFc(Tem) was found to be homogeneous and to have residue Ile-253 at its *N*-terminus [the numbering system used by Edelman *et al.* (1969) for protein Eu is used throughout]. Sequencing of peptide Ab from fragment upFc(Tem) showed a lack of homogeneity, although the peptide present in greatest yield had the same sequence as peptide Ac throughout the ten-residue stretch analysed. Peptides Ac from both fragments upFc(Tem) and upFc(McC) were homogeneous and identical. They were found to correspond to the region of the heavy chain with residue Thr-307, within the C_H2-domain disulphide loop, as its *N*-terminal residue. Peptide B was found to consist of the *C*-terminal end of the heavy chain, with residue Thr-411 at the *N*-terminus. Peptide B (Tem) was homogeneous; peptide B (McC) was found to contain 60–70% of a peptide identical with peptide B (Tem), but minor sequences present indicated that about 30% of the heavy chains of IgG(McC) had been cleaved at one or more of residues

Phe-405, Leu-406 or Tyr-407. At three positions, residues 312, 315 and 419, the γ chains of IgG(Tem) and IgG(McC) were found to differ from the sequence reported for the γ chain of IgG(Eu).

Amino acid analyses

The amino acid compositions of peptides Aa, Ac and B from IgG(Tem) were determined. The results are shown in Table 2, in which the amino acid compositions of corresponding regions from the γ chain of IgG(Eu) (Edelman *et al.*, 1969) are given for comparison. The finding of one residue of *S*-carboxymethylcysteine in peptide Aa and two residues in peptide Ac is in agreement with the ratio of radioactivity to u.v. absorbance observed after the separation of these peptides by ion-exchange chromatography (Fig. 2). Peptides Aa and Ac each

Fig. 2. Separation by ion-exchange chromatography on CM-cellulose of the peptides eluted in pool A from the Sephadex G-50 column

The sample applied was 6ml. The flow rate was 22.5 ml/h and 1.5 ml fractions were collected. Further details of the elution are given in the text. —, E_{280} ; ---, radioactivity.

Fig. 3. Preparation and electrophoresis in sodium dodecyl sulphate/polyacrylamide-gel of peptides from fragment upFc

Experimental details are given in the text. Gels contained 0.1% (w/v) sodium dodecyl sulphate and 12.5% (w/v) acrylamide.

Table 1. *Sequencer data obtained for peptides derived from fragment upFc*

The sequences of regions of H chain(Eu) (Edelman *et al.*, 1969) are also given for comparison. —, indicates identity of sequences. N.D., Not determined.

Peptide	Repetitive yield (%)	Sequence
Aa (Tem) H chain(Eu) 253–262	96	Ile-Ser-Arg-Thr-Pro-Glu-Val-Thr-Cys-Val-
Ab (Tem)	N.D.	Heterogeneous
Ac (Tem)	94	Thr-Val-Leu-His-Gln-Asp-Trp-Leu-Asn-Gly-
Ac (McC) H chain(Eu) 307–316	92	—Asn—Asp—
B (Tem)	>85	Thr-Val-Asp-Lys-Ser-Arg-Trp-Gln-Gln-Gly-
B (McC) H chain(Eu) 411–420	92	—Glu—

Table 2. *Amino acid compositions of peptides isolated from fragment upFc(Tem)*

Values are residues per molecule of peptide. About 20nmol of each peptide were hydrolysed in 200 μ l of constant-boiling HCl for 24 h at 110°C. The compositions of the peptides are compared with the compositions of theoretical peptides from various regions of the γ chain of IgG(Eu) (Edelman *et al.*, 1969). Abbreviations: CmCys, *S*-carboxymethylcysteine; N.D., not determined.

Amino acid	Peptide Aa	IgG(Eu) residues 253–306	Peptide Ac	IgG(Eu) residues 307–376	Peptide B	IgG(Eu) residues 411–446
Lys	2.4	3	7.0	9	1.7	2
His	1.9	2	1.0	1	2.5	3
Arg	2.5	3	1.8	2	0.9	1
Asp	6.0	6	5.9	5 (6)*	3.3	3
Thr	4.4	4	5.3	5	2.2	2
Ser	4.9	4	5.2	5	5.0	6
Glu	7.2	7	8.4	9 (8)	4.5	4
Pro	3.7	3	7.9	7	1.0	1
Gly	1.1	1	3.1	3	2.2	2
Ala	1.6	1	3.2	3	1.1	1
Val	9.2	12	4.9	5	3.2	3
Met	0.1	0	0	1 (0)	1.0	1
Ile	0.9	1	2.1	2	0	0
Leu	1.7	1	7.0	6 (7)	3.1	3
Tyr	3.0	3	2.9	3	1.1	1
Phe	1.3	1	1.0	1	1.0	1
CmCys	1.0	1	1.8	2	1.0	1
Trp	N.D.	1	N.D.	1	N.D.	1
Carbohydrate	+	+	—	—	—	—

* The numbers of residues in parentheses are those that would be observed in a heavy chain of Gm(1) allotype (Thorpe & Deutsch, 1966).

contain one residue of tryptophan, three residues of tyrosine and one residue of phenylalanine, so that their molar extinction coefficients can be assumed to be the same.

