Differential Susceptibility of Human IgA Immunoglobulins to Streptococcal IgA Protease

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ABSTRACT IgA protease, a proteolytic enzyme found in human saliva and colonic fluid, hydrolyzes human serum IgA immunoglobulins to yield Fab$_\alpha$ and Fc$_\alpha$ fragments. The enzyme is produced by organisms in the normal human microflora and can be purified from culture filtrates of the common human oral organism *Streptococcus sanguis* (American Type Culture Collection no. 10556). IgA protease is inactive against all other protein substrates examined including the other classes of human immunoglobulins. The role of this enzyme in affecting the function of the secretory IgA immune system is unknown.

To further characterize and explain this unusual substrate specificity, the susceptibility of 31 human IgA myeloma proteins of both subclasses was investigated. 16 IgA1 and 15 IgA2 myeloma paraproteins were treated with enzyme and the extent of proteolysis was determined by cellulose actate electrophoresis, immunoelectrophoresis, polyacrylamide gel electrophoresis, and column chromatography. All IgA1 proteins were enzymatically cleaved to Fab$_\alpha$ and Fc$_\alpha$ fragments, but all IgA2 proteins were resistant, yielding no fragments after prolonged enzymatic treatment. N-terminal amino acid sequence analysis of the purified Fc$_\alpha$ fragment of a single IgA1 paraprotein was as follows: Thr-Pro-Ser-Pro-?-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser. Comparison of this sequence to that reported for the IgA1 heavy chain shows that the enzyme-susceptible peptide bond is a Pro-Thr in the IgA1 hinge region. The most likely explanation of the resistance of the IgA2 subclass to IgA protease is a deletion in the heavy chain which commences with the critical threonine of the susceptible Pro-Thr bond.

INTRODUCTION

IgA protease is an enzyme elaborated by streptococci into the human oral cavity and gastrointestinal tract. At neutral pH the enzyme proteolytically cleaves human immunoglobulin A to yield intact Fab$_\alpha$ and Fc$_\alpha$ fragments (1, 2). Since the alimentary tract has an immune system mediated by antibodies of the IgA class, the presence of this protease in secretory fluids would seem to represent an important biological interaction between bacteria and locally synthesized antibody. Studies of the function of IgA protease have revealed several unusual and useful properties. First, it is the only known proteolytic enzyme which yields an intact Fc$_\alpha$ fragment from human serum and secretory IgA. Since secretory IgA contains secretory component and J chain attached to its Fc region by disulfide bridges (3), structural studies are particularly important and IgA protease has been useful in making available intact Fc$_\alpha$ fragment for this purpose. Moreover, the study of the biological properties of isolated Fc regions of other immunoglobulins has been useful in understanding correlations between structure and function within these molecules.

A second unusual and potentially important property of IgA protease is its specificity for human IgA. Although it readily hydrolyzes human secretory and serum IgA it has been shown incapable of cleaving the IgA of other mammalian species including those of the monkey, dog, rabbit, and mouse. In addition, many other

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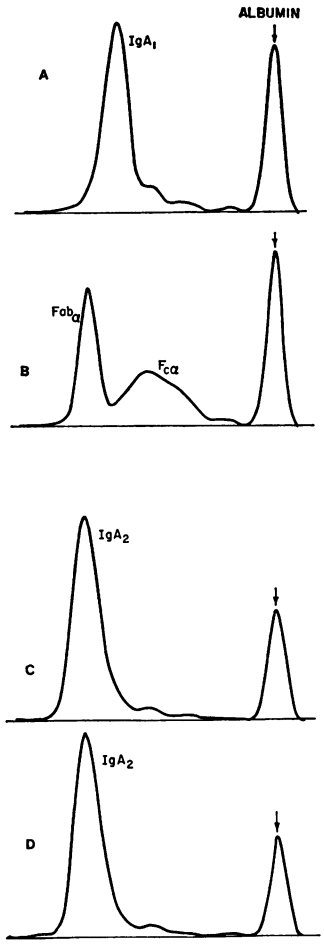

