Fb'₂, a New Peptic Fragment of Human Immunoglobulin G

By DOROTHY M. PARR, GEORGE E. CONNELL, DAVID I. C. KELLS
and THEO HOFMANN

Department of Biochemistry, University of Toronto, Toronto, Ont. M5S 1A8, Canada

(Received 26 August 1975)

1. The digestion of a human IgG1/ κ myeloma protein with pepsin in the presence of 8M-urea was observed to produce a fragment, designated Fb'₂, which differed from the products of aqueous peptic digestion and from other characteristic immunoglobulin digestion products. 2. Fragment Fb'₂ was also found when two other IgG1/ κ proteins were treated similarly. 3. Sedimentation-equilibrium studies showed the mol.wt. of fragment Fb'₂ to be 56800. 4. On reduction, two equivalents of each of three peptides were released from fragment Fb'₂; these were characterized by N- and C-terminal determinations and by amino acid sequencing. 5. Fragment Fb'₂ was shown to consist of the constant regions of both light chains, from residue Ile-117 to the C-terminus, and the C_H1 domains and hinge region of the heavy chains, from residue Val-113 to residue Met-252, with a gap of five residues within the intrachain disulphide loop, between residues Leu-174 and Tyr-180.

Since the fragmentation of rabbit IgG* with papain, by Porter (1959), proteolytic enzymes have been widely used in the determination of immunoglobulin structure and in the investigation of relationships between structure and function.

It was shown by Nisonoff *et al.* (1960) that digestion of rabbit IgG with pepsin resulted in the formation of a single large fragment, now known as fragment F(ab')₂, with the loss of most of the Fc region. Further studies of the products of peptic digestion led to the identification of fragment Pep III' (Utsumi & Karush, 1965), a fragment from the C-terminal end of the rabbit IgG molecule. An analogous fragment from human IgG, described as component II by Heimer *et al.* (1967) and as pFc' by Bennich & Turner (1969), has been isolated and shown to correspond closely to the C_H3 domain (Edelman *et al.*, 1969). Pepsin has also been used in the preparation of variable and constant halves of Bence-Jones proteins by Seon *et al.* (1972), who found that the susceptibilities of the two halves of the protein to the enzyme were dependent on the temperature at which digestion was carried out. Peptic digestion of whole human IgG at high temperatures (Seon & Pressman, 1975) has led to the isolation of a fragment that includes the C_H2 domain.

Examination of peptic digests of human IgG in solutions containing high concentrations of urea

showed that the products formed were different from those of peptic digestion in aqueous buffers, and also appeared to differ from other recognized immunoglobulin digestion products. Experiments were therefore carried out to characterize the main fragment produced by peptic digestion of human IgG in the presence of urea.

Materials and Methods

Aqueous pepsin digestion

IgG(Tem), a human myeloma protein with heavy chains of the γ_1 subclass and κ light chains, was prepared from serum as described previously (Connell *et al.*, 1973). A 750mg portion of IgG(Tem) was digested with pig pepsin (2 $\times$ crystallized; Worthington Biochemical Corp., Freehold, NJ, U.S.A.) at 37°C for 24h in 0.1M-sodium acetate adjusted to pH4.3 with acetic acid, with an enzyme/substrate ratio of 1:100 (w/w). The reaction was stopped by raising the pH to 9.0 with 2M-Tris/HCl, pH9.0, and 1M-NaOH. Fragment F(ab')₂ was isolated by gel filtration on a column of Sephadex G-100 (5cm $\times$ 90cm) eluted with 0.1M-Tris/HCl, pH9.0.

Urea/pepsin digestion

Immunoglobulins in aqueous solution (approx. 20mg/ml) were dialysed against two changes of 0.1M-sodium acetate buffer, pH4.3. Urea was dissolved in the dialysed sample to make the concentration slightly above 8M, and the pH was adjusted to 3.6 with HCl. Distilled water was added if necessary to adjust the final urea concentration to 8M. Sufficient pepsin was

* Abbreviations: IgG, immunoglobulin gamma; fragment F(ab')₂, both light chains and the N-terminal halves of both heavy chains, with interchain disulphide bonds intact; fragment Fc, the C-terminal half of the heavy chain; C_H1, C_H2 and C_H3, constant-homology regions of the heavy chain; V_H, variable region of the heavy chain; C_H, constant region of the heavy chain; C_L, constant region of the light chain.

added to give an enzyme/substrate ratio of 1:100 (w/w), and the samples were kept at 23°C. At various times samples were removed in which digestion was stopped by the addition of 2 drops of 2M-Tris/HCl, pH 9.0, followed by NaOH to bring the pH to 9.0.