C-Terminal determinations

The *C*-terminal amino acid residues of peptides Aa and Ac from fragment upFc(Tem) were determined. After 1 h digestion, carboxypeptidase S_1 had

released 43.4, 14.6, 13.3, 9.9 and 3.9 nmol of valine, serine, leucine, arginine and tyrosine respectively from 18 nmol of peptide Aa. This *C*-terminal analysis confirms the conclusion drawn from the amino acid analysis cited above, that the *C*-terminal sequence of peptide Aa is -Tyr-Arg-Val-Val-Ser-Val-Leu, residues 300–306 inclusive of the γ chain. After 1 h digestion, carboxypeptidase S_1 had released 43.3 nmol of aspartic acid from 44 nmol of peptide Ac, but less than 8 nmol of any other amino acid. The

closest correlation between the observed amino acid composition of peptide Ac and a theoretical peptide based on the sequence of γ chain (Eu) was observed when the C-terminal residue of the theoretical peptide was assumed to be Asp-376 (Table 2). If Asp-376 is indeed the C-terminal residue of peptide Ac, then the C-terminal sequence expected would be -Pro-Ser-Asp. It has been observed (Hui *et al.*, 1974) that penicillocarboxypeptidase S₁ does not readily cleave bonds between neighbouring residues with small side chains, so that the liberation only of aspartic acid from this peptide is a finding consistent with the analysis.

Yields of peptides

When characterization of peptides Aa, Ac and B was completed and their molecular weights could be calculated, the yields of fragment upFc and its constituent peptides which had been obtained were determined. By using an extinction coefficient ($E_{1\text{cm}}^{1\%}$) at 280nm of 14.0 for fragment upFc, the recovery of the fragment from the digestion mixture after gel filtration was found to be approx. 90% of the theoretical value. Since peptides Aa, Ac and B each contain one residue of tryptophan, it was assumed that their molar extinction coefficients would be similar, and, from this assumption, the recoveries of the purified peptides from whole IgG were found to be as follows: peptide Aa, 59% of the theoretical value; peptide Ac, 21% of the theoretical value; and peptide B, 89% of the theoretical value. The high yield of peptide B is presumably owing to the fact that only a single purification step was required to obtain this peptide from fragment upFc. The low yield of peptide Ac in peak

Ac from the ion-exchange column can be accounted for by the fact that peak Ab from the same column (Fig. 2) contained a large amount of a very closely related peptide. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the peptides in peak Ab were the same size as those of peak Ac (i.e. 70 residues) so the most likely explanation for the separation of peak Ab from peak Ac, and also for the presence of several very small peaks eluted from the CM-cellulose column (Fig. 2), seems to be that peptide Ac has undergone various extents of charge modification, such as deamidation, resulting in a 'family' of peptides of slightly different charges.

Discussion

Consideration of the results obtained from sequence studies, amino acid compositions and N- and C-terminal analyses enabled us to identify peptides Aa, Ac and B as fragments of the heavy chain as follows: peptide Aa, residues Ile-253 to Leu-306; peptide Ac, residues Thr-307 to Asp-376; and peptide B, residues Thr-411 to Gly-446. The characterization of these three peptides has enabled us to identify the whole of fragment upFc and to locate its position in the IgG molecule (Fig. 4). Most of the Fc fragment is present, but the hinge region is absent, and a section of 34 residues has been lost from the disulphide loop of the C_H3 domain. Characterization of fragment Fb'₂(Tem) (Parr *et al.*, 1976) has shown that the digestion of IgG1/ κ with pepsin in the presence of 8M-urea results in cleavage at the C-terminal side of the hinge region, so that the C-terminal residue of fragment Fb'₂ is Met-252. The identification of Ile-253

Fig. 4. Schematic representation of the structure of fragment upFc

The solid lines indicate the parts of the IgG molecules found in the upFc fragment; the broken lines indicate the remainder of the molecule. The amino acid residues marked are the N-terminals of peptides isolated from reduced and alkylated fragment upFc, except for residue Ile-377, which is the N-terminal residue of the missing section.

Fig. 5. Schematic representation of the sites of peptic cleavage in a γ chain in aqueous buffer and in urea

Fragments produced by digestion in urea are shown at the top of the diagram, those produced by aqueous digestion at the bottom. Broken arrows indicate regions of multiple cleavages at uncertain sites. Amino acid residues marked are the *N*-terminal sides of cleavage sites. PCA, pyrrolidone carboxylic acid.

as the *N*-terminal residue of fragment upFc shows that no small peptides have been lost at this cleavage site.

Fig. 5 compares the sites within a γ 1 heavy chain which are cleaved with pepsin in an aqueous medium and in 8M-urea. The most striking differences illustrated by Fig. 5 are the loss of the variable region, which is also lost from the κ chain, and the retention of the C_H2 domain during digestion in urea. By contrast, after aqueous peptic digestion the variable region is conserved in the $F(ab')_2$ fragment, and the C_H2 domain is lost as small peptides. The loss of the variable region during digestion in urea can be accounted for by assuming that the unfolding of the molecule in urea causes the exposure of more sites susceptible to enzyme action than are exposed under aqueous conditions. Similarly the C_H3 domain shows increased susceptibility to pepsin digestion, being cleaved between residues Asp-376 and Ile-377 and between residues Leu-410 and Thr-411. In addition, in IgG(McC) further partial cleavages were observed in this region. This observation, too, can be explained by assuming an unfolding of the molecule in urea. Preservation of the C_H2 domain, with the only cleavage site being between residues Leu-306 and Thr-307, is a more unexpected finding, many sites that are susceptible to pepsin in aqueous buffers remaining intact. It is probable that this result is due to changes brought about by the action of the urea on the pepsin, rather than to changes in the substrate. It seems clear that the differences in digestion products are due to changes in the enzyme specificity, as well as to unfolding of the substrate, since no identical cleavage sites have been

observed under the two conditions. It is also noteworthy that the sites of cleavage are different even in the hinge region, which is believed to be in an extended conformation even in aqueous solution.

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