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Specific Cleavage between Variable and Constant Domains of Rabbit Antibody Light Chains by Dilute Acid Hydrolysis†

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ABSTRACT: Rabbit antibody light chains were subjected to limited acid hydrolysis and the degree of cleavage assessed by gel filtration and quantitative Edman degradation. Hydrolysis was carried out in the presence of 10% acetic acid–pyridine (pH 2.5) or in this solution in the presence of 25% 1-propanol or 7 M guanidine·HCl, for periods up to 5 days. The range of cleavage achieved under these conditions was 15–90%, optimal cleavage occurring after 4 days in the guanidine solution. The appearance of half-molecules was demonstrated by gel filtration on Sephadex G-75 in acid–urea following full reduction and alkylation. There was no evidence for cleavage at other sites since smaller peptides were not demonstrated. A unique site of cleavage was shown to occur near the beginning of the constant region by Edman degradation. In order to demonstrate a unique sequence beginning at the cleavage point without separation of the two halves of the chain, the amino terminus of the light chain was selectively blocked. After removal of the amino-terminal alanine on one light chain (3315) by Edman degradation, the second residue, glutamine, was cyclized to pyrrolidonecarboxylic acid and the chain simultaneously cleaved in acid in the presence of guanidine. A single sequence of 26 residues was obtained which, on the basis of homology with human and other rabbit light chains was placed at position 109–135, close to the beginning of the constant region. This indicated that the cleavage probably occurred between Asp-109 and Pro-110, a bond previously shown to be acid labile. This approach should greatly aid not only in the sequencing of rabbit antibody light chains but also in defining the roles of the constant and variable domains in the structure and function of antibody.

Cleavage at the switch region between variable and constant domains of immunoglobulin light chains has been described by use of enzymes (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969). However, the yield of half-molecules produced by these methods is low, of the order of 10–22% and there is great variability in conditions required among various light chains. The rabbit light chain appears to be more resistant to the action of endopeptidases and the more vigorous conditions required result in cleavage at other sites (Poulsen *et al.*, 1972). The separation of variable and constant region segments (V_L and C_L) for structural studies, activity determinations and studies of the distribution of allotypic determinants requires that the cleavage be specific in the switch region and occur in reasonable yield.

Attention has recently been drawn to cleavage of aspartylproline peptide bonds in various proteins exposed to low pH and temperatures in the range of 25–40° during enzymic and chemical hydrolysis (Piszkiewicz *et al.*, 1970). The suggestion (Freedlender and Haber, 1972) that such a cleavage occurred during demaleylation after tryptic digestion of pneumococcal antibody light chains and the placement of the labile Asp–Pro bond in the constant part of the switch region (Strosberg *et al.*, 1972) led us to investigate dilute acid hydrolysis as a means for specific cleavage of the rabbit antibody light chain into variable and constant halves.

Materials and Methods

Preparation of Light Chains. Homogeneous antibodies to the type VIII pneumococcal polysaccharide were obtained using established procedures (Pincus *et al.*, 1970; Cheng and Haber, 1971). The antibodies were subjected to mild reduction and alkylation (Fleischman *et al.*, 1962) and heavy and light chains were separated by gel filtration on Sephadex G-100 using 1 M propionic acid as the eluent.

Aliquots of one light-chain preparation (2388) were di-

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alyzed against 0.01 N acetate buffer (pH 5.5) and then labeled with ^{125}I (New England Nuclear Co.) to a specific activity of 2 Ci/g (Hunter and Greenwood, 1962).

Dilute Acid Hydrolysis. Hydrolysis of the light chain (0.05–5 mg/ml) was carried out in 10% (v/v) acetic acid (Pierce Buffer Grade) adjusted to pH 2.5 with pyridine (Pierce Sequanal Grade). Various denaturing conditions were also used. The above acid was made 25% (v/v) in 1-propanol (Pierce Sequanal Grade) (Ooi and Scheraga, 1964) or 7 M in guanidine hydrochloride (Schwarz-Mann, Ultra Pure grade). All digestions were at 40° for periods from 24 to 120 hr.

Degree and Site of Cleavage. The degree of cleavage was determined either by separating the products of hydrolysis on gel filtration, after reduction and alkylation or by determining the amount of the new amino-terminal group liberated.

