An Active Derivative of Rabbit Antibody Light Chain Composed of the Constant and the Variable Domains Held Together Only by a Native Disulfide Bond

(amino-acid sequence/antibody activity/limited peptide cleavage/antibody combining site)

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ABSTRACT The immunoglobulin light chain is thought to be organized into two structural domains, the constant and the variable regions, linked by a switch region of lesser tertiary organization. Light chains of homogeneous rabbit antibodies to pneumococcal polysaccharides labeled with ^{125}I were subjected to trypsin digestion at different temperatures. At 43° molecular size (as determined by gel filtration) remained unchanged until the chain was subjected to full reduction and alkylation, when half molecules were demonstrated. One light chain resisted tryptic digestion at 43° . At 50° extensive digestion to small peptides occurred. These experiments confirm the susceptibility of the switch region to enzymatic attack as well as the relative resistance of the constant and variable region domains. They also demonstrate that the third disulfide bridge in rabbit light chain extends between the two structural domains and maintains the integrity of the molecule even when a peptide bond in the switch region is cleaved. When the light chain was subjected to dilute acid hydrolysis [10% acetic acid-pyridine (pH 2.5) in 7 M guanidine-HCl for 24-90 hr] selective cleavage at the switch region was demonstrated in yields up to 90%. The site of cleavage was shown to be the acid labile Asp 109-Pro 110 bond by automated Edman degradation. After cleavage the molecule remained intact until full reduction and alkylation, when half molecules were again demonstrated. A light chain cleaved in this way with its inter-domain disulfide bond intact, was able to recombine with a homologous heavy chain and restore antigen binding. This indicates that an intact switch region is not needed for antibody activity. The availability of a functional variable region now allows for planning an examination of the antibody combining site by the technique of peptide synthesis.

Edelman *et al.* (1) have proposed a domain theory of the organization of the three-dimensional structure of immunoglobulins in which the constant and the variable parts of the antibody light (L) chain are independent densely folded structures. Recently, an x-ray crystallographic model of a myeloma Fab fragment by Poljak and coworkers (2) has supported this hypothesis.

In this study, rabbit antibody L-chain was subjected to limited cleavage of the polypeptide chain between constant and variable domains. The properties of the cleaved molecule with respect to molecular size and antibody reactivity were then examined.

METHODS

Specific anti-polysaccharide antibody was isolated (3, 4). One of these antibodies (2388) had previously been subjected

to fractionation by the use of affinity chromatography (5). After mild reduction and alkylation (6) of antibody, heavy (H) and L chains were isolated by gel filtration with 1 M propionic acid (6). H and L chains prepared in this manner were "renatured" by extensive dialysis against 0.01 M sodium acetate buffer (pH 5.5) (7-9).

^{125}I -Labeling of Light Chain at low specific activity ($2\ \mu\text{Ci}/\mu\text{g}$) was performed according to the method of Hunter and Greenwood (10). Labeled L-chain was separated immediately from unreacted free iodine on a $0.5 \times 7\text{-cm}$ anion-exchange resin. (Ag 1- $\times$ 8, 200-400 mesh chloride from Bio-Rad Lab) equilibrated with 0.05 M phosphate buffer (pH 7.5).

Trypsin Digestion was performed at a trypsin concentration of 0.1 mg/ml (TRTPCK, Worthington) and an L-chain concentration of 0.2 mg/ml or less in 1.0 M Tris-HCl buffer (pH 8.0) for 0.5-24 hr at 43° or 50° . The reaction was stopped by addition of di-isopropylfluorophosphate to 1 mM.

Papain Digestion was performed with 5 μg of mercuripain/ml (Nutritional Biochem. Corp.) and 0.2 mg of light chain/ml or less in 0.1 M sodium phosphate buffer (pH 7.0) containing 1.0 mM cysteine and 0.2 mM ethylene diamine tetraacetate (EDTA) for 5-30 min at 37° .

Dilute Acid Hydrolysis (11) was used for selective cleavage of the L chain. This was performed as described (submitted for publication). Briefly, L-chain 2388 (5) in 0.01 M Na acetate buffer (pH 5.5) was added to 10% acetic acid at pH 2.5 in 7 M guanidine-HCl (Schwarz-Mann ultra pure), to a concentration of 0.2 mg/ml. Incubation was at 40° for times varying between 24 and 96 hr. L chain was renatured slowly by dialysis against a small volume of 0.01 M Na acetate buffer (pH 5.5) without stirring for 18 hr before extensive dialysis for an additional 30 hr.

Sequence Determinations. Amino-acid sequences were determined in the protein sequenator as described*.

Gel Filtration. After the cleavage procedures described previously, mildly reduced and alkylated L chain was subjected to gel filtration, either directly, after incubation in 7 M guanidine-HCl (Schwarz-Mann ultra pure) for 4 hr, or after full reduction and alkylation. Complete reduction and

* Strosberg, A. D., Fraser, K. J., Margolies, M. N. & Haber, E. (1972) submitted for publication.

