Isolation of an Active Heavy-Chain Variable Domain from a Homogeneous Rabbit Antibody by Cathepsin B Digestion of the Aminoethylated Heavy Chain†

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ABSTRACT: Cathepsin B from bovine liver has been used to cleave the heavy chain of partially reduced and aminoethylated rabbit allotype a1 IgG. Three major cleavages have been identified, one of which appears to be at the peptide bond carboxy terminal to the two adjacent (aminoethyl)cysteine residues at positions 133 and 134. The variable domain of the heavy chain (V_H) was isolated by gel filtration from both pooled heterogeneous rabbit IgG and a homogeneous rabbit antitype III pneumococcal polysaccharide antibody. This V_H inhibited the binding of ^{125}I-labeled (allotype a1) IgG to anti-a1 allotypic antibodies. The recombinant molecule consisting of V_H and light chain from the homogeneous antibody is active in an antigen binding assay.

The study of the structure and function of several proteins has been facilitated by the use of proteases which cleave the native protein between domains of tightly folded structure. This has been especially true for immunoglobulins (Fleischman et al., 1963; Hochman et al., 1973). Papain, pepsin, and trypsin have been the most commonly used proteases (Nisonoff et al., 1975). Individual immunoglobulin domains are usually isolated by this technique (Hochman et al., 1973; Sharon & Givol, 1976; Mole et al., 1975; Smith & Dorrington, 1972). The structure and function of proteins could be more finely probed if a class of proteases existed which cleave peptide bonds at a limited number of sequence-specific sites in a manner analogous to the cleavage of nucleic acids by restriction endonucleases. We have utilized the sequence specificity of the protease cathepsin B to prepare the variable domain of the heavy chain of rabbit IgG. Cathepsin B cleaves synthetic peptide substrates containing two adjacent basic amino acids at the peptide bond carboxy terminal to these residues with much greater efficiency than peptides containing only a single basic amino acid (McDonald & Ellis, 1975). A nonhormonal protein substrate, proalbumin, is converted to albumin by cleavage at the peptide bond following an arginine–arginine sequence (Quinn & Judah, 1978). However, this specificity has not been observed in peptide prohormones which contain two adjacent basic amino acid residues (Aronson & Barrett, 1978; MacGregor et al., 1979; Bond & Barrett, 1979) presumably due to steric hindrance or digestion conditions. Cathepsin B has also been reported to possess a dipeptidyl carboxypeptidase activity (Aronson & Barrett, 1978; MacGregor et al., 1979; Bond & Barrett, 1979) and a papainlike activity (Etherington, 1974). The heavy chain of rabbit IgG has two cysteine residues at positions 133 and 134 (Cebra et al., 1968; Fruchter et al., 1970; O'Donnell et al., 1970) which are converted to (aminoethyl)cysteine upon partial reduction and reaction with ethylenimine (Raftery & Cole, 1963; Rosemblatt & Haber, 1978). The aminoethylated heavy chain possessing the two adjacent basic amino acids in an interdomain region should be accessible to a proteolytic enzyme. Since the rabbit heavy chain contains no other dibasic amino acid sequence, the variable domain (V_H)[1] should be easily isolated if cathepsin B retains the specificity shown with peptide substrates and proalbumin. We report that digestion with cathepsin B results in three major cleavages of rabbit IgG heavy chain, one of which appears to be at the dibasic amino acid sequence. The V_H produced following the proteolytic cleavage can be isolated readily by gel filtration.

The variable domains of immunoglobulins have been shown in several cases to fully replace their homologous chains in antigen binding assays (Hochman et al., 1973; Sharon & Givol, 1976; Ehrlich et al., 1979). The variable region of the light chain (V_L) from an elicited rabbit antibody can react with the homologous heavy chain to form a fully active recombinant molecule (Ehrlich et al., 1979). The variable region of the heavy chain has been isolated from the IgG of several species (Mole et al., 1975; Rosemblatt & Haber, 1978; Rodwell & Karush, 1978). We present here the preparation of V_H by a novel method from both pooled heterogeneous rabbit IgG and homogeneous rabbit antitype III pneumococcal polysaccharide (S3) antibody. The V_H from the heterogeneous IgG can inhibit the reaction between a1 IgG and anti-a1 allotypic antibodies. The V_H from the homogeneous antibody is shown to bind antigen after recombination with homologous light chain.

