Analysis of the mechanism, rate, and sites of proteolytic cleavage of human immunoglobulin D by high-pressure liquid chromatography

(Fab and Fc fragments/protein structure/hinge region/immunoassay/circumsporozole antigen)

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ABSTRACT The high susceptibility of human immunoglobulin D to proteolytic degradation affects its biological function, metabolism, and immunoassay. High-pressure liquid chromatography was used to investigate the mechanism and rate of limited proteolytic cleavage of IgD and also to identify, isolate, and quantify the reaction products. Within 1 to 5 min, tryptic digestion of native IgD almost quantitatively yields a labile Fab fragment, a stable Fc fragment, and a highly charged peptide derived from the hinge region. A galactosamine-rich glycopeptide from the hinge region increases inversely as the Fab is largely degraded to a series of peptides within 1 hr. In contrast, the Fc and the high-charge peptide resist proteolysis for more than 24 hr. The initial sites of cleavage of IgD occur in the hinge region at exposed secondary structures predicted to be residues. Concomitant with removal of the galactosamine-rich glycopeptide at its carboxyl terminus, the Fd fragment is rapidly and rather randomly degraded, but the light chain is somewhat more resistant than the Fd section of the heavy chain. This study of the rapid rate of proteolysis of IgD explains the rarity with which intact IgD is found in human sera. It also raises questions about immunoassay of IgD, which is usually measured with antisera against Fc. In vivo, proteolytic cleavage initiates the catabolism of circulating IgD, and it also affects the role and fate of IgD as an antigen receptor on the B-cell membrane.

Unlike other immunoglobulins, human IgD has been little studied because of its low concentration in normal serum and its great susceptibility to proteolytic degradation. It is difficult to purify intact IgD for structural study. Nonetheless, the complete amino acid sequence of a human myeloma IgD protein has been determined in our laboratory. The sequence of the constant (C) region of the heavy chain was subsequently confirmed by analysis of the genomic sequence by White et al. The principal biological function attributed to IgD is to serve as a membrane-bound receptor for antigen when it is coexpressed with IgM on the surface of differentiating B lymphocytes. This process IgD is believed to undergo proteolysis to form Fab and Fc fragments; then Fab is degraded, whereas Fc is endocytosed. Because sensitivity to proteolysis is a hallmark of IgD and apparently is associated with its biological function, we have investigated the mechanism and the rate of limited cleavage of human IgD by trypsin and other proteolytic enzymes. This article illustrates the application of HPLC to the quantitative study of the time course of the proteolysis of undesaturated IgD, to the isolation and structural analysis of the reaction products, and to the identification of the sites of initial cleavage. HPLC gives quantitative values and permits isolation of fragments in quantities suitable for sequence analysis; it also allows visualization and recovery of smaller peptides that are not detectable by NaDodSO₄/PAGE and similar methods.

MATERIALS AND METHODS

Materials. Human IgD was prepared from the plasma of patient WAH as described. In some experiments a second IgD, designated HUD, was used. The sources of trypsin and other enzymes and reagents (1, 3, 8) and equipment, columns, and reagents for HPLC (9, 10) have been reported.

Methods. Methods used in our laboratory for the determination of the primary structure of proteins and for their physical and immunochemical characterization (1, 3, 7–10) and the methods for computer analysis of sequence data (11) and for HPLC (9, 10) have been described.

Most experiments on limited digestion were done under nonadenaturing conditions with the WAH IgD and trypsin at an enzyme-to-substrate weight ratio of 1:100 in 0.1 M Tris-HCl or 0.1 M ammonium bicarbonate (pH 8.0) at 37°C. In other experiments the temperatures ranged from 4° to 50°C. Digestion was stopped by addition of soybean trypsin inhibitor. In kinetic experiments the digestion times varied from 3 sec to 24 hr. In experiments with other proteolytic enzymes such as pepsin, plasmin, and kallikrein, conditions such as the pH and time of incubation were changed as seemed appropriate. Each digest was analyzed by NaDodSO₄/PAGE (12–30% gradient) with and without the addition of mercaptoethanol and with the use of densitometry to evaluate the relative concentrations of the enzymatic fragments produced.

In some experiments an HPLC system described elsewhere was used. The appearance and disappearance of fragments and to obtain samples suitable for amino acid and sequence analysis. For reversed-phase chromatography a Synchropak RP-P column (25 × 0.41 cm i.d.) was used; elution was with a linear gradient of 0–60% of 1-propanol in 0.1% trifluoroacetic acid over a period of 75 min, with measurement of the eluate absorbance at 230 nm. The high-charge peptide and other small peptides that eluted early were separated with a reversed-phase Ultrasphere ODS column (25 × 0.4 cm i.d.). In other experiments the limited tryptic digest was separated by gel-permeation HPLC on a Spherogel TSK-2000SW column, connected in tandem with a TSK-3000SW column (both 60 × 0.75 cm i.d.) (10); elution was with 0.15 M sodium phosphate, pH 8.0/0.15 M NaCl over a period of 150 min, with measurement of eluate absorbance at 280 nm. The purified peptides were analyzed with the amino acid analyzer, and in most cases the sequences were determined by automated Edman degradation.