FIGURE 1 Cellulose acetate electrophoresis of sera containing IgA myeloma proteins treated with IgA protease. The albumin peaks have been aligned. (A) Serum containing an IgA1 paraprotein. (B) The same serum as in A after enzyme treatment for 16 h at 37°C. The IgA1 paraprotein has been split to Fab_α and Fc_α which form new peaks as indicated. (C) Serum containing an IgA2 paraprotein. (D) The same serum as in C after enzyme treatment for 16 h at 37°C. Control sera (A and C) had been incubated with Tris buffer instead of enzyme. Anode is to the right.

protein substrates have been found resistant, including the other four classes of immunoglobulins (4). The specificity of the enzyme for the two subclasses of human IgA is the subject of this report. These subclasses, IgA1 and IgA2, have been identified in several laboratories by immunologic study of myeloma components (5, 6). Through the use of appropriate antisera, each IgA myeloma protein can readily be identified as belonging to one or the other subclass. While IgA1 and IgA2 proteins exhibit extensive antigenic cross-reactions, they do have significant structural differences. For example, IgA2 proteins have been shown to have a 12 amino acid deletion in the hinge region of the heavy chain with an associated difference in glycopeptide composition when compared to IgA1 (7, 8). In addition, the IgA2 subclass exists in two allelic forms; these genetic variants are designated "$Am_2(+)$" and "$Am_2(-)$" (9). $Am_2(+)$ IgA2 proteins lack disulfide bonds between their heavy and light polypeptide chains, while the rare ($Am_2(-)$) IgA2 proteins have heavy-light disulfide bonds of the type found in IgA1.

This report demonstrates that IgA protease specifically hydrolyzes myeloma proteins of the human IgA1 but not those of the IgA2 subclass. Amino acid sequence studies of the Fc_α fragment of a single IgA1 myeloma protein indicates that the pronounced difference in susceptibility of the two subclasses may be due to the deletion in the hinge peptide of IgA2 proteins.

METHODS

Preparation of IgA protease. Lancefield group H *Streptococcus sanguis* (American Type Culture Collection Bacterial Strain no 10556) was incubated in 10 ml Todd-Hewitt broth (Baltimore Biological Laboratories, Cockeysville, Md.) for 6 h at 37°C. The organisms in a log phase of growth were inoculated into 4 liters Todd-Hewitt broth and incubated in stationary culture for 48 h at 37°C. Agitation of the culture flask was found to markedly reduce enzyme production. After removal of the bacteria by centrifugation the enzyme was isolated from the cell-free supernatant medium by sequential treatment with 60% ammonium sulfate, 70% acetone, and anion exchange chromatography as previously described (2). This purification method removes 98% of extraneous protein in the culture fluid as determined by absorbancy at 280 nm. When incubated at 37°C with an equal volume of human myeloma IgA at 10 mg/ml in Tris-HCl buffer, 0.05 M, pH 8.1, the enzyme preparation can leave all substrate in 2 h.

Preparation of human myeloma protein substrate. 31 human IgA myeloma sera were selected for study. The subclass of each paraprotein was identified by Ouchterlony analysis using sheep anti-human IgA (Meloy Laboratories Inc., Springfield, Va., lot no. 103-6) which can differentiate the subclasses by the spurring of the IgA1 precipitin band over the IgA2. 16 paraproteins were IgA1 and 15 IgA2. Among the IgA2 proteins was one known to carry the rare $Am_2(-)$ allotype; such IgA2 proteins are characterized by the presence of disulfide bridges between the light and heavy polypeptide chains. All other IgA2 proteins were assumed to be of the $Am_2(+)$ allotype which lack light-heavy bridges (9). One IgA1 serum was biclonal in that an IgG paraprotein was also present.

Enzymatic treatment of IgA. All myeloma sera were dialyzed against Tris-HCl buffer, 0.05 M, pH 8.1, mixed with an equal volume of enzyme, and incubated for 16 h at 37°C. Controls contained buffer instead of enzyme. Whole sera were used since IgA protease has been found to be unaffected by serum protease inhibitors.[1] The digests were examined by cellulose acetate electrophoresis (Microzone electrophoresis, Beckman Instruments, Inc., Fullerton, Calif.). Digested paraproteins show the disappearance of the M component and the appearance of an anodally mi-

[1] One of the 55 normal human sera examined in our laboratory has been found to inhibit the enzyme. Preliminary experiments indicate this inhibition is due to antibody.