Materials and Methods

General. Protein concentrations were determined by amino acid analysis following 24-h hydrolysis at 110 °C in constant boiling HCl in sealed evacuated tubes. Analyses were performed on a Durrum D-500 amino acid analyzer.

Iodination of proteins was performed according to Marchalonis (1969).

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[1] Abbreviations used: V_H, variable region of Ig heavy chain; Fv, Ig fragment composed of the variable region of light chain and the variable region of heavy chain; V_L, variable region of Ig light chain; Fd, amino-terminal half of the Ig heavy chain; DNP, 2,4-dinitrophenyl; PTH, phenylthiohydantoin; $NaDodSO_4$, sodium dodecyl sulfate; HAS, *N*-hydroxysuccinimide-activated Sepharose; DTT, dithiothreitol; PBS, phosphate-buffered saline (0.005 M potassjum phosphate, 0.15 M NaCl, and 0.02% NaN_3, pH 7.0); PCAase, pyroglutamate aminopeptidase; S3, type III pneumococcal polysaccharide.

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Automated Edman degradation was performed in a Beckman 890C sequencer using a 0.1 M Quadrol program described previously (Brauer et al., 1975). Phenylthiohydantoin (PTH) amino acids were identified and quantified by gas–liquid chromatography and high-pressure liquid chromatography (Margolies & Brauer, 1978). Thin-layer chromatography on polyamide sheets was also used. Criteria for PTH amino acid identification and quantitation have been reported elsewhere (Steiner et al., 1979).

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel (12.5%) electrophoresis in 6 M urea under reducing conditions was performed according to Swank & Munkres (1971).

Digestion with pyroglutamate aminopeptidase (PCAase) (Boehringer Mannheim) was performed according to Podell & Abraham (1978).

Preparation of Heavy Chains. The homogeneous antitype III pneumococcal polysaccharide (S3) antibody 3T74 was purified by affinity chromatography as described previously (Ehrlich et al., 1978). The homogeneity of this antibody was tested by amino acid sequence analysis of the light chains and by alkaline urea gel electrophoresis after complete reduction and carboxymethylation (Chen et al., 1976). Pooled allotype a1 IgG was isolated from nonimmune serum of New Zealand white rabbits by ion-exchange chromatography on columns of diethylaminoethylcellulose (DE-52, Whatman). Ig was eluted by using 0.01 M potassium phosphate and 0.02% sodium azide (pH 7.5).

Disulfide bonds of antibody or heterogeneous IgG were reduced by incubation at 37 °C for 90 min in 0.5 M Tris-HCl and 0.02 M dithiothreitol (DTT), pH 8.2, and then aminoethylated for 30 min at 20 °C with ethylenimine (K and K Fine Chemicals) which was added in three aliquots until there was a fivefold molar excess over DTT. IgG was then dialyzed at least 4 h against 1 M acetic acid and then 18 h against 5 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5. Heavy and light chains were then separated on a Sephadex G-100 column (2.5 × 90 cm) equilibrated in 5 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5.

Digestion of Aminoethylated Heavy Chain and Purification of V_H. Cathepsin B was purified to homogeneity from bovine liver (Husain, 1976) and stored at 5 °C in 0.02 M sodium acetate, 0.1 M sodium chloride, and 0.5 mM mercuric chloride, pH 4.5.