GEL FILTRATION. Subsequent to cleavage the labeled light chain was subjected to full reduction and alkylation. The solution was made 0.4 M with respect to Tris·HCl buffer (pH 8.2) and 7 M with respect to guanidine·HCl. The solution was adjusted to pH 8.2 with 5 N NaOH if necessary. The solution was then made 5 mM with respect to dithiothreitol (Sigma) and incubated at 37° for 1 hr. A 50% molar excess of iodoacetamide was then added. The alkylation was allowed to proceed for 15 min at 0°. During this procedure the three intrachain disulfide bonds of the rabbit light chain are reduced and alkylated. One of these bonds has been shown to link the variable and constant domains (Poulsen *et al.*, 1972; Strosberg *et al.*, 1972). The solution was then applied to a Sephadex G-75 column (1 × 190 cm) (Pharmacia) equilibrated with 6 M urea–0.1 M acetic acid which had previously been calibrated with respect to molecular size by use of a number of markers (Freedlender and Haber, 1972). The column effluent was passed through a scintillation counter, connected *via* a Nuclear-Chicago rate meter to a chart recorder (Poulsen *et al.*, 1972).

SEQUENCE DETERMINATION. In those experiments in which the degree and site of cleavage were determined by quantitative Edman degradation (following hydrolysis) the protein solution was either immediately lyophilized or subjected to preliminary dialysis to remove guanidine and then lyophilized. Quantitative sequential degradation using either the manual or automated technique was then carried out.

Manual Edman degradations were performed using the three-stage procedure described by Blomback *et al.* (1966). Automated degradations (with the Beckman Sequencer 890B) were carried out using a Quadrol program essentially the same as that described by Edman and Begg (1967). All reagents and solvents used in the sequence determination studies were obtained from Pierce Chemical Co. and were of "Sequanal" grade. The Pth derivatives of the amino acids[1] were identified using either gas chromatography (Pisano and Bronzert, 1969) or thin-layer chromatography (Edman, 1970). Quantitative yields of Pth-amino acids were computed by comparing the peak heights of the sample to the relevant standard Pth derivative.

Results

Sequence Data. Following limited acid hydrolysis of light chain 3315, a mixed sequence on Edman degradation of the unfractionated material was expected. The results of an experiment in which this light chain was incubated in 10% acetic acid (pH 2.5) for 5 days are presented, with quantitative data, in Table I.

TABLE I: Manual Edman Degradation of Acid-Treated Light Chain (3315).

Step no.		1 2 3
Residues found		Ala Gln Ile Pro Val Ala
Expected N-terminal sequences		
I[a]		Ala-Gln-Ile-
II[b]		Pro-Val- Ala-
Sequence I	Pth-Amino Acid (μM)	Yield Rel to 0.25 μmol of Light Chain Analyzed (%)
Ala	0.095	38
Gln	*c*	*c*
Ile	0.088	36
Sequence II		Yield of Sequence II Rel to Sequence I (%)
Pro	0.027	28
Val	0.023	
Ala	0.020	23

[a] N-Terminal sequence of 3315 as determined by Edman degradation. [b] Due to cleavage between Asp-109 and Pro-110. [c] Pth-glutamine was not quantitated by gas–liquid chromatography.

The results are consistent with a single point of cleavage under the conditions of hydrolysis used. The absolute yield of alanine on Edman degradation is in keeping with that usually found with immunoglobulin light chains. The degree of cleavage, based on the yield of proline at the first step was 28%, expressed as a percentage of the absolute yield of alanine. The assumption is made that the same factor which caused the low initial absolute yield of alanine also operates on the new N-terminus proline released during the hydrolysis. The degree of cleavage as estimated in this way is confirmed in the chromatographic experiments discussed subsequently.

In order to simplify the analysis of the sequence following the cleavage point the amino-terminal half of 3315 was blocked at the time of the cleavage reaction. It has been shown that in acid conditions N-terminal glutamine cyclizes to form pyrrolidonecarboxylic acid (Melville, 1935) which will not react with phenyl isothiocyanate. As glutamine was demonstrated at position 2 of 3315 light chain the N-terminal residue alanine, was first removed by a single manual Edman degradation. The residual protein was dissolved in 10% acetic acid (pH 2.5) made 7 M in guanidine·HCl and incubated at 40° for 96 hr. The protein, after being freed of reagents by dialysis, was then degraded in the protein sequenator for 26 steps. The results are presented in Table II and compared to the sequence of peptide III (Strosberg *et al.*, 1972), a peptide from the beginning of the constant region. The identity of the two sequences indicates that the cleavage site is between Asp-109 and Pro-110.

Chromatographic Data. Gel filtration of the reaction mixture following dilute acid hydrolysis and full reduction and

[1] Abbreviations: Pth-amino acid, 3-phenyl-2-thiohydantoin derivative; PCA, pyrrolid-2-one-5-carboxylic acid.

TABLE II: Automated Degradation of Acid-Treated *N*-Pyroglutamyl-3315.