FIG. 1. Gel filtration (Sephadex G-100 in *a* and *b* and G-75 in *c* and *d*) of trypsin-digested L-chain 2398 in neutral salt solutions (0.02 M HPO_4^{2-} , 0.1 M NaCl, pH 7) (*a* and *b*) and after full reduction and alkylation in acid urea (6 M urea–0.1 N acetic acid) (*c* and *d*). Incubation time at 43° was 3 hr (*a* and *c*) and 21 hr (*b* and *d*). MW, molecular weight of molecules used in calibration of the column; FRA, fully reduced and alkylated. I^- , free $^{125}\text{I}^-$.

alkylation was performed at an L chain concentration of 5–200 $\mu\text{g}/\text{ml}$ in a solution containing 6 mM dithiothreitol (Sigma)–7M in guanidine·HCl–0.4 M Tris·HCl buffer (pH 8.2) for 1 hr at 37°. This was followed by carboxymethylation with a 50% molar excess of iodoacetamide for 15 min at 0° in the same solution. The mixture was directly subjected to gel filtration either on a 1 $\times$ 100-cm Sephadex G-100 column equilibrated with 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl or on a 1 $\times$ 190-cm Sephadex G-75 column equilibrated with 0.1 N acetic acid and 6 M urea.

Columns were calibrated with blue dextran, IgG, [^{125}I]IgG, fully reduced and alkylated L chain, ^{125}I -labeled L chain, cytochrome C, ^{125}I -labeled parathyroid hormone, and [^{125}I]glucagon and free ^{125}I as described (12).

Antibody Activity of the Cleaved L Chain was measured after recombination with the homogeneous homologous H chain. The binding of the recombined antibody to the Sepharose immunoabsorbent was then determined. A 1 $\times$ 5-cm glass test tube was coated with 50 μl of 0.2 mg/ml solution of non-immune rabbit L chain. 200 μl of a 0.5 mg/ml solution of non-immune H chain or antibody H chain was added and the mixture was incubated 15 min at room temperature (22–24°). Since both the L chains and the H chains were in 0.01 M Na acetate buffer (pH 5.5) they would be expected to recombine and reform an IgG molecule (7–8). This unlabeled protein was allowed to coat the tube. Next, tracer amounts of ^{125}I -labeled L chain (5–25 μl) (from nonimmune gammaglobulin or antibody) was added to the tube and the mixture was incubated for 18 hr at 4°. Then 200 μl of 0.1 M phosphate buffer (pH 7.2) containing 1 mg of nonimmune rabbit gammaglobulin per ml (Pentex) and 10 μl of immunoabsorbent suspension (5) was added. An increase in the ionic strength and pH of the buffer as well as in the concentration of gammaglobulin were necessary

in order to decrease nonspecific adsorption to the immunoabsorbent.

The tubes were then continually agitated for 18 hr at 4° in order to keep the immunoabsorbent in suspension. Tubes were then counted in a Nuclear Chicago crystal scintillation counter.

The immunoabsorbent was then sedimented by gravity, and the supernatant was removed with a constriction pipette. The sediment was washed three times with 3 ml of 0.15 M sodium chloride and was allowed to settle by gravity between each wash. The sediment was then counted. The fraction of counts bound to immunoabsorbent relative to total counts was then computed.

RESULTS

Demonstration of the interdomain disulfide bond

Trypsin Cleavage. Fig. 1*a* and *b* show the gel filtration patterns obtained after trypsin hydrolysis of L chain 2398 for 30 min and 24 hr at 43°. It is apparent that the molecular size of the L chain is unchanged in neutral buffer after the shorter trypsin digestion, though after prolonged digestion, evidence for small fragments is seen. However, upon full reduction and alkylation, followed by gel filtration in acid-urea, half molecules are seen (Fig. 1*c* and *d*). The yield of half molecules is increased by prolonged trypsin digestion, though simultaneously more small fragments are released. This suggests that the peptide chain has been cleaved at about its middle and that the two halves may be held together either by a disulfide bond or by noncovalent interactions.

Fig. 2*a* shows gel filtration patterns in acid urea after trypsin hydrolysis and full reduction and alkylation of L-chain 2388 at 43°. It is apparent that even after 18 hr, there is no evidence for any cleavage of the polypeptide chain. Fig. 2*b* shows that at 50° considerable tryptic hydrolysis takes place, yielding small fragments, but no evidence for half molecules. This indicates that the production of half molecules requires a residue susceptible to trypsin somewhere near the switch region at the center of the L chain and that this enzyme is not universally applicable to the production of half molecules from rabbit L

FIG. 2. Gel filtration (Sephadex G-75) in acid urea of L-chain 2388 digested with trypsin that had been fully reduced and alkylated after trypsin digestion. Symbols as in Fig. 1.

chains. Since all these L chains are K_B allotype b4, the residue that is susceptible to trypsin must be in the variable region.

