The Position of Various Cleavages of Rabbit Immunoglobulin G*

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SUMMARY

The 5 S fragments of rabbit immunoglobulin G were prepared by digestion with pepsin or with CNBr. Cleavage of these fragments with trypsin led to the localization of the positions of these splits on the heavy chain. The COOH-terminal residue released by carboxypeptidase A from the pepsin-produced 5 S fragment was leucine. The pepsin-5 S fragment was reduced and aminoethylated to yield the 3.5 S fragment. Trypsin digestion of this fragment yielded two decapeptides which represent the COOH terminus of the heavy chain part of the 3.5 S fragment. The major one, obtained in 65% yield, had the sequence

Ser-Lys-Pro-Thr-Asp-Cys-Pro-Pro-Pro-Glu-Leu.

The other decapeptide which was obtained in a smaller yield (20%), had the same amino acid composition but a different carbohydrate composition.

When the CNBr-5 S fragment was digested with pepsin and then reduced and aminoethylated, it yielded a 3.5 S fragment. Digestion of the latter product with trypsin released the same decapeptides mentioned above. The COOH-terminal sequence of the CNBr-5 S fragment was shown to be Asp-Thr-Leu-homoserine. Thus, the order of the size of the various fragments produced by different cleavages is: CNBr > pepsin > trypsin.

The positions of the various cleavages were correlated with the known sequence of the heavy chain.

Immunoglobulin G is composed of four polypeptide chains, two heavy and two light, and the antibody-combining sites are found in the Fab fragments which consist of one light chain and the NH₂-terminal half of the heavy chain (4). Four methods are available for the cleavage of rabbit IgG into fragments which carry biological activity. (a) Papain cleaves the molecule to yield two identical 3.5 S Fab fragments and a crystallizable Fc (5), which is a dimer of the COOH-terminal half of the heavy chain. These fragments are derived from a papain proteolysis at approximately the middle of the heavy chain (6) and accompanying reduction of disulfide bonds (7). (b) Pepsin hydrolyses IgG into a divalent 5 S (Fab')₂ fragment and to smaller pieces derived from Fc (8). Upon reduction, the 5 S (Fab')₂ cleaves into two 3.5 S Fab', slightly bigger than the papain-produced Fab (9). (c) CNBr also cleaves the molecule into a 5 S fragment (10) denoted (Fab")₂ (11) which, upon reduction, yields active 3.5 S Fab" and it has been suggested that the CNBr cleavage occurs between the position of papain and pepsin cleavages (10). (d) Trypsin cleaves mildly reduced and aminoethylated IgG into two active 3.5 S Fab fragments and a crystallizable Fc fragment, presumably by cleavage at an AE-Cys-Ser bond in the heavy chain (12).

The similar size of the Fab fragment produced by the different methods (mol wt approximately 50,000) suggests that all of these cleavages occur in the same area of the heavy chain which is probably more accessible to the different cleaving agents.

We have used the hydrolysis by trypsin, mentioned above, as a tool to localize the position of the various cleavages. Pepsin-Fab was found to be 10 residues longer than trypsin-Fab, since trypsin digestion of the aminoethylated, pepsin-produced Fab released one decapeptide. The position of CNBr cleavage of IgG was established by the finding of a homoserine-containing tetrapeptide which was released upon tryptic digestion of CNBr-produced (Fab")₂. The CNBr-(Fab")₂ was digested with pepsin and then reduced and aminoethylated. Trypsin digestion of this Fab again released the above-mentioned decapeptide. Hence the order of size of the different Fab fragments according to the cleaving agent is CNBr > pepsin > trypsin, whereas the trypsin-produced Fc is 4 residues longer than the papain-Fc.