Aminoethylated heavy chain, after separation from light chain, was dialyzed for 18 h at 5 °C against 0.01 M sodium acetate, pH 5.5. Cathepsin B (1.2 mg/mL) was activated by diluting the enzyme into 10 volumes of 0.01 M sodium acetate, pH 5.5, 0.6 mM DTT, and 1.2 mM disodium ethylenediaminetetraacetic acid and heating for 5 min at 30 °C. Activated cathepsin was then added to the heavy-chain solution (0.3–2 mg/mL) (enzyme/substrate ratio 1:50 by weight) and digestion proceeded for 3.5 h. In analytical experiments aliquots of the digestion mixture were removed at various times. After 1% $NaDodSO_4$ was added to each aliquot, the digest samples were dialyzed against $NaDodSO_4$–polyacrylamide gel sample buffer in preparation for subsequent electrophoresis. In preparative experiments, the cathepsin B digestion was stopped after 3.5 h by addition of 0.5 mM iodoacetic acid and incubation at room temperature was continued for 15 min. The digestion mixture was then dialyzed for 18 h at room temperature against 5 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5. Gel filtration on an Ultrogel AcA 54 column (2.4 × 90 cm) equilibrated in 3 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5, was then performed. Fractions containing peptides which were to be sequenced were dialyzed exhaustively against water and lyophilized.

Assay of Recombined Antibody Fragments for S3 Binding Activity. For use in the S3 antigen binding assay, an insolubilized goat antirabbit light-chain antibody was prepared in the following way. Light chain (7 mg) from pooled heterogeneous rabbit IgG [isolated according to Ehrlich et al. (1978)] was reacted with 13 mL of CNBr-activated Sepharose (Pharmacia) by incubation for 3 h at 20 °C in 0.1 M $NaHCO_3$ and 0.5 M NaCl. Goat antirabbit IgG serum (gift of Dr. V. Zurawski) was applied to a 6.5-mL column of the light chain–Sepharose equilibrated in phosphate-buffered saline (PBS). The column was washed extensively with PBS and then with PBS plus 3 M NaCl until the absorbance at 280 nm was less than 0.07. The anti-light-chain antibodies were then eluted with 3 M NH_4SCN in PBS. Fractions containing eluted antibody were pooled and dialyzed against PBS for 18 h followed by dialysis against 0.1 M potassium phosphate, pH 6.5, for 4 h. The anti-light-chain antibody (0.5–1.5 mg/mL) was then reacted with hydroxysuccinimide-activated Sepharose (HAS) (25–40 mg of antibody per g of HAS) according to Gottleib et al. (1975) to provide the desired reagent.

The radiobinding assay for anti-S3 antibodies was analogous to the assay described by Chen et al. (1976) and Ehrlich et al. (1978). The activity of heavy chains or putative V_H from homogeneous anti-S3 antibody 3T74 was assayed by mixing the chains with a two- to threefold excess of light chains in a final concentration of 4 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5. The protein was then dialyzed against 0.01 M sodium acetate and 0.02% sodium azide, pH 5.5, for 18 h at 5 °C. The recombined antibody or antibody fragments (50 μL) were added to ^{125}I-labeled soybean trypsin inhibitor–S3 conjugate in 0.005 M potassium phosphate, 0.15 M sodium chloride, and 0.02% NaN_3, pH 7.0 (PBS) (100 μL) and incubated at 20 °C for 20 min. A suspension of goat antirabbit light chain–Sepharose was then added (50 μL), and the mixture was kept in suspension by placing the assay tube on a rotating platform. This incubation continued for 4 h at 20 °C with one resuspension of the Sepharose after 2 h. The mixture was centrifuged for 1.5 min at 12000*g* at 20 °C after an incubation of 5 min at 5 °C. One hundred microliters of supernatant was removed, and the supernatant and Sepharose including the residual supernatant were counted. The percent of antigen bound was expressed as counts per minute in the Sepharose fraction minus counts per minute in the supernatant divided by their sum and multiplied by 100. The background was less than 5%, and the maximum binding varied from 50 to 65%.

Determination of Allotype. The a1 allotypic determinants of V_H were determined by using a radioimmunoassay analogous to that reported by Gottleib et al. (1975). Samples to be assayed for allotype (0.25–1.25 mg/mL) were dialyzed for 17 h at 4 °C against 0.01 M sodium acetate and 0.02% sodium azide, pH 5.5. Serial dilutions were made in 0.01 M sodium acetate, 0.02% sodium azide, and 0.1 mg/mL bovine serum albumin, pH 5.5. The V_H, heavy chain, or IgG dilutions (125 μL) were added to [^{125}I]IgG (allotype a1) (25 μL) in PBS containing 1 mg/mL bovine serum albumin. Anti-a1–HAS (50 μL) (gift of M. Mudgett-Hunter) (which had been determined previously to bind 60% of the [^{125}I]IgG) in PBS was added and the tube stirred vigorously. The Sepharose was kept in suspension by rotation for 4 h at 20 °C. The assay tube was then incubated for 10 min at 4 °C and centrifuged at 12000*g* for 1 min. After one-half of the supernatant (100 μL) was removed, the precipitate and supernatant were counted