Abbreviations: V, variable; C, constant; GαN, galactosamine.

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Hexosamine analysis was done with the amino acid analyzer, after acid hydrolysis (1–3, 11).

RESULTS

Limited Cleavage of IgD with Trypsin. Undenatured WAH IgD was digested with trypsin at 37°C, and samples were removed for analysis beginning at 0.5 min and at progressively increasing times up to 24 hr. At zero time, some fragments of low molecular weight were present in small amount, probably because of autolytic degradation, but most of the IgD was in the form of the monomer (Mr ≈ 175,000) and higher aggregates that did not penetrate the gel. There was some heterogeneity probably due to differences in degree of glycosylation; for example, we have shown that Asn-445 in the δ chain of IgD is only partially glycosylated (9). Within 0.5 min most of the IgD was cleaved into Fab and Fc fragments and a small amount of peptides of lower molecular weight (Fig. 1). In NaDodSO4/PAGE in the absence of mercaptoethanol, the Fab migrated a little slower than the Fc. In the presence of mercaptoethanol, the reduced Fab separated into the amino-terminal half of the δ chain (Fd) (Mr ≈ 25,000) and the light chain (Mr ≈ 23,000), and the reduced Fc moved as a double band (Mr ≈ 34,000) because it consists of two forms that differ in degree of glycosylation. However, the Fd fragment was immediately digested to a series of polypeptides that decreased progressively in size, whereas the light chain was digested more slowly. When the same experiment was done with trypsin at 50°C where the enzyme is partially inactivated, the fragmentation of IgD occurred more slowly, and the Fc fragment still resisted proteolysis. Within 2 min much of the IgD was degraded, and no intact IgD or δ chain remained at 5 min. Experiments under other conditions and also with IgD protein HUD confirmed that the Fc fragment resists trypsin digestion for periods up to 96 hr, whereas the Fab fragment is rapidly digested to form a series of discrete polypeptides and a somewhat more stable light chain.

Proteolysis of IgD with Other Enzymes. The digestion pattern of WAH IgD obtained with plasmin was very similar to that for trypsin, but the rate of cleavage into Fab and Fc and the degradation of the Fab were much slower. Kallikrein gave a different pattern, in that four fragments were formed initially. Two of these (Mr ≈ 100,000 and 88,000) remained after digestion for 24 hr, but the other two (Mr 64,000 and 57,000) were degraded. It appeared that kallikrein cleaved IgD at two different positions in the C2 domain. Pepsin rapidly degraded IgD but also gave a different fragmentation pattern. By 5 min most of the IgD was split into a number of small fragments and a large one (Mr ≈ 125,000). Reduction showed that the latter was a dimer with monomer molecular weight of about 60,000; on further digestion this yielded a Mr 24,000 fragment that was stable up to 24 hr. The structure of the fragments produced by plasmin, kallikrein, and pepsin was not investigated by sequence analysis as was done for the trypsin fragments (see below); however, analysis of the fragmentation patterns indicated that all four proteolytic enzymes first attacked one or more sites in the C2 domain and hinge region of the δ chain and produced a stable fragment derived from the Fc region.

Preparative Isolation of IgD Tryptic Fragments and Identification by Sequence Analysis. To identify the sites of cleavage and the major fragments produced, IgD was digested on a preparative scale with trypsin for 24 hr at 37°C. A precipitate formed, which mainly contained low molecular weight peptides. The precipitate was washed, dissolved in 8 M urea/0.1% trifluoroacetic acid, and applied to a Synchropak RP-P column. Several peptides were isolated and were identified by sequence analysis. These tryptic peptides were rich in aromatic amino acids, which explains their insolubility. They originated mainly from the hypervariable, or complementarity-determining, regions of the δ and λ chains, for example, the segment from Gly-46 to Arg-66 of the δ chain, and also a peptide in which the segment from Val-18

![Fig. 1](image-url)
to Arg-31 of the \( \lambda \) chain is disulfide-bonded to the segment from Ser-81 to Lys-112 of the \( \lambda \) chain.

The supernatant containing the soluble fragments was applied to Sephadex G-100. The major fragments in each peak were separated by HPLC and were identified by amino acid analysis and sequence analysis. The predominant component and the first to separate on Sephadex was the Fc fragment; this begins at position 287 in the WAH \( \delta \) chain and has the sequence Thr-Pro-Glu-Cys-Pro-Ser-His . . . The next peak eluted on Sephadex and purified by HPLC was the 32-residue GalN-rich hinge glycopeptide that begins at Ala-235 and extends through Arg-266.