Papain Digestion. Half molecules did not appear after papain digestion until the L chain was subjected to full reduction and alkylation, but their yield was low, never exceeding 30%. The ratio of small fragments to half molecules was far greater than in trypsin hydrolysis.

Limited Acid Hydrolysis. Aspartyl-proline peptide bonds are especially sensitive to hydrolysis under mildly acidic conditions (11, 12). Since prior sequence analysis* showed the sequence Asp 109-Pro 110, we subjected antibody L chains to limited acid hydrolysis and demonstrated a unique cleavage at this bond (submitted for publication). Fig. 3 shows gel filtration of L-chain 2388 after incubation under these conditions for 24 hr at 40°. In Fig. 3a, it is apparent that even in the presence of 6 M urea, the partly cleaved L chain migrates in the position of uncleaved L chain, whereas after full reduction and alkylation, half molecules are readily demonstrated (Fig. 3b). Prior incubation of the acid-cleaved L chain in 7 M

FIG. 3. Gel filtration (Sephadex G-75) in acid urea (6 M urea-0.1 N acetic acid) of light chain 2388 subjected to dilute acid hydrolysis; applied to the column without prior full reduction and alkylation (a), after full reduction and alkylation (b), after exposure to 7 M guanidine·HCl without full reduction and alkylation (c). Yield of half molecules was 25%. Symbols as in Fig. 1.

FIG. 4. Gel filtration (Sephadex G-75) of L-chain 2388 after dilute acid hydrolysis in 7 M guanidine·HCl at 40° for 4 days followed by full reduction and alkylation. Symbols as in Fig. 1.

guanidine·HCl does not release half molecules as shown in Fig. 3c.

These studies indicate that the half molecules are probably not held together by noncovalent bonds and that after cleavage near the switch region they may only be dissociated by disulfide bond reduction.

We have also shown (submitted for publication) that the yield of cleavage during limited acid hydrolysis could be improved by incubation of L chain in 7 M guanidine·HCl-10% acetic acid-pyridine (pH 2.5). Fig. 4 shows a typical separation of half molecules, after full reduction and alkylation of L-chain 2388 that had been incubated in acid-guanidine for 4 days. The major product is half molecules (90%); only minor amounts of uncleaved light chain and small peptide fragments were detected.

The same L-chain 2388 was also subjected to automated Edman degradation. Table 1 compares the residues obtained in the first 10 steps of degradation with those found in a tryptic peptide from the switch region* and a sequence obtained from chain 3315 that had an amino terminus blocked by pyrrolidone carboxylic acid (submitted for publication). From N-terminal studies an Asp-Pro sequence was expected at positions 1 and 2. It is possible to deduce from the known sequence of the amino terminal and switch regions that two cleavages occurred, in L-chain 2388, one after position 1, and a second after position 109.

Antigen Binding Activity. Antibody L-chain 2398, subjected to 24 hr trypsin hydrolysis (Fig. 1b), when combined with homologous H chain showed no antigen binding. This may indicate that under these conditions of hydrolysis, additional peptide bonds were cleaved in regions other than the switch region.

Fig. 5 summarizes results of antigen binding experiments with L-chain 2388 cleaved in acid-guanidine. In computing these data the ratio of counts bound to immunoadsorbent to total counts for uncleaved antibody L chain, after recombination with antibody H chain was arbitrarily set at 100%. This bound-to-total ratio under these experimental conditions was 0.59 (0.58-0.64). The zero point was arbitrarily set as that

ANTIBODY ACTIVITY OF CLEAVED RABBIT LIGHT CHAIN

FIG. 5. Antigen binding activity of recombined L and H chains as discussed in the text. Each point indicated an independent experiment. H_{AB} , heavy chain of antibody, H_N , heavy chain of nonspecific IgG, L_{AB} , light chain of antibody, L_N , light chain of nonspecific immunoglobulin.

value obtained by recombining labeled nonimmune light chain with nonimmune heavy chain. The observed bound-to-total ratio was 0.08 (0.07–0.11).

It is apparent from Fig. 5 that antibody H chain when recombined with nonspecific L chain possesses some antigen binding activity, 23(12–33)%, consistent with previous observations (13, 14). After exposure to 7 M guanidine-HCl, antibody L chain when recombined with H chain shows 92(76–100)% antigen binding with respect to unexposed L chain. Progressive acid cleavage results in a slight drop in binding activity, falling to 78(75–82)% at 90% cleavage. The antigen-binding activity of cleaved antibody L chain when combined with nonimmune heavy chains was 4(0–22)%. As controls, nonimmune L-chains were cleaved and combined with antibody H chains. Results were similar to those observed on combination of antibody H chains with nonimmune uncleaved L chains. Nonimmune H chains when combined with nonimmune cleaved L chains showed only minimal antigen binding. These results indicate that L chain,

even though cleaved at its switch region, is able to recombine with H chain to yield a molecule capable of antigen binding. Control experiments indicate that this binding is specific. Cleaved nonimmune L chains when combined with antibody H chains are unable to enhance the binding of H chain, nor are specific L chains either intact or cleaved able to enhance specific binding of nonimmune H chain.