After digestion on a preparative scale, products were separated by gel filtration in a column (5cm×90cm) of Sephadex G-150 [Pharmacia, (Canada), Dorval, Que., Canada] eluted with 0.1M-sodium acetate buffer, pH 5.0, containing 6M-urea. Protein peaks were located by determining u.v. absorption at 280nm, and pooled fractions were concentrated by ultrafiltration in Diaflo cells (Amicon Corp., Lexington, MA, U.S.A.).

Reduction and alkylation

The fragment, as eluted from the gel-filtration column, was dissolved in 6M-urea/acetate buffer, pH 5.0; to this solid urea and 2M-Tris/HCl, pH 8.2, were added to give a final urea concentration of 8M and a final pH of 8.2. Dithiothreitol was added to give a final concentration of 10mM. After 2½h at 23°C, the products were alkylated by the addition of sufficient 1M-iodo[2-¹⁴C]acetic acid (250µCi/ml) to make the final concentration 22mM. Peptides prepared thus were separated by gel filtration on a column (1.8cm×210cm) of Sephadex G-50 eluted with 6M-urea/0.1M-sodium acetate buffer, pH 5.0. If necessary, further purification was achieved by repeated gel filtration through the same column until the peptides each showed a single band when examined by electrophoresis in starch gel or polyacrylamide gel, and a single N-terminal residue.

Starch-gel electrophoresis

Starch-gel electrophoresis was carried out at room temperature (23°C) in 8M-urea/0.1M-sodium formate buffer, pH 3.6, as described by Smithies *et al.* (1962). The buffer was made up without 2-mercaptoethanol. Gels were stained with 1% (w/v) Amido Black in acetic acid/methanol/water (1:5:5, by vol.) and de-stained with 1% (v/v) acetic acid.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out as described by Weber & Osborn (1969), with gel rods 0.5cm×9cm. The volume of acrylamide solution used was increased to give an acrylamide concentration in the gel of 12.5%, but the other components of the gel mixture were used as described. Samples, which were already totally reduced and alkylated, were prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by mixing together 10µl of sample, 50µl of 0.01M-

sodium phosphate buffer, pH 7.0 (containing 7.8g of NaH₂PO₄·2H₂O and 21.57g of Na₂HPO₄/l), and 10µl of the same buffer containing 4% (w/v) sodium dodecyl sulphate. The proteins were denatured by immersing the tubes containing these dilutions in boiling water for 1 min. A drop of a solution containing equal parts of aq. 0.05% Bromophenol Blue and glycerol was added to each sample, and the entire volume was applied to the gel column. Gels were stained as described by Weber & Osborn (1969), except that staining was done at 70°C for 1 h. De-staining was done by diffusion, the first stage of which, usually overnight, was done at 70°C.

Ultracentrifugation

Sedimentation-velocity and -equilibrium studies were carried out in a Spinco model E ultracentrifuge, at 20°C, with 1M-propionic acid as solvent. The sedimentation coefficient was calculated from sedimentation-velocity data obtained with schlieren optics, at a rotor speed of 60000 rev./min. Sedimentation-equilibrium data were obtained at 37600 rev./min by using the meniscus-depletion method of Yphantis (1964).

N-Terminal determination

N-Terminal residues were determined by the dansyl chloride method for proteins as described by Percy & Buchwald (1972).

C-Terminal determination

C-Terminal residues were determined after digestion with penicillocarboxypeptidase S1 (Hui *et al.*, 1974) in 0.3ml of 0.03M-pyridine/acetate buffer, pH 4.7, at 37°C with enzyme/substrate ratios of about 1:100 (mol/mol). Digestion was stopped by immersing the tubes in boiling water for 5 min. The samples were applied to the Beckman 120C amino acid analyser and run with the standard hydrolysate system, except for peptides that might contain glutamine and/or asparagine, in which case the accelerated system for physiological fluids with a lithium citrate buffer system (Benson *et al.*, 1967) was used.

Sequenator analysis

Sequence analyses were carried out in a Beckman sequencer, model 890C, with the volatile N,N-dimethylbenzylamine buffer described by Hermodson *et al.* (1972). The programme used for this system was essentially the same as that described by Hermodson *et al.* (1972). The identification of the phenylhydantoin derivatives of amino acids was carried out by g.l.c. after their conversion into silylated derivatives. Half-cystine residues, in the form of S-carboxy-

[methyl-¹⁴C]cysteine, were also identified by liquid-scintillation counting.