	110	115	120	125	130
Ag[c]	Arg-Thr-Val-Ala-Ala-Pro-Ser-Val-Phe-Ile-Phe-Pro-Pro-Ser-Asn-Glu-Gln-Leu-Lys-Ser-Gly-Thr-Ala-Ser-Val-Val-Cys-Leu				
PCA 3315[a]	Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro-Pro-Ala-Ala-Asp-Gln-Val-Ala-Thr-Gly-Thr-Val-Thr-Ile-Val(Cys)Val				
III[b]	Gly-Asp-Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro-Pro-Ala-Ala-Asp-Gln-Val-Ala-Thr-Gly-Thr-Val-Thr-Ile-Val(Cys)Val				

[a] See text for details PCA 3315. [b] Strosberg *et al.* (1972). [c] Bence-Jones κ chain, Ag (Putnam *et al.*, 1966). (Cys), not identified.

TABLE III: Percentage Cleavage as Determined by Gel Filtration.

	Time (Days)			
	1	2	3	4
10% Acetic acid–pyridine (pH 2.5)	15	27	24	26
10% Acetic acid–pyridine (pH 2.5)–	18[a]	24[a]	29[a]	38[a]
25% propanol				47
10% Acetic acid–pyridine (pH 2.5)–	30[a]	47[a]	68[a]	84[a]
7 M guanidine·HCl		65		90

[a] Refers to a single experiment in which daily aliquots were obtained and analyzed. Where two numbers appear, two separate hydrolyses were performed.

alkylation produced two peaks, one of which had an identical elution volume with uncleaved light chain and corresponded to a molecular weight of 22,000. The other peak eluted in the position of cytochrome *c* (mol wt 12,000) and was symmetrical. There was little evidence for lower molecular weight peptides which would indicate other cleavage sites. The results calculated on the basis of peak area for different conditions and reaction times are presented in Table III.

Discussion

By homology with other rabbit light-chain sequences (Strosberg *et al.*, 1972) and with human κ chains it is apparent that the cleavage point is close to the beginning of the constant region which probably begins at a position analogous to 107 as in many myeloma light chains (Edelman, 1970).

The percentage of cleavage in dilute aqueous acid as determined by quantitative Edman degradation is in close agreement with the value obtained by the chromatographic methods (see Tables I and III). The yield of N-terminal proline released by acid cleavage of another light chain discussed previously (Poulsen *et al.*, 1972) was 80% which compared favorably to the 84–90% yield of half molecules on gel filtration. Since the results obtained using the two methods were in close agreement, further quantitative studies were performed with the chromatographic method. Multiple analyses under a variety of conditions were possible by use of labeled light chain which allowed for economic utilization of homogeneous antibody preparations. Several conditions for acid cleavage were explored. In the initial experiments the conditions were those used by Freedlander and Haber (1972) for demaleylation, *i.e.*, pH 2.5 at 40° for 5 days.

However, in order to minimize the possibility of hydrolysis at other sites an attempt was made to define the minimal conditions for the cleavage reaction. It is evident from Table III that maximal cleavage using the original conditions occurred in 2 days. However, when the hydrolysis is performed in the presence of guanidine·HCl maximal cleavage is not observed until 4 days and approaches 90°. This may be related to unfolding of the molecule with increased accessibility of the Asp–Pro bond.

Following the maximal period of incubation in guanidine·HCl no other end groups were detected by Edman degradation and only minor amounts of labeled peptides revealed by gel filtration. This indicates that the cleavage occurs specifically at the Asp–Pro bond.

Our results demonstrate that dilute acid hydrolysis of rabbit light chains specifically cleaves the molecule close to its switch region in very high yield. This approach should be very useful in further sequence analysis as well as in functional studies delineating the respective roles of intact constant and variable regions (Poulsen *et al.*, 1972). The domain hypothesis of Edelman *et al.* (1969) as well as the crystallographic model of Poljak *et al.* (1972) predict ready accessibility of this region to enzymes and reagents. In rabbit light chain optimal yields were not obtained, however, until denaturation in 7 M guanidine·HCl was carried out. This suggests a relative lack of accessibility of the switch region in the rabbit. It may well be that the presence of the intrachain disulfide bond linking the variable and constant domains sufficiently stabilizes the conformation so as to hinder accessibility to the switch region.

Cleavage at Asp–Pro bonds may be of value as a general method for generating large peptides by specific cleavage at a rarely occurring peptide bond. It may also create a certain degree of confusion in the use of CNBr cleavage in protein sequencing, because the acid conditions used with this reagent may also cause cleavage at Asp–Pro bonds (Piszkiewicz *et al.*, 1970).

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