grating Fc_α and a cathodal Fab_α fragment. A typical cellulose acetate analysis for each IgA subclass is shown in Fig. 1. All digests were also examined by immunoelectrophoresis performed in 2% agar or agarose and developed with rabbit anti-human IgA (10). In the case of cleaved IgA proteins immunoelectrophoresis revealed an anodally migrating Fc_α fragment and the disappearance of the initial paraprotein precipitin band. To confirm the findings in whole sera, the paraproteins were isolated (11) from six sera containing IgA1 and six containing IgA2 proteins. The concentrations of these proteins was adjusted to 10 mg/ml in Tris-HCl buffer, pH 8.1, and their purity confirmed immunologically. The purified IgA preparations were digested and studied by cellulose acetate electrophoresis and immunoelectrophoresis; in addition, these digests were examined by polyacrylamide disk gel electrophoresis in 5% gels in pH 9.5. Corresponding unstained whole polyacrylamide gels were imbedded in 2% agar and appropriate antisera applied to parallel troughs. This technique permits immunologic identification of fragments as they elute from the polyacrylamide into the agar.

20 mg each of one IgA1 and one IgA2 protein was treated with enzyme for 16 h at 37°C and chromatographed on Biogel P-200 (Bio-Rad Laboratories, Richmond, Calif.) to isolate and characterize the proteolytic fragments. The column had previously been calibrated with marker proteins of known molecular weight. The purity of isolated fragments was confirmed immunologically and by polyacrylamide disk gel electrophoresis. As previously reported (1), the molecular weight of the 3.0S monomeric form of Fc_α fragment derived from myeloma IgA is 41,500. The Fab_α fragment corresponds in size to the trypsin-derived Fab fragment ($\sim$46,000) as determined by elution position in calibrated molecular seive chromatography columns.

Amino acid sequence study. A limited amino acid sequence on 0.2 μM of Fc_α fragment was determined by Edman degradation using a Beckman model 890A sequencer as previously described (12, 13).

RESULTS

IgA protease added to whole serum readily cleaved all IgA1 paraproteins to Fab_α and Fc_α fragments as determined by the combined criteria outlined. In contrast, no IgA2 proteins were digested by the enzyme as shown by the absence of change in electrophoretic mobility on cellulose acetate and immunoelectrophoresis (Figs. 1 and 2). The results obtained with the six purified IgA1 and six IgA2 paraproteins were entirely consistent with these obseravtions, and polyacrylamide gel electrophoresis confirmed that IgA2 proteins were not hydrolyzed (Fig. 2). Despite the apparent resistance of IgA2 to enzymatic cleavage, it was necessary to exclude the possibility that cleavage had occurred without yielding recognizable fragments. This phenomenon has been observed in digests of protein substrates having multiple disulfide bridges which may prevent fragment release. Accordingly, previously digested IgA2 proteins in 4 M urea were reduced with 0.01 M dithiothreitol at pH 8.1, alkylated with 0.025 M iodoacetamide, and the digests restudied by polyacrylamide gel electrophoresis. This treatment induced the anticipated change in electrophoretic mobility of IgA2 but no fragments were liberated, indicating that unrecognized proteolysis had not occurred.

The serum containing the biclonal M components showed proteolysis of the IgA protein, but the IgG paraprotein was unaffected. The single IgA2 protein known to be of the $Am_3(-)$ allotype was also unaffected by enzyme treatment.

Immunologic analysis of protein eluted from Biogel P-200 also showed that IgA2 proteins were unchanged by enzyme treatment. Fab_α and Fc_α fragments of enzymatically treated IgA1 proteins were readily purified by column chromatography and no residual uncleaved substrate could be demonstrated. Limited amino acid sequence analysis of the Fc_α fragment of one of these proteins revealed the following: Thr-Pro-Ser-Pro-?-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-. This sequence corresponds to that reported for the IgA1 hinge region by Frangione, Wolfenstein-Todel, and Mihaesco (8, 14), and includes the entire 12 residue sequence which is deleted in IgA2 proteins. Interestingly, the N-terminal threonine of the Fc_α fragment is the first residue missing in the hinge region deletion of IgA2. Because of the deletion, the enzyme-susceptible Pro-Thr peptide bond in IgA1 proteins is absent in the IgA2 hinge. These relationships are shown in Fig. 3. The fifth residue in the sequence was not identified, presumably because it is the serine bearing the carbohydrate moiety of the IgA1 hinge peptide and would require additional techniques for its identification.