Extinction coefficient

Extinction coefficients were determined in duplicate by drying to constant weight a known volume of a protein solution whose absorption at 280 nm had previously been determined in a 1 cm cell. The solvent used was 1 M-propionic acid.

Results

Urea/pepsin digestion of IgG(Tem)

Starch-gel electrophoresis showed that after 30 min digestion IgG(Tem) was cleaved into two major fragments. A further 90 min of digestion changed the electrophoretic pattern only slightly; no new bands appeared, but the two major ones already present became more diffuse (Plate 1). A time of 45 min was therefore used for the urea/pepsin digestion of IgG(Tem) on a preparative scale. The gel-filtration profile obtained from this digest is illustrated in Fig. 1. Protein peaks were pooled as shown, and starch-gel electrophoresis showed that peaks A and B corresponded to the major digestion products. When characterization of the fragment isolated in peak A had been completed (Table 1), it was found that recovery of this fragment, after digestion and gel filtration, was over 90% of the theoretical value obtained by using an extinction coefficient ($E_{1\%}^{1\text{cm}}$) of 5.8 determined at 280 nm. When the digestion was repeated, with fragment F(ab')₂(Tem) as substrate instead of IgG(Tem), the recovery of peak A was essentially the same, whereas the yield of peak B was zero, indicating that the fragment(s) eluted from the column in peak B were derived from the Fc region.

Isoleucine, valine and tyrosine were found as *N*-terminal residues in pool A, and isoleucine, together with threonine, serine and phenylalanine, were found as *N*-terminal residues in pool B.

When two other myeloma proteins with heavy chains of the γ_1 subclass and κ light chains were treated in the same way, their elution patterns were found to be very similar. The *N*-terminal residues found in pools A and B from these proteins were also the same as those found in pools A and B from IgG(Tem).

Sedimentation coefficient and molecular weights

A sedimentation-velocity study in neutral aqueous buffer showed that product A had aggregated when dialysed against this buffer. The experiment was repeated, with 1 M-propionic acid as solvent, when a single apparently homogeneous peak was seen with schlieren optics. The value of $s_{20,w}$ in this system was 1.4S.

In a sedimentation-equilibrium study, also in 1 M-propionic acid, plots of $\ln C$ versus r^2 (Yphantis, 1964) were non-linear; the slope decreased with increasing values of r^2 . This appearance is characteristic of solutes that are behaving in a non-ideal fashion (Roark & Yphantis, 1969). The data were re-examined by using a computer program developed by Roark & Yphantis (1969). Plots of various reciprocal apparent point-average molecular weights (number-average, weight-average, Z-average) against concentration all converged linearly at infinite dilution, giving a value of 56800. This study provides further evidence of the homogeneity of the preparation.

Product A, after total reduction and alkylation, gave rise to three bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The mobilities of these bands were compared with those of several known-molecular-weight markers. The mobilities of the standard markers, relative to that of Bromophenol Blue, were: (i) IgG heavy chain, 0.21; (ii) IgG light chain, 0.44; (iii) ribonuclease, 0.66; and (iv) cytochrome *c*, 0.71. The mobilities of the three components derived from product A were 0.73, 0.79 and 0.86. These values were judged, by linear extrapolation of a graph of log mol.wt. against mobility, to be those for peptides of mol.wts. 11000, 9500 and 7500 respectively.

The minimum mol.wt. of the parent fragment was estimated from the sum of these values to be 28000. Comparison of this estimate with the molecular weight determined by sedimentation equilibrium suggests that product A yields, on reduction, two equivalents of each of the three constituent polypeptides.

Fig. 1. Separation of the products of urea/pepsin digestion of IgG(Tem) by gel filtration

The column (5 cm × 90 cm) was packed with Sephadex G-150 and eluted with 0.1 M-sodium acetate buffer, pH 5.0, containing 6 M-urea. The flow rate was 48 ml/h and 400 ml was eluted before fraction collecting began; 8 ml fractions were collected.

Sequence studies

N-Terminal determinations on the peptides after separation by gel filtration showed that the largest peptide, peptide I, had isoleucine as its *N*-terminal residue, the middle peptide, peptide II, tyrosine, and the smallest peptide, peptide III, valine. These results were in agreement with the *N*-terminal findings obtained for the intact fragment. Sequence analysis showed that peptide I consisted of the constant region of the κ chain, with isoleucine 117 at its amino terminus. [The numbering system for amino acid residues used by Edelman *et al.* (1969) for the κ and γ chains of protein Eu is used throughout.] The tyrosine found as the *N*-terminal residue of peptide II was residue 180 of the heavy chain, within the intra-chain disulphide loop of the C_H1 domain. Peptide III was found to begin with Val-113 of the heavy chain, at the switch between the V_H and C_H regions. The analysis by sequenator gave unambiguous identification of 17, 19 and 33 residues respectively for peptides I, II and III. The repetitive yields were 93, 93 and 90% respectively. No differences were found between these sequences and the published sequences for κ and γ 1 chains.