MATERIALS AND METHODS

Rabbit IgG and IgG Fragments—Rabbit IgG prepared as previously described (7) was concentrated by vacuum dialysis and stored at -20°C. Pepsin digestion of rabbit IgG was performed according to Mandy, Rivers, and Nisonoff (13) and the (Fab')₂...
fragment was isolated by chromatography on a Sephadex G-75 column (14) equilibrated with 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4. The CNBr-(Fab')₂ fraction was prepared according to Cahnmann, Amion, and Sela (10). In order to avoid extensive denaturation we attempted to minimize the time of exposure of the products to acid conditions; therefore, incubation with CNBr (0.14 M in 0.3 M HCl) was for only 2 hours, and the purification of the (Fab')₂ fragment was performed in neutral buffer as described in the results. Reduction and aminemethylation (15) were performed by the incubation of the different (Fab') preparations in 0.2 M Tris-HCl (pH 8.2), 0.005 M dithiothreitol for 1 hour at room temperature, followed by the addition of ethylene imine (final concentration, 0.15 M). After a further 30 min of incubation the mixture was applied to a Sephadex G-75 column (equilibrated with 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4) and the 3.5 S Fab fragment was isolated. Protein concentration was calculated from the measurements of optical density at 280 nm, with an extinction coefficient ε 1% cm -1 of 14.0 for the IgG or the Fab fragments.

Enzymes—Pepsin (twice crystallized), carboxypeptidase (three times crystallized), and trypsin (twice crystallized) were purchased from Worthington. Trypsin was treated with chloromethyl-L-(2-phenyl-1-toluene-p-sulfonamide)ethyl ketone according to Kostka and Carpenter (16). Digestion with trypsin was carried out in 0.15 M NaCl, 0.02 M sodium phosphate buffer (pH 7.4), for 2½ hours at room temperature, with the use of 1% by weight of trypsin to substrate. Digestion with papain was performed in 0.1 M NH₄HCO₃, 0.01 M 2-mercaptoethanol, 0.002 M EDTA.

Reagents—CNBr and ethylene imine were obtained from Fluka (Switzerland) and dithiothreitol was obtained from Calbiochem.

Amino Acid Analyses—Hydrolysis was performed in constant boiling HCl in sealed, evacuated tubes, at 110° for 18 to 24 hours. A Beckman model 120B amino acid analyzer, equipped with a 6.3-volt EEL model 106/20 high sensitivity unit, was used for the amino acid analyses (17). Hydrolysates of peptides obtained from CNBr-(Fab')₂ were treated with pyridine acetate buffer, pH 6.5, for 1 hour at 100° before the analysis, in order to convert the homoserine lactone to homoserine (18). Qualitative detection of homoserine in different fractions was performed on an acid hydrolysate of about 0.1 pmole of each fraction by high voltage electrophoresis. Amino acid composition of the aminoethylated Fab' showed that it contained 3.20 moles of AE-Cys per mole of Fab. Therefore, trypsin digestion near this AE-Cys will release free serine and glycine. Therefore, trypsin digestion near this AE-Cys will release free serine and glycine.

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Edman Degradation—The procedure described by Margoliash (21) was used. An aliquot of the residual peptide was taken after each step for amino acid analysis. After the last step the free COOH-terminal residue was analyzed without acid hydrolysis. Considerable losses of lysine (22) and aminemethyl cysteine were observed during the procedure. The phenylthiohydantoin derivative of lysine was identified by thin layer chromatography (23).

Physical Methods—Spectrophotometric measurements were made with a Zeiss PMQ1 spectrophotometer, with quartz cuvettes with 10-mm light path.