DISCUSSION

The human alimentary tract has a well-developed secretory immune apparatus and supports the growth of a luxuriant microbial flora, yet little is known of their mutual interaction. Studies in contaminated germ-free mice have shown that microbial growth in the intestine stimulates the development and promotes the maturation of immunocompetent cells in the lamina propria (15). In adult life, a balance is apparently reached since bacteria in larger number proliferate freely amid the tissues of the gut and the oral cavity. Recent experiments have emphasized that each species of bacteria preferentially attaches to and proliferates among tissues of a specific type (16). In the case of *S. sanguis* the ecological niche is the enamel surface of the tooth, although this species is a predominant organism in mixed saliva (17) and has also been found in cultures of human feces. While itself not cariogenic, *S. sanguis* is a major component among the densely packed organisms in dental plaque, the percursor to caries formation (17). The data reported here show that *S. sanguis* elaborates a proteolytic enzyme of extraordinary substrate specificity in that it acts only on human IgA proteins of the IgA1 subclass.

The presence of this enzyme in alimentary fluid such

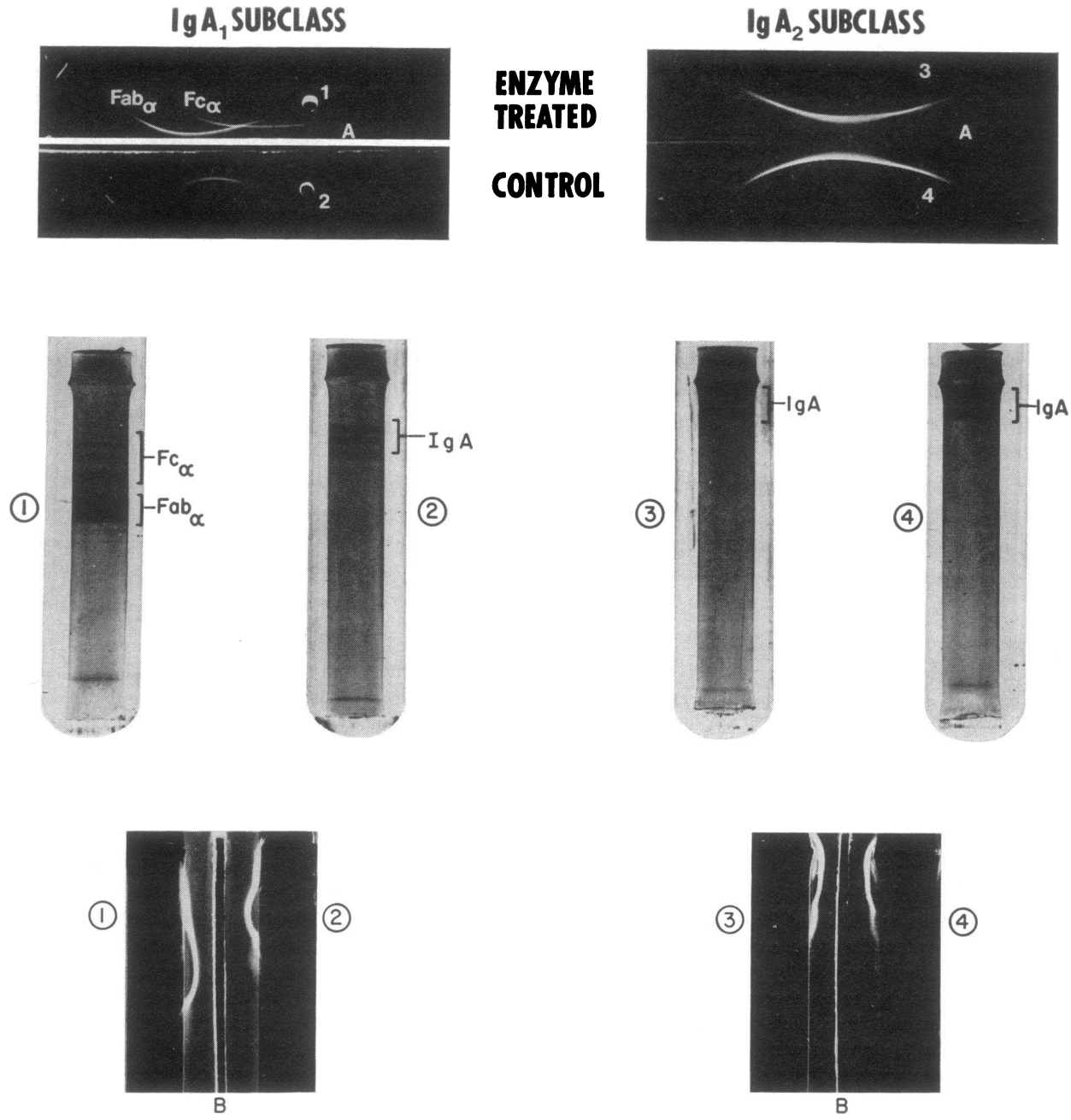

FIGURE 2 Composite photograph showing susceptibility of IgA1 and resistance of IgA2 myeloma proteins to IgA protease. *Immunoelectrophoresis* (top): Purified IgA1 (well 2) is split by IgA protease to Fab_α and Fc_α fragments (well 1), while IgA2 protein (well 4) shows no fragmentation after exposure to enzyme (well 3). Antiserum in troughs A is unabsorbed rabbit anti-human IgA. Anode is to the right. *5% Polyacrylamide gel electrophoresis* (center): Purified IgA1 protein (gel 2) yields Fab_α and Fc_α fragments after enzyme treatment (gel 1). The periodic banding of substrate IgA1 and its Fc_α fragment reflects polymerization, a typical occurrence with IgA proteins. The double band configuration of the Fab_α has not been explained. Purified IgA2 protein (gel 4) shows no fragmentation after enzyme treatment (gel 3). Anode is at the bottom; stain is for protein with Coomasie Blue. *5% Unstained polyacrylamide gel electrophoresis* (bottom): Gels corresponding to those in the center panel were imbedded in agar. Double diffusion analysis with rabbit anti-human IgA (troughs B) shows the difference in migration of IgA1 (gel 2) and its Fc_α fragment (gel 1). IgA2 (gel 4) after enzymatic treatment (gel 3) shows no Fc_α, change in mobility, or loss of its antigenic reactions with anti-IgA antiserum.