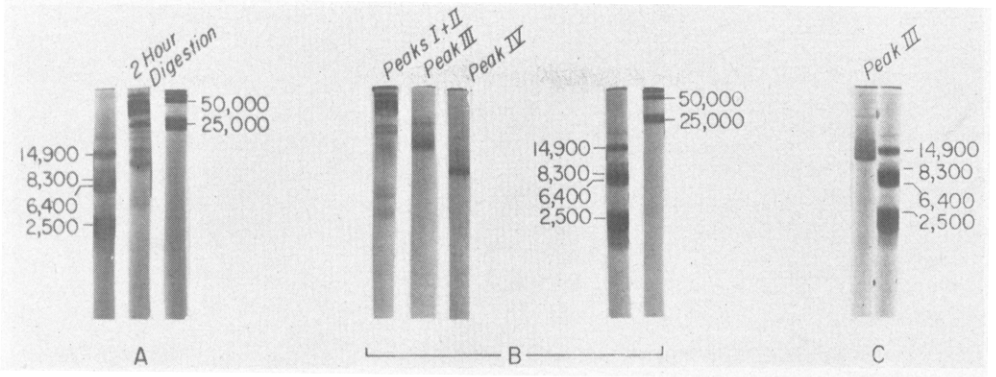

FIGURE 1: (A) $NaDodSO_4$–urea–polyacrylamide gel (12.5%) of aminoethylated heavy chain from homogeneous rabbit antibody 3T74 following digestion with cathepsin B (2% by weight of the amount of heavy chain) for 2 h. Molecular weights of standards (CNBr fragments of myoglobin and rabbit IgG) are indicated. (B) $NaDodSO_4$–urea–polyacrylamide gels of peaks analogous to peak I–IV (see Figure 2) from cathepsin digestion of homogeneous 3T74 aminoethylated heavy chain. Molecular weights of standards (CNBr fragments of myoglobin and IgG) are shown. (C) $NaDodSO_4$–urea–polyacrylamide gels of peak III (see Figure 2) from cathepsin digestion of aminoethylated heavy chain from pooled heterogeneous IgG. Molecular weights of standards (CNBr fragments of myoglobin) are shown.

Table I: Amino Acid Sequence of Peptide(s) Isolated from Cathepsin B Digests of Aminoethylated Pooled Rabbit IgG

	yields[a] of PTH amino acid from cycle no.										position of assigned sequence in H chain
fraction	1	2	3	4	5	6	7	8	9	10	
pool I	L (11.0) G (6.4) V (5.9)	S (3.0) D (7.1)	S (2.8) T (2.5)	V (7.9) P (3.0)	V (7.5) S (2.4) Q (1.4)	S (2.8) (S)	V (7.7) T (3.9)	T (1.8) V (5.7)	S (1.2) T (2.3) A (1.0) P (1.3)	S (0.6) L (5.6)	184→ 135→ unassigned
pool II	*b*	S (8.2) D (11.6) V (7.7)	S (10.4) T (5.6) A (2.3)	V (19.3) P (5.5) E (1.4)	V (15.1) S (4.0) F (1.1)	S (6.9) (S) Q (1.0)	V (13.2) T (3.3) L (6.4)	T (4.0) V (9.0)	S (5.3) T (4.6) K (5.5)	S (3.9) L (7.5) P (0.8)	184→ 135→ unassigned
pool III[c]	S (24.5) L (3.8)	V (23.0) L (10.3)	E (19.5)	E (18.9)	S (20.2) F (3.1)	G (11.4)	G (12.0)	R (2.4)	L (18.6)		2→ unassigned
pool IV[d]	M (18.3) L (5.5) G (4.0) Y (3.4)	G (7.2) D (4.4)	P (2.4) T (1.6)	P (2.4) V (2.7) Y (1.3)	E (1.8)	E (8.5) (1.2)	E (5.0) L (1.5) T (1.1)	L (8.6) Y (1.0)	S (4.5)	S (5.0)	350→ unassigned