RESULTS

Site of Pepsin Cleavage to Produce (Fab')₂—The elution pattern obtained from a Sephadex G-75 column of a pepsin digest of rabbit IgG (450 mg) is shown in Fig. 1A. The recovery of optical density at 280 nm was 95% in the first peak, 16.5% in the second peak, and 14.5% in the last peak. The material under the first peak is the (Fab')₂ (14) and upon sedimentation in the ultracentrifuge it shows one symmetrical peak with s₂₀,w = 4.9 S. Carboxypeptidase A digestion of this material (3.7 mg) released only leucine (0.068 pmole) in a yield of 1.95 moles per mole of (Fab')₂, assuming for the latter a molecular weight of 106,000 (9). After reduction and aminemethylation, the Fab' was purified again on the Sephadex G-75 column (Fig. 1B), and the major component obtained which represents Fab' had a sedimentation coefficient s₂₀,w = 3.5 S. The material under the last peak in Fig. 1B contained reagents from the reduction mixture and no amino acids in significant yield. Amino acid analysis of the aminoethylated Fab' showed that it contained 4.0 moles of AE-Cys per mole of Fab', as calculated on the basis of 8.4 arginine residues in Fab (25). The aminoethylated Fab' (20 mg per ml in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4) was digested with trypsin (1% by weight of Fab') for 2½ hours at room temperature, and the digest was applied to a Sephadex G-75 column, equilibrated with 0.05 M NH₃ (Fig. 1C). Amino acid analysis of the material emerging at the position of Fab' showed that it contained 3.20 moles of AE-Cys per mole of Fab. The other fractions (PRT1, PRT2, PRT3) were analyzed for their amino acid composition with the amino acid analyzer and for their content of peptides by high voltage electrophoresis.

Fraction PRT3 contained only free serine (15% yield) and free glycine (18% yield). These amino acids might represent COOH-terminal residues of the light chain of Fab' since it has been shown by hydrazinolysis that rabbit immunoglobulin light chain has cysteine, serine, and glycine as COOH-terminal residues.³ If, by analogy with human immunoglobulin light chain, the amino acid next to the COOH-terminal serine (or glycine) is cysteine (26), it is converted in our experiment to COOH-terminal residues (serine or glycine) and its composition was: Lys, 1.00; AE-Cys, 0.85; Thr, 1.00; Ser, 0.97; Glu, 1.07; Pro, 3.96; Leu, 1.00. The sequence of this decapeptide was established as follows. Carboxypeptidase A digestion released only leucine (90% yield), and hy-

³ D. Givol, unpublished results.
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Fig. 1. Gel filtration of pepsin digest of rabbit IgG and of trypsin digest of reduced, aminoethylated Fab'. A, elution pattern of pepsin-digested IgG; 450 mg in 10 ml were applied to a Sephadex G-75 column (3 x 125 cm), equilibrated, and developed with 0.15 M NaCl, 0.02 M phosphate buffer (pH 7.4), at room temperature. Fractions of 10 ml were collected. B, chromatography of the reduced, aminoethylated pepsin-Fab'. The material emerged at the front of the column in tube 45 (N.D., not determined; PTH, phenylthiohydantoin) as follows: Step 1: Lys, 0.60; AE-Cys, 0.60; Thr, 1.00; Ser, 0.60; Glu, 1.10; Pro, 4.00; Leu, 1.00. Step 2: Lys, N.D.; AE-Cys, N.D.; Thr, 0.80; Ser, N.D.; Glu, 1.00; Pro, 3.20; Leu, 1.00. Step 3: Lys, N.D.; AE-Cys, N.D.; Thr, 0.31; Ser, N.D.; Glu, 1.00; Pro, 2.80; Leu, 1.00. Step 4: Lys, N.D.; AE-Cys, N.D.; Thr, 0.31; Ser, N.D.; Glu, 1.00; Pro, 1.70; Leu, 1.00. Peptide PRT2pA: AE-Cys-Pro-Pro-Pro-Glu-Leu. Step 1: AE-Cys, 0.13; Glu, 1.20; Pro, 1.60; Leu, 1.00. Step 2: AE-Cys, 0.00; Glu, 1.17; Pro, 2.00; Leu, 1.00. Step 3: AE-Cys, 0.00; Glu, 1.20; Pro, 1.60; Leu, 1.00. Step 4: AE-Cys, 0.00; Glu, 1.12; Pro, 0.32; Leu, 1.00. Step 5 (without hydrolysis): Leu, 0.73.