Since the light-chain peptide was released from the fragment in peak A only after reduction and alkylation, the *C*-terminus of this peptide must be the *C*-terminal half-cystine residue through which the light-heavy interchain bond is formed. Digestion of 15.7 nmol of peptide II with carboxypeptidase S1 caused the release of methionine, leucine, threonine, aspartic acid and lysine in amounts of 9.5, 8.5, 6.2, 6.0 and 2.9 nmol respectively. The sequence of the γ 1 chain for residues 248–252 is known to be Lys-Asp-Thr-Leu-Met (Edelman *et al.*, 1969). There is no other comparable sequence in the κ or γ 1 constant regions, and Met-252 is the only methionine residue between the beginning of the constant region and residue 357 in the γ 1 chain. This evidence identifies

peptide II unambiguously as a product of the γ 1 chain extending from residue 180 (tyrosine) to residue 252 (methionine). After digestion with carboxypeptidase S1 for 2 h, 22.6 nmol of peptide III released 17.6 nmol of valine and 16.2 nmol of leucine. No other residue was present in significant yield. When the digest was repeated with about 50 nmol of peptide III, a similar result was obtained, 24.2 nmol of valine and 27.5 nmol of leucine being released after 1 h. This pair of amino acids occur in consecutive sequence only at two positions between Val-113 and Tyr-180 of the γ 1 chain. The sequence Leu-145–Val-146 cannot be the *C*-terminal of this peptide because the calculated molecular weight of the peptide Val-113–Val-146 would be much lower than that observed for peptide III. We conclude that the *C*-terminal sequence must be Val-173–Leu-174. The residue third from the *C*-terminal end, Ala-172, is evidently not released from the Pro–Ala linkage by carboxypeptidase S1 under the conditions used.

Discussion

The results given above and summarized in Table 1 characterized the fragment obtained in peak A of the elution diagram as a new fragment of IgG, consisting of the constant regions of the light chains, the C_H1 domains of the γ chains and the entire hinge region (Fig. 2). The similarity of the relationship between this fragment and the Fb fragment described by Gall & D'Eustachio (1972) to that between fragments Fab and F(ab')₂ has led us to propose the name fragment Fb'₂ for this fragment, despite the loss from the heavy chain of the pentapeptide Gln-175–Leu-179.

The site of cleavage between Phe-116 and Ile-117 in the κ chain caused by peptic digestion in the presence of 8 M-urea is the same as that reported by Seon *et al.* (1973) after digestion of Bence-Jones proteins with pepsin at various temperatures, including 37°C.

Table 1. Properties of peptides obtained by reduction of the fragment isolated in peak A (Fig. 1)

Peptide	Sequence		Postulated site	10 ⁻³ × Mol.wt.	
	<i>N</i> -Terminal	<i>C</i> -Terminal		Observed*	Calculated†
I	Ile-Phe-Pro- (17)‡	-Cys§	κ (Ile-117-Cys 214)	11	10.8
II	Tyr-Ser-Leu (19)‡	-Lys(Asp,Thr)-Leu-Met	γ 1 (Tyr-180-Met 252)	9.5	7.8
III	Val-Thr-Val- (33)‡	-(Val,Leu)	γ 1 (Val-113-Leu 174)	7.5	6.6

* From sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

† Calculated from the amino acid sequences of Eu light chains and heavy chains (Edelman *et al.*, 1969) corresponding to the postulated sites.

‡ The values in parentheses indicate the number of consecutive residues identified in the *N*-terminal sequence analysis of each peptide.

§ The identity of the *C*-terminal amino acid of the κ chain peptide was inferred from the integrity of the light-heavy interchain disulphide bond in the parent fragment.

EXPLANATION OF PLATE I

Urea/formate starch-gel electrophoresis of a time-study of the digestion of IgG(Tem) with pepsin in the presence of 8M-urea

Experimental details are given in the text.

Fig. 2. Schematic representation of the structure of fragment Fb'₂

The continuous lines indicate the parts of the IgG molecule found in the Fb'₂ fragment, and the broken lines indicate the remainder of the molecule. The amino acid residues marked are the *N*-terminals of peptides isolated from the urea/pepsin digestion products of IgG(Tem).