[a] Yield in nanomoles of PTH amino acids calculated from high-pressure liquid chromatography and/or gas–liquid chromatography. PTH amino acids are given in the single letter code (IUPAC–IUB Commission on Biochemical Nomenclature, 1968). [b] This fraction was lost. [c] Pool III was sequenced after digestion with PCAase. In addition to the PTH amino acids listed, at several positions minor PTH amino acids of less than 10% of the major sequence were detected. Pooled heterogenous rabbit H chain allotype a1 has two amino acid alternatives at position 3 (cycle 2) (Wilkinson et al., 1966). [d] Sequence analysis on this fraction was repeated following digestion with PCAase. An additional sequence was detected corresponding to V_H beginning at residue 2.

in a γ counter for 1 min. The results are expressed as percent counts per minute bound which is equal to counts per minute in the Sepharose fraction minus counts per minute in the supernatant divided by their sum; the range of binding was 0 to 62%.

Results

Cathepsin B Digestion and Isolation of V_H from Pooled Heavy Chains. In a series of analytical experiments, $NaDodSO_4$–urea gel electrophoresis indicated that a 2–4-h digestion with cathepsin B produced fragments which corresponded to the size of V_H. The results of a 2-h digestion are shown in Figure 1A. In addition to a small amount of heavy chain, at least five low molecular weight fragments were observed. Clearly, the cathepsin B digestion was not restricted to the 134–135 peptide bond as anticipated. This was confirmed by a sequenator analysis of a 30-min cathepsin B digest. Two major amino-terminal sequences were found which began at residues 135 and 184.

Thereafter in a preparative experiment, 73 mg of aminoethylated nonimmune heavy chains was digested with cathepsin B for 3.5 h. After inactivation of the enzyme with iodoacetic acid, the digest was dialyzed against 3 M guanidine hydrochloride and 0.1 M NaOAc, pH 5.5, and applied to an Ultrogel AcA 54 column. The results of the gel filtration experiment, shown in Figure 2, indicated the presence of at least four peptides. When sequenator analyses of each peptide fraction (peaks I–IV, 20–80 nmol) were performed, only three amino-terminal sequences were found, two of which were identical with those found in the analytical experiment. As shown in Table I, a few minor sequences were also seen, in addition to the three major amino-terminal sequences beginning at residues 135, 184, and 350. These minor sequences could not be correlated with any region of the rabbit heavy-chain molecule as delineated by Cebra et al. (1968), Fruchter et al. (1970), Pratt & Mole (1975), and M. N. Margolies (unpublished experiments).[2] When the peptide in peak III was treated with PCAase, the amino-terminal sequence of the heavy chain starting at residue 2 was revealed. This was consistent with the removal of the amino-terminal pyroglutamic acid residue at position 1 by the action of PCAase. In the same sequence

[2] The reported sequence beginning at residue 184 is LSSVVSVPVTSSS (Pratt & Mole, 1975). However, the sequence of this region has proved to be LSSVVSVTSSS based on experiments on three different homogeneous antibodies (M. N. Margolies, unpublished experiments).