as saliva and colon contents should be considered when secretory immunity is under study. Immunofluorescent techniques using antisera specific for IgA are frequently used in examining tissues of the gut and such antisera would be unable to differentiate between Fc_α and intact IgA involved in immunologic reactions. Although enzyme activity is relatively low in mixed saliva, it is high in suspensions of dental plaque, suggesting that accumulation of bacteria at tissue surfaces may create an environment in which IgA antibody function is significantly affected. The enzyme is clearly active physiologically since initial experiments have shown that Fc_α fragments are present in the human colon; it was this observation which initially led to the identification of the enzyme (1). In order to define in more detail those organisms other than *S. sanguis* which elaborate IgA protease, all commonly occurring bacteria in the human oral cavity are currently being evaluated in vitro for enzyme production. Preliminary data indicate that streptococci alone are capable of elaborating the enzyme, although it is possible that in vivo conditions may allow the other organisms to produce similar substances which influence immune function.

The influence of IgA protease on the function of secretory antibody is unknown. Grey, Abel, Yount, and Kunkel have reported (5) that human secretory IgA in colostrum may reach 50% IgA2 in type in contrast to human serum where IgA2 represents only 10% of total IgA immunoglobulins. If one assumes that enzymatic hydrolysis adversely affects the antibody function of IgA1, enzyme-resistant IgA2 antibody could have selective advantage at secretory sites populated by masses of bacteria. Thus the evolution of the IgA2 subclass itself may have been influenced by IgA protease in secretory fluids and on mucus membranes. Along similar lines, evolutionary pressures may have led to the development of epithelial cell membrane transport pathways which favor the passage into secretions of IgA2 type antibody as opposed to IgA1. There are data which show that locally synthesized IgA antibody can be transported to either the plasma or the gut lumen (18), but there is no direct evidence that these transport mechanisms are subclass related. Confirmation of any such hypothesis will require greater understanding of how antibody released from cells is partitioned between plasma and lumen. There is no evidence indicating that the two subclasses of IgA serve different biological functions, but biologic variation among IgA subclasses may be expected since the IgG subclasses show differences in their capacity to fix complement, traverse the placenta, and fix to cells (19). It is noteworthy that many biological differences among the IgG subclasses can be localized to the Fc fragment. Since IgA protease is now available, functional studies will be possible using the Fc_α fragment derived from IgA.