Hence the sequence of Peptide PRT2 is:

Trypsin

\[ (AE-Cys)\text{Ser-Lys-Pro-Thr-AE-Cys-Pro-Pro-Pro-Glu-Leu} \]

Papain

\[ \text{PRT2pA} \]

Since the pepsin-produced (Fab')_2 has COOH-terminal leucine and the trypsin-produced Fe has NH2-terminal serine (12), Decapeptide PRT2 represents that part of the heavy chain which is between the sites of hydrolysis of these two enzymes. Peptide PRT1 was obtained in 20% yield. It was purified by paper electrophoresis at pH 3.5 (mobility with respect to lysine, 0.40) and its amino acid composition is the same as that of Peptide PRT2, but in addition galactosamine was detected on the amino acid analyzer.

A sample of Peptide PRT1 (0.2 μmole) was hydrolyzed in 2 M HCl at 100° for 2 hours. The hydrolysate was brought to dryness, redissolved in water, and brought again to dryness. The residue was chromatographed in two different solvents—butanol-acetic acid-water (4:1:5, upper phase) and butanol-pyridine-water (6:4:3)—and the chromatogram was developed with the alkaline silver-nitrate reagent (27) for the detection of reducing compounds. Four positive spots were detected, corresponding to galactosamine, galactose, glucose, and xylose. Similar analysis of Peptide PRT2 showed that it contained only glucose and xylose. No sequence analysis of Peptide PRT1 was carried out and it is assumed that its amino acid sequence is the same as for Peptide PRT2.

Position of CNBr Cleavage to Produce (Fab')_2—Rabbit IgG (950 mg) was digested with CNBr (0.14 M CNBr, 0.3 M HCl) for 2 hours (10). The reaction mixture was dialyzed against 0.1 M sodium acetate, pH 7.8, and the precipitate formed during the dialysis was centrifuged and discarded. The supernatant (72.5% of total optical density at 280 μM of the starting material) was precipitated with 18% Na2SO4 (13). The precipitate was centrifuged at room temperature and was dissolved in 10 ml of water. This solution (65% of optical density at 280 μM of starting material) was applied to a Sephadex G-150 column equilibrated with 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4. Fig. 2 shows the elution pattern from this column. The material under the first peak contains aggregates. The material under the second peak represents (Fab')_2, and it was recovered in a yield of 45% of the optical density at 280 μM of the starting material. The (Fab')_2 was rerun on the same column and concentrated for further studies. It sedimented as a symmetrical...
peak with $s_{20,w} = 4.9$ S, whereas upon mild reduction and amino- 
ethylation only one component with $s_{20,w} = 3.3$ S was observed. 

Trypsin digestion was used in order to isolate from the CNBr- 
(Fab')$_2$ a peptide containing homoserine which represents the 
COOH terminus of this fragment. Fig. 3 shows the elution 
pattern of such a digest from a Sephadex G-50 column equili-
 brated with 0.05 M NH$_3$. An aliquot of 15% from Fractions 
CT1, CT2, and CT3 was brought to dryness, hydrolyzed in 6 N 
HCl, and analyzed for its homoserine content. Only Fraction 
CT2 contains homoserine, which was found to be mainly (75%) 
in an acidic peptide which was purified by paper electrophoresis 
at pH 6.5 (mobility, 0.52 with respect to aspartic acid). The 
composition of this peptide was Asp, 1.02; Thr, 1.00; homoserine, 
1.00; Leu, 0.96. Analysis after one step of Edman degradation 
showed: Asp, 0.16; Thr, 0.93; homoserine, 1.00; Leu, 1.00. The 
tetrapeptide was incubated in 2 M NH$_3$ at room temperature for 
16 hours in order to convert homoserine lactone to homoserine, 
and after lyophilization was digested with carboxypeptidase A 
for 3 hours. The amino acid analysis of the digest showed 
homoserine, 0.95; Leu, 0.95. Hence the sequence of Tetrapeptide 
CT2 is: Asp-Thr-Leu-homoserine.