Amino acid sequence analysis of a series of other peptides purified by HPLC showed that they were all derived from the V region of the \( \delta \) chain, which had been completely digested by trypsin. This work also established that the major fragments and tryptic peptides produced by brief digestion of native IgD with trypsin could be identified by their retention time in HPLC. For example, the retention time of the GalN-rich glycopeptide under the standard elution conditions was 26 min. Thus, a kinetic study of the course of tryptic cleavage of native IgD was undertaken with use of HPLC to identify the chief products by their retention time and to measure their concentration at various times of digestion.

**HPLC Study of the Time Course of Tryptic Cleavage of IgD.**

The time course of tryptic digestion was studied quantitatively over the period from 0 to 24 hr by use of two different columns for HPLC. In each case the peptides and Fab and Fc fragments were identified by their retention times in HPLC and by amino acid analysis. Also, where appropriate the digestion products were identified by NaDodSO4/PAGE, by immunodiffusion, and by amino acid sequence analysis. Separation of the Fab and Fc fragments from each other was achieved by use of the Spherogel column, but in this system the peptides derived from Fab aggregated (Fig. 2). Reversed-phase chromatography on the Synchropak RP-P column separated the smaller peptides and the hinge peptides from the Fab and Fc fragments, but the latter were eluted close together in this system (Fig. 3).

Fig. 2 illustrates the almost instantaneous cleavage of IgD into the Fab and Fc fragments, followed by rapid degradation of Fab. The Fab fragment essentially disappeared within 1 hr and was converted to peptides that aggregated. Hence, reversed-phase chromatography was used to follow the time course of the appearance of the peptides; these were largely derived from Fab and the hinge region (Fig. 3). The first peptides to appear came from the hinge region. These consisted of the high-charge peptide and other small, largely basic peptides that were eluted together after 7 min and also the GalN-rich peptide, which had a retention time of 26 min. The two major peptides originating from the hinge region reached a maximum at 30 min and persisted for 24 hr (Fig. 3). Fab was rapidly degraded into a series of peptides; the bulk of these had a retention time of about 35 min and attained their maximum at about 60 min of digestion, by which time Fab had disappeared.

The results of the experiments illustrated in Figs. 2 and 3 are plotted as a composite graph in Fig. 4. This shows the yields of Fab and Fc as a function of time; it also shows the yields of the GalN-rich peptide and the high-charge peptide derived from the hinge region. The high-charge peptide and the Fab and Fc fragments reached a maximum at about 1 min, whereas the maximum yield of the GalN-rich peptide required about 1 hr. This suggests that the initial tryptic cleavage of IgD occurred on both sides of the high-charge peptide, with the release of several small peptides (see Fig. 1). Initially, the GalN-rich peptide probably remained attached to the Fd portion of Fab, for the rapid degradation of Fab paralleled the appearance of the GalN-rich peptide, as is shown by the inverse relationship of their yields (Fig. 4).

**DISCUSSION**

The sites of tryptic cleavage of the IgD hinge region are consistent with its predicted secondary structure. In the predicted conformation a \( \beta \)-turn (the sequence GRGG in Fig. 1) separates the random structure of the GalN-rich peptide from the highly charged \( \alpha \)-helical region (EEKKKEKEKEEQEERE in Fig. 1). Trypsin cuts in the middle of the \( \beta \)-turn to yield the highly resistant GalN-rich peptide, which is shielded from further hydrolysis by the bulky GalN oligosaccharides adjacent to the peptide’s only bond susceptible to
The amino-terminal portion of the α-helix is cleaved to yield the high-charged peptide. Despite its three basic residues, this peptide is shielded from further tryptic action by the proximity of the six negatively charged glutamic acid residues. In a brief period many tryptic cuts are made in the Fd portion of Fab and to a lesser extent in the light chain, so that Fab is rapidly disintegrated in an apparently random fashion. The Fc portion, however, is resistant to tryptic degradation because its conformation remains intact, owing to the persistence of the disulfide bridge at its amino terminus and the maintenance of the lateral and longitudinal interactions of the Cγ2 and Cγ3 domains.

Although limited proteolytic cleavage is widely used in research to produce Fab and Fc fragments of antibodies and other immunoglobulins, the structural and biological implications of the varying susceptibility of different classes of immunoglobulins to such cleavage are rarely considered. Undenatured human immunoglobulins of different classes and subclasses and immunoglobulins of other species vary greatly in their susceptibility to limited cleavage by proteolytic enzymes, including trypsin, papain, pepsin, and plasma serine esterases such as plasmin (12). In general, IgG1 is readily cleaved to yield Fab and Fc fragments, IgG2 and IgM are more resistant, IgA1 is split only by an IgA1 protease from pathogenic bacteria, and IgA2 is not cleaved by any known protease. The unique structure of each immunoglobulin type and the protease specificity determine the susceptibility to cleavage, the size and stability of the fragments formed, and the sites of cleavage. These factors affect the biological functions and the catabolism of antibodies and also of their membrane counterparts, the antigen receptors of the B cell.