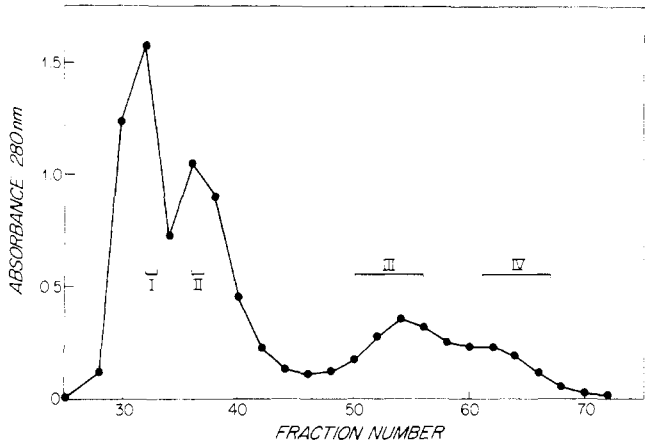

FIGURE 2: Isolation of V_H from pooled heterogeneous rabbit aminoethylated IgG heavy chain which had been digested with cathepsin B. Aminoethylated heavy chain (73 mg) was digested with 1.46 mg of cathepsin B for 3.5 h at 20 °C in 0.01 M sodium acetate, pH 5.5. After the reaction was stopped with iodoacetic acid, the digest was dialyzed against 3 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5. The protein was then chromatographed on an AcA 54 column (2.6 × 90 cm) equilibrated in 3 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5. Fractions were 3.9 mL. The results of gel filtration of the digest were similar to the pool heavy-chain digestion except that peaks I and II were not resolved.

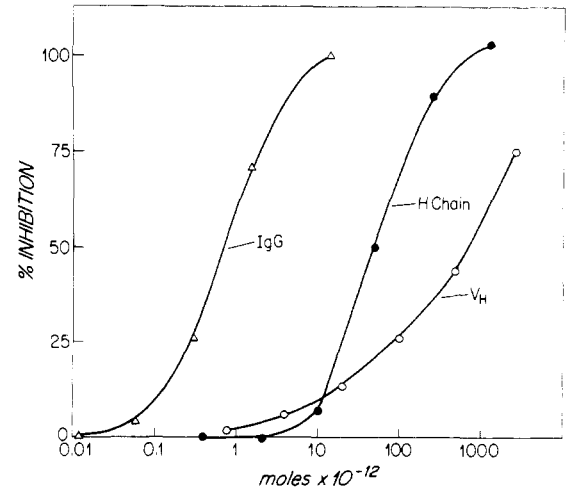

FIGURE 3: Inhibition of anti-a1 allotypic antibodies. Varying amounts of IgG, heavy chain, and V_H in 125 μL of 0.01 M sodium acetate and 0.02% sodium azide, pH 5.5, were added to [^{125}I]IgG (25 μL in PBS). HAS–anti-a1 (50 μL suspension in PBS), in an amount which could bind 60% of the [^{125}I]IgG, was added and the assay tube rotated at 20 °C for 4 h. After centrifugation, 100 μL was removed and the Sepharose and supernatant fractions were counted. Binding of less than 60% indicates inhibition of the reaction between IgG and the antiallotypic antibodies. Each point is an average of a duplicate assay.

analysis, two amino acids, valine and leucine, were found at residue 3, but this microheterogeneity is characteristic of pooled rabbit heavy chains (Wilkinson et al., 1966). The peptide in peak IV also possessed the characteristic heavy-chain amino-terminal sequence following treatment with PCAase. However, the major sequence in peak IV corresponded to the heavy-chain sequence which starts at residue 350.

As shown in Figure 1C, a molecular weight range of 12000 to 15000 was obtained for the peptide in peak III. The peptide in peak III was assigned as V_H (1–134) on the basis of the molecular weight and the amino-terminal sequence as shown in Table I. The yield of this putative V_H was 13% starting from the aminoethylated heavy chain.

Inhibition of a1 Allotype by V_H. As it is known that the a1 allotypic determinants reside on V_H (Mole et al., 1975), the putative V_H was assayed for a1 allotype. As shown in Figure 3, the protein in peak III inhibits the allotypic binding of ^{125}I-labeled a1 IgG to anti-a1 antibodies. This inhibition was 1 order of magnitude less effective than intact heavy chain. The shape of the inhibition curve of V_H is different from the shapes of the inhibition curves of IgG and heavy chain.