Peptic digestion of intact IgG at 37°C does not cause any cleavage in the light chain, so presumably it is a conformational change in the heavy chain brought about by the urea that permits cleavage between κ -chain residues 116 and 117. Denaturation of the IgG by urea or by high temperatures appears to create the same susceptibility in the variable regions of both κ and γ chains, since these regions of whole IgG were destroyed by the urea/pepsin digestion, as they were by the high-temperature pepsin digestion procedure of Seon & Pressman (1974). These same authors (Seon & Pressman, 1975) also claim to have isolated the C_H2 domain, beginning at Ile-253, from a peptic digestion fragment obtained at 70°C. This also indicates that labile sites made available by urea or heat denaturation are similar, since the C-terminal residue of peptide II of fragment Fb'₂ is Met-252.

The value of 5.8 for the extinction coefficient of fragment Fb'₂ is markedly lower than that of intact IgG, but is in accordance with the amino acid composition of the fragment. Only one tryptophan residue is found in each of the C_L and C_H1 domains, instead of the two found in the other homology regions, and the hinge region contains none. Further, the entire Fb'₂ fragment contains only seven tyrosine and five phenylalanine residues, so that the absorption at 280nm would be expected to be low. The absorption of the hinge-region peptide (peptide II) isolated from fragment Fb'₂ was so low at this wavelength that the peptide was located in column eluates by the position of the radioactivity incorporated into *S*-carboxy[methyl-¹⁴C]cysteine rather than by u.v. absorption.

Fragment Fb'₂ therefore may provide a source of peptides suitable for sequencing that can fill the gaps remaining in the C_H1 and hinge regions of some γ chains. Also, since the fragment extends a short distance into the C_H2 domain, it may be of use in identifying more closely the sites within this domain that are responsible for antibody-molecule effector functions.

The authors are grateful to Dr. W. Pruzanski for sera from patients with multiple myeloma, and to Mrs. A. Cunningham, Mrs. I. Csermely, Mr. S. Rhee and Mr. C. Yu for technical assistance. This work was supported by the Medical Research Council of Canada.

References

- Bennich, H. & Turner, M. W. (1969) *Biochim. Biophys. Acta* **175**, 388–395
- Benson, J. V., Jr., Gordon, M. J. & Patterson, J. A. (1967) *Anal. Biochem.* **18**, 228–240
- Connell, G. E., Freedman, M. H., Nyburg, S. C., Painter, R. H., Parr, D. M., Pinteric, L. & Pruzanski, W. (1973) *Can. J. Biochem.* **51**, 1137–1141
- Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U. & Waxdal, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 78–85
- Gall, W. E. & D'Eustachio, P. (1972) *Biochemistry* **11**, 4621–4628
- Heimer, R., Schnoll, S. S. & Primack, A. (1967) *Biochemistry* **6**, 127–134
- Hermanson, M. A., Ericsson, L. H., Titani, K., Neurath, H. & Walsh, K. A. (1972) *Biochemistry* **11**, 4493–4502

- Hui, A., Rao, L., Kurosky, A., Jones, S. R., Mains, G., Dixon, J. W., Szewczuk, A. & Hofmann, T. (1974) *Arch. Biochem. Biophys.* **160**, 577-587
- Nisonoff, A., Wissler, F. C., Lipman, L. N. & Woernley, D. L. (1960) *Arch. Biochem. Biophys.* **89**, 230-244
- Percy, M. E. & Buchwald, B. M. (1972) *Anal. Biochem.* **45**, 60-67
- Porter, R. R. (1959) *Biochem. J.* **73**, 119-126
- Roark, D. E. & Yphantis, D. A. (1969) *Ann. N.Y. Acad. Sci.* **164**, 245-278
- Seon, B.-K. & Pressman, D. (1974) *J. Immunol.* **113**, 1190-1198
- Seon, B.-K. & Pressman, D. (1975) *Immunochemistry* **12**, 333-337
- Seon, B.-K., Roholt, O. A. & Pressman, D. (1972) *J. Biol. Chem.* **247**, 2151-2155
- Seon, B.-K., Grossberg, A. L., Roholt, O. A. & Pressman, D. (1973) *J. Immunol.* **111**, 269-271
- Smithies, O., Connell, G. E. & Dixon, G. H. (1962) *Am. J. Hum. Genet.* **14**, 14-21
- Utsumi, S. & Karush, F. (1965) *Biochemistry* **4**, 1766-1779
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297-317