Many proteolytic enzymes cleave IgA immunoglobulins

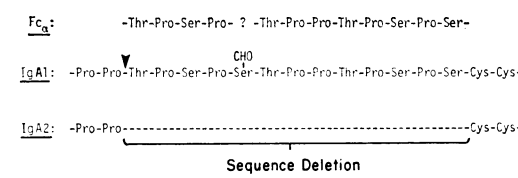

FIGURE 3 Amino acid sequence of a single IgA1 Fc_α fragment compared to published data (8, 14) on the IgA heavy chain hinge regions. The Fc_α sequence (top) is aligned with the IgA1 hinge sequence (center) to show that IgA protease attacks at a Pro-Thr peptide bond (arrow). This peptide bond is absent in the IgA2 hinge region (bottom) because the critical threonine residue is involved in the internal deletion of the IgA2 heavy chain. The ? residue in the Fc_α corresponds to the serine in IgA1 to which the carbohydrate (CHO) is thought to be covalently linked.

at their enzyme-sensitive hinge region to yield antigen-binding Fab fragments and low molecular weight peptides. While there is some variation in the efficiency and site of cleavage, proteases which hydrolyze one immunoglobulin class invariably hydrolyze all classes to some extent and are also active against other protein substrates. This is clearly not the case with *S. sanguis* IgA protease, which not only is specific for proteins of the IgA1 subclass but unlike other proteases it produces an intact Fc_α fragment which is resistant to further enzymatic digestion.

The difference in the susceptibility of the IgA subclasses can be explained if the amino acid sequence data reported here are compared to those reported by others (8, 14). The IgA protease-susceptible peptide bond in IgA1 lies immediately N-terminal to the 12 residue deletion in the corresponding amino acid sequence of the IgA2 heavy chain. Thus, the critical threonine residue of the Pro-Thr sequence is absent in IgA2. Because deletions in primary structure do not actually leave gaps in the polypeptide chain, the sequence in IgA2 proteins resumes with cystine, creating a Pro-Cys bond at the site corresponding to the Pro-Thr of IgA1 (Fig. 3). It is interesting to note the presence of another Pro-Thr bond in the IgA1 hinge peptide (Fig. 3) which is apparently unaffected by the enzyme under our conditions of digestion. This indicates that other factors contribute to the unique susceptibility of the IgA1 hinge peptide such as the presence of a carbohydrate component, which is not present in IgA2, and the extent of folding in the heavy chain hinge region. Because the folding of a polypeptide chain is determined by its primary structure (20), the IgA1 and IgA2 hinge regions are likely to differ substantially in conformation and thereby in susceptibility to enzyme attack. It is unlikely that the difference in susceptibility is directly related to the presence or absence of disulfide bonds between the light chains and heavy chains, since the single protein studied of the $Am_3(-)$ allotype presumably has light-heavy

disulfide bonds (21) but is also resistant to enzymatic cleavage.

The differential susceptibility of immunoglobulin subclasses to proteolytic digestion has been noted by others. Knight, Lichter, and Hanly (22) have reported detailed studies on secretory IgA of the rabbit, showing that molecules bearing the g genetic allotype are susceptible to papain cleavage, while those of the f allotype are highly resistant. Differences in susceptibility to proteases have also been found among human IgG subclasses (23). Such differential susceptibility may be a useful property when examining biologic activities of antibody in complex systems such as secretory fluids. The value of IgA protease as a reagent for this purpose must await confirmation that the subclasses of human secretory IgA shows the same differential susceptibility as do serum IgA paraproteins.

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