Isolation of an Active V_H from Homogeneous Rabbit Antibody 3T74. The digestion with cathepsin B was repeated on aminoethylated heavy chain from homogeneous anti-S3 pneumococcal polysaccharide antibody 3T74. The results of gel filtration of the digest were similar to those of the heterogeneous heavy-chain digestion except that only one high molecular weight peak was observed. $NaDodSO_4$–urea–polyacrylamide gels shown in Figure 1B revealed that peak IV from the homogeneous 3T74 heavy-chain digestion had a molecular weight of ~8000 while the protein in peak III had several bands in the region from 12500 to 15000. The contaminant in peak III with a molecular weight of 21000 represented less than 10 mol % of the total protein as determined by densitometry of the polyacrylamide gel. The results of the sequence analysis on the peak fractions were analogous to those shown for the pool in Figure 2.

The putative V_H from peak III was recombined with 3T74 light chain and assayed for S3 binding. In Figure 4 the amount of protein required to bind the antigen is compared for the two

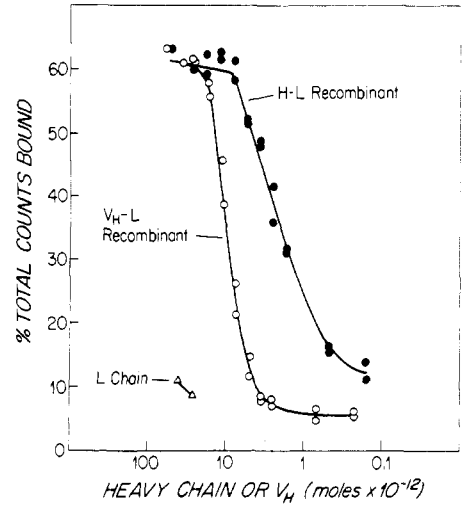

FIGURE 4: Double antibody radioimmunoassay for binding to type III pneumococcal polysaccharide. 3T74 V_H (252 pmol) and 3T74 light chain (567 pmol) or 3T74 heavy chain (177 pmol) and 3T74 light chain (567 pmol) were dialyzed against 0.01 M sodium acetate and 0.02% sodium azide, pH 5.5, for 18 h at 5 °C. Serial dilutions (50 μL) were added to 100 μL of [^{125}I]S3-SBTI in PBS. After a 20-min incubation at 20 °C, antirabbit light chain–HAS of (50 μL in PBS) was added and the assay tubes were rotated for 4 h at 20 °C. After centrifugation, 100 μL was removed and the Sepharose and supernatant were counted. Open circles, V_H–light chain recombinant; filled circles, heavy chain–light chain recombinant; open triangles, light chain alone.

types of recombinant molecules. The V_H–light chain recombinant was approximately 21% as active as the heavy chain–light chain recombinant. However, the binding curve for the V_H–light chain recombinant has a different shape than the binding curve for the heavy chain–light chain recombinant.

Discussion

The V_H domain from rabbit a1 IgG can be easily isolated from aminoethylated heavy chain following cleavage with cathepsin B. A single digestion step and gel filtration step are required. In addition, since the digestion is performed on isolated heavy chain, the likelihood of contaminating light

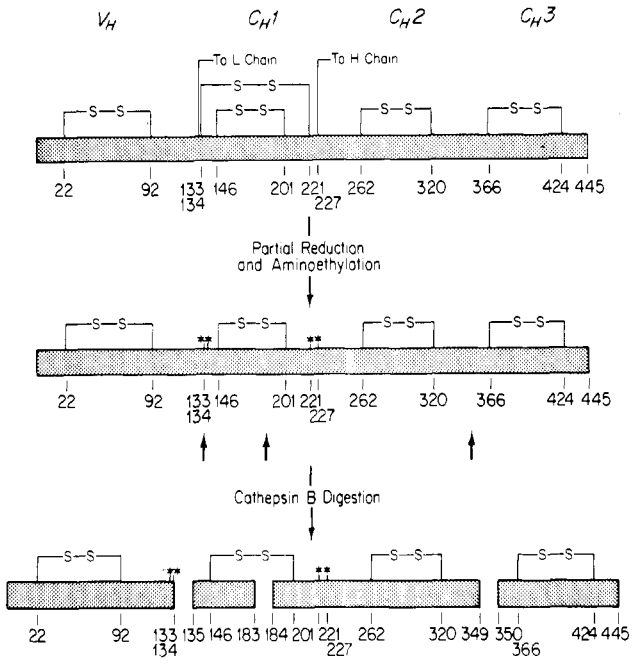

FIGURE 5: Structure of partially reduced and aminoethylated rabbit heavy chain and the fragments which are produced by digestion with cathepsin B. Numbering is by homology with the human protein DAW IgG1 (Press & Hogg, 1970). Arrangement of disulfide bonds is according to O'Donnell et al. (1970). An asterisk indicates (aminoethyl)cysteine.