Of all known classes of immunoglobulins, human IgD is the most susceptible to proteolytic degradation. Yet we have found that the Fc fragment and two peptides derived from the hinge region of IgD are obtained in high yield on incubation with trypsin at 37°C and are very stable, whereas the Fabδ fragment is degraded almost instantaneously. This time study of the proteolysis of IgD explains the rarity with which intact IgD is found in human sera, for proteolytic cleavage to Fab and Fc initiates the catabolism of immunoglobulins. Our findings also cast doubt on the immunoassay for IgD by use of certain antisera. They are also relevant to the role and fate of IgD as a membrane receptor during antigen-driven differentiation of B lymphocytes.

Because intact IgD is very difficult to isolate from normal human serum, the usual source is the serum from the infrequent patients with multiple myeloma who produce a great excess of IgD (1–3). In a separate study of the sera of about 50 such patients, we found fragmented IgD present in all cases, and usually it was the predominant form. We then sent a questionnaire to all listed commercial suppliers of animal antisera for measurement of IgD in the clinical laboratory. With two exceptions, we found that the antiserum had been prepared against the Fcδ fragment rather than against intact IgD. This suggests that most clinical laboratories assay Fcδ rather than IgD.

Although IgD is reported to be present in the sera of many species (13), intact IgD containing the four domains shown in Fig. 1 (VH, Cδ1, Cδ2, and Cδ3) has been isolated in significant amounts only from the human. Little is known about the IgD in most species. Gene sequencing has indicated that the IgD of both the mouse (6) and the rat (14) have a three-domain structure with a deletion of Cγ2 and a shortened hinge region. The rodent IgDs are unique among all the immunoglobulins because they lack the Cγ2 domain and the corresponding Cγ2 gene segment. This raises the question whether rodent IgDs
are functional in any way, except perhaps as a B-cell antigen receptor (15).

The evolutionary origin of the human δ hinge has been puzzling because of its division into two segments encoded by separate exons (3, 4) and because it has no homology to the hinge region of other immunoglobulin isotypes (2, 6). Furthermore, although the δ hinges of the mouse (4) and the rat (14) are homologous to the human GalN-rich segment, the rodents lack the second hinge exon and the corresponding high-charge segment. There is no precedent for such a missing exon in other immunoglobulin isotypes. On the basis of computer searches of the protein sequence data base, we and our coworkers had suggested (1–3) that the first hinge exon, encoding the GalN-rich segment, may have been derived from the exon encoding the second C-region domain of the μ chain (Cγ2). Two further observations have resulted from the present work: the first is the stability of the two hinge peptides described above, and the second is the surprising homology of these hinge peptides to malaria parasite antigens and to sequences in the gag polypeptide of oncogenic viruses (Fig. 5). The stability of the two hinge peptides lends support to our suggestion that these peptides, when released, may exert some hormone-like or triggering effect on either T or B cells (2). There is increasing evidence that peptides derived by proteolysis of immunoglobulins may have effects on the immune response (12).

The significance of the homology of the δ hinge peptides to malarial and oncogene sequences is more speculative. One possibility is that the second hinge exon may represent translocation of a foreign gene sequence into the genomic sequence of the human δ chain. Translocations of myc and other oncogene sequences to the immunoglobulin genomic structure are being reported with increasing frequency (12). Because of the recombinant nature of immunoglobulin gene expression, such inserted sequences could be productive, and the second δ hinge exon may represent an example of this phenomenon. This idea is also supported by the fact that all of the RNA-splicing signals at the exon boundaries of the genomic sequence of the δ chain follow the GT/AG rule, but the donor site of the second δ hinge exon is most unusual in having three pyrimidine residues after the GT (4). A similar pyrimidine-rich sequence is seen only in exon 4 of adenovirus (4).

This work illustrates the application of HPLC to the analysis of the mechanism and kinetics of enzymatic cleavage of a protein that produces both stable fragments and transient intermediates. Quantitative recovery and structural study of a variety of products that differ greatly in size, charge, and structure are difficult. Usually several different methods are required, and these often entail progressive loss of material. In this study, HPLC offered many advantages, including quantification of the yield of the proteolytic products as a function of time and isolation of both large fragments and small peptides in an amount and form suitable for structural study. Widespread application of HPLC to study of similar problems may be anticipated.

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