Relative Size of CNBr-(Fab')$_2$ and Pepsin-(Fab')$_2$—In order to 
establish the relative size of the CNBr-(Fab')$_2$, and the pepsin-
(Fab')$_2$ we used Decapeptide PRT2 as a marker. If after pepsin 
cleavage of CNBr-(Fab')$_2$ this decapeptide could be obtained, 
then the CNBr-(Fab')$_2$ would be larger than the pepsin-(Fab')$_2$. 
The CNBr-(Fab')$_2$ was dialyzed against 0.1 M sodium acetate for 
8 hours. The pH of the solution was adjusted to pH 4.5 and 
pepsin (1% by weight of (Fab')$_2$) was added. After 16 hours of 
incubation at 37°C the solution was dialyzed against 0.2 M Tris-
HCl, pH 8.2. This material was reduced and aminoethylated 
and was passed through a Sephadex G-75 column. The 3.5 S 
Fab obtained from the column was digested with trypsin and was 
chromatographed on a Sephadex G-50 column. Fig. 4 shows a 
comparison between the elution pattern of this column and that 
of an analogous experiment in which trypsin digestion was per-
fomed on aminoethylated pepsin-Fab'. The two elution 
patterns are very similar indeed. Fraction CPRT3 contains 
only free serine (20% yield) and free glycine (16.7% yield), 
since both were obtained in the same yield with or without acid 
hydrolysis. Fraction CPRT2 contained mainly one peptide
FIG. 5. Diagrammatic representation of the position of cleavages in rabbit IgG and its relation to the known sequence of that region. The four-chain model is according to Porter (31) except that the disulfide bonds linking the heavy chains are not marked. The sequence below the model is according to the sequence of Fe by Hill et al. (29) and the sequence of Fraction of CNBr-Cl by Cebra, Porter, and Steiner (30). The positions of cleavages of the intact molecule by CNBr, pepsin, and trypsin are marked according to the data presented in this paper. The peptides which have the same mobility by paper electrophoresis as Fraction PRT2 and the same amino acid composition (Lys, 0.99; AE-Cys, 0.85; Thr, 0.97; Ser, 0.88; Glu, 1.10; Pro, 4.20; Leu, 1.00). It was obtained in 55% yield. Similarly, Fraction CPRT1 contained the same peptide as fraction PRT1.

The identity of the peptides obtained from aminoethylated pepsin-Fab' and from reduced-aminoethylated, pepsin-digested CNBr-(Fab')z provides evidence that CNBr cleavage is further than the pepsin cleavage toward the COOH terminus of the heavy chain.

DISCUSSION

The results reported in this paper deal with the position of the cleavages by pepsin, CNBr, and trypsin in the heavy chain of rabbit IgG which produces a divalent or monovalent Fab fragments. The finding of NH2-terminal serine in trypsin-Fc (12) and of COOH-terminal leucine in pepsin-(Fab')z fits well with the terminal residues of Decapeptide PRT2 which has been isolated from a trypsin digest of aminoethylated Fab'. The two peptides PRT1 and PRT2 differ only in their carbohydrate content and the yield of both peptides together is 85%. Since these peptides contain 1 AE-Cys residue this yield is in agreement with the decrease of AE-Cys content from 4.0 in aminoethylated Fab' to 3.2 in the trypsin-Fab'. The conditions used in the reduction of the peptide-(Fab')z are sufficient to reduce the disulfide bonds between the heavy and light chains. Hence, from the 4.0 AE-Cys residues of Fab', 1 is probably present on the light chain (25) and 3 are on the heavy chain part of Fab'. Since trypsin cleaves the AE-Cys-Ser bond and Peptide PRT2 has 1 AE-Cys residue, then 2 of the AE-Cys residues on the heavy chain must be in positions 6 and 11 from the COOH-terminal leucine of Fab'. This implies that in the original IgG molecule isolated from the various Fab fragments are placed along the known sequence. The position of papain cleavage to produce Fe is marked according to Hill et al. (29) and the extension of papain cleavage toward Fab is deduced from the work of Utsumi and Karush (28). The numbers represent the number of residues between the various cleavages. T, trypsin; T*, trypsin cleavage in aminoethylated Fab'; P, pepsin; Pap, papain; L, light chain; H, heavy chain.