chain or light-chain fragments is small (Ehrlich et al., 1978). The evidence that peak III contains predominantly V_H includes the following: (1) $NaDodSO_4$–urea–polyacrylamide gel electrophoresis revealed that the peptide in peak III was of domain size; (2) automated sequence analysis following PCAase digestion revealed a major sequence beginning at residue 2; (3) the recombinant molecule consisting of peak III peptide and light chain (both from a homogeneous antibody) bound antigen; (4) the peptide in peak III inhibited the binding of anti-a1 allotypic antibodies to a1 IgG.

The structures of rabbit heavy chain and the fragments produced by digestion with cathepsin B are schematically illustrated in Figure 5. Cathepsin B produced three major cleavages of aminoethylated heavy chain as evidenced by sequenator studies. The desired cleavages occurred between Cys-134 and Gly-135 in the sequence -Cys(AE)-Cys(AE)-Gly-Asp- (AE = aminoethyl) to provide V_H. Other cleavages occurred at the second peptide bond in the sequences which follow: Ser-Leu-Ser-Ser (residues 183–186) and Tyr-Thr-Met-Gly (residues 348–351). Although cathepsin B recognized the sequence consisting of two adjacent (aminoethyl)-cysteine residues, minor sequences were found in each fraction, indicating that additional cleavages occurred. Other attempts at utilizing this sequence specificity of cathepsin on proteins (MacGregor et al., 1979; Bond & Barrett, 1979; Aronson & Barrett, 1978) have not been successful except in the case of proalbumin (Quinn & Judah, 1978). As the two adjacent (aminoethyl)cysteine residues are located in an interdomain region, this site might be especially susceptible to cleavage by cathepsin B. In addition, the cleavage within the C_H1 domain (at residue 184) which occurs early in digestion may result in a conformational change in the interdomain region between V_H and C_H1, rendering this site more accessible to cleavage.

As the sequence analysis of PCAase-digested V_H peptide revealed a single major amino terminus, the heterogeneity in the size of V_H as evidenced by $NaDodSO_4$ gel electrophoresis suggests variation in length at the carboxy terminus. This is consistent with the known dicarboxypeptidase activity in cathepsin B preparations (MacGregor et al., 1979; Bond & Barrett, 1979; Aronson & Barrett, 1978). The presence of both endopeptidase activity and dicarboxypeptidase activity may be the result of the pH at which the digestion was performed (pH 5.5). S. S. Husain (unpublished experiments) has shown that cathepsin B has optimum endopeptidase activity at pH 6.5 while the maximum dicarboxypeptidase activity occurs at pH 3.

Although V_H binds antigen (after recombination with specific light chain) and inhibits the reaction between allotype a1 IgG and anti-a1 allotypic antibodies, it does not quantitatively replace heavy chain. This was observed previously in the case of inhibition of a1 allotypic antibodies by Mole et al. (1975) in that V_H was a less effective inhibitor of a1 allotype than Fd. Furthermore, Rosemblatt & Haber (1978) have shown that a V_H fragment (V_H lacking nine amino-terminal residues) upon recombination with light chain was only 12% as active as a heavy chain–light chain recombinant. By contrast, V_L domains, which have been isolated from immunoglobulins, appear to be equipotent to the intact homologous light chain in an antigen binding assay (Hochman et al., 1973; Sharon & Givol, 1976; Ehrlich et al., 1979). There are several possible explanations for the lower activity of V_H as isolated following cathepsin cleavage. First, many minor internal cleavages may have been produced by cathepsin [or by papain in the case of Mole et al. (1975)] which individually are not detected by sequence analysis or $NaDodSO_4$–polyacrylamide gel electrophoresis but represent in the aggregate a significant proportion of the protein. In addition, some size