DISCUSSION

It has been demonstrated previously that the switch region of human myeloma light chains is susceptible to preferential enzymatic cleavage, resulting in halving of the molecular weight (15, 16). Enzymatic hydrolysis of rabbit light chains with either trypsin or papain, or limited acid hydrolysis, did not produce half molecules until the cleaved chain was subjected to full reduction and alkylation. The appearance of a new amino-terminal residue permitted localization of the site of limited acid hydrolysis by sequencing techniques to the switch region. It is likely that the cleaved light chain is held

TABLE 1. Automated Edman degradation of acid-cleaved L-chain 2388 compared to known switch-region sequence

Known switch region sequences:	
1 Switch-region peptide of L-chain 2377*	110 115 120 Gly-Asp-Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro-Pro
2 Acid-cleaved L-chain 3315 with blocked N-terminus	110 115 120 Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro-Pro
Found sequences:	
1 Amino-terminal sequence of L-chain 2388	1 5 10 Asp-Pro-Val-Leu-Thr-Gln-Thr-Pro-Pro-Ser
2 Residues found at position 1 to 10 of acid cleaved L-chain 2388†	Asp Pro Val Leu Thr Gln Thr Pro Pro Ser Pro Val Leu Thr Gln Thr Pro Ile Ser Val Ala Pro Val Leu Phe Pro
Deduced N-terminal sequences:	
1 N-terminal sequence 2388	Asp-Pro-Val-Leu-Thr-Gln-Thr-Pro-Pro-Ser
2 Cleavage between Asp (1) and Pro (2)	Pro-Val-Leu-Thr-Gln-Thr-Pro-Pro-Ser-Val
3 Cleavage between Asp (109) and Pro (110)	Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro

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† The ratio Val:Leu:Ala at Step 3 was 1:0.5:1.2.

together by a disulfide bond between the variable and constant domains, since denaturing conditions alone fail to effect a reduction in molecular size. The rabbit K_B light chain contains two intrachain disulfide bonds in positions homologous to human and murine K chains but appears to have an additional pair of half-cystines. Recently one of these half cystines has been localized in the constant region and the other in the variable region*, and a disulfide bond between them was documented (Lamm, M. and Frangione, B., personal communication).

Fig. 3 indicates that the fully reduced and alkylated intact light chain appears at a smaller elution volume through Sephadex G-75 in acid urea than does L-chain with intact intrachain disulfide bonds, whether or not it has undergone proteolytic cleavage at its switch region. This observation was confirmed in separate control experiments with uncleaved light chain. The reason for an alteration in the apparent elution volume may relate to an increase in Stokes radius due to more complete unfolding of the fully reduced L-chain in acid urea. Similar observations have been made previously by Utsumi and Karush (17). The shoulder seen on the major peak in Figs. 1a and 3a may reflect a slight increase in Stokes radius consequent to cleavage of the peptide bond, since both of these peaks are shown to be mixtures of intact and cleaved light chains.

The asymmetry of the half-molecule peak produced by tryptic hydrolysis of L-chain 2398 (Fig. 1) probably indicates an asymmetric cleavage of the light chain. In view of the previous discussion, this cleavage is likely to be N-terminal to the switch region, producing a larger C-terminal fragment with a smaller N-terminal fragment. A similar pattern was observed by Bjork and Tanford (9) in rabbit light chains hydrolyzed by trypsin. It should be noted that cleavage nearly at the center of the chain, at Asp 109 by limited acid hydrolysis produced a symmetrical peak (Figs. 3 and 4) as expected.

L-chain cleaved at Asp 109, but with variable and constant regions held together by the presumed interdomain disulfide bridge, can enhance antigen combining activity of homologous antibody heavy chain in a manner similar to that of uncleaved L-chain. This indicates that a continuous polypeptide chain is unnecessary for proper combination with the heavy (H) chain to form an active antibody molecule. Smith and Dorrington's (18) observations that the variable and constant domains of human light chains may recombine with

the heavy chain independently are in agreement with this observation.

The demonstration of an inter-domain disulfide bridge indicates a constraint exercised on the variable region sequence by the constant region. If there were indeed two genes governing the sequences of the constant and the variable regions (19), the code for one-half cystine would of necessity have to be conserved in all genes governing variable regions.

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