Recent studies on the papain-produced Fc by Hill et al. (29), as well as on the CNBr-produced Fc (Cl) by Cebra, Steiner, and Porter (30), have established the amino acid sequence of the region of the heavy chain which evidently includes all of the positions of cleavages which produce active fragments. This sequence, together with a diagrammatic representation of the positions of various cleavages in IgG, is given in Fig. 5. It is shown that Peptide PRT2 and CT2 reported here fit parts of the known sequence in the positions indicated by arrows. The last 6 residues of Peptide PRT2 have the same sequence as the first 6 residues of papain-Fc (29) and the 4 NH2-terminal residues of Peptide PRT2 fit the extension provided by sequence analysis of the COOH-terminal peptide (T2) of CNBr-C1 (30). Thus Peptide PRT2 provides the overlap between the pepsin-Fab', the papain-Fc, and the trypsin-Fc. The CNBr-Fab' is 18 residues longer than the pepsin-Fab', whereas the latter is 10 residues longer than the trypsin-Fab'. The trypsin-Fc obtained from aminoethylated IgG (12) has an extension of 4 residues over that of papain-Fc. It is worth noting that papain cleaves the bond Thr–AE-Cys in Peptide PRT2 (to yield Hexapeptide PRT2pA) in the same position that it cleaves the intact molecule to yield Fe with NH2-terminal Cys (29). Mage and
Harrison (32) have shown that a short digestion of peptide (Fab')2 by water-insoluble papain (7), followed by reduction and carboxymethylation, released a fraction which contains predominately S-carboxymethyl Cys, 1.1; Glu, 1.1; Pro, 2.3; Leu, 1.1 (32). This fraction is very similar in amino acid composition to hexapeptide PRT2βA isolated from Peptide PRT2 after papain digestion. This might suggest that the initial papain cleavage in the IgG molecule is in the Thr-Cys bond. However, it seems very likely that papain digestion proceeds further toward the NH₂-terminal part of the heavy chain since Utsumi and Karush reported a fraction of about 3500 molecular weight which is released upon papain hydrolysis of pepsin-(Fab')2. This fraction contains 1.1 moles of S-carboxymethyl Cys and 0.9 mole of half-cystine per mole of Fab', implying that prolonged papain digestion continues toward the NH₂-terminus of the heavy chain beyond the position of trypsin cleavage reported here.

The finding of COOH-terminal leucine in pepsin-(Fab')2 and the high yield of Peptide PRT2A imply that papain cleaves the heavy chain in a unique position (Leu-Leu bond) to yield (Fab')2. This is probably the position of the initial cleavage by papain since short digestion of IgG with pepsin yields, in addition to (Fab')2, a 3.3 S fragment which is antigenically indistinguishable from papain-Fc-C (14). Further peptic digestion cleaves this 3.3 S fragment into smaller pieces (14).

These chemical studies provide also some insight into the size of the flexible region in the heavy chain which is more accessible to enzymic digestion. The methionine residue is available for CNBr cleavage only under denaturing conditions of 0.3 N HCl (10). Intact rabbit IgG is not digested by trypsin (12) since the only lysine residues present in this region are followed by prolines. However, there is one Lys-Asp bond 4 residues from the methionine (see the sequence in Fig. 5) and one Lys-Thr bond 7 residues from the first Cys toward the NH₂-terminus (30). These bonds are not available for trypsin in the intact molecule and must therefore be buried in the globular part of the Fe and the Fab, respectively. Therefore the maximal length of the region accessible to enzymes is around 25 to 30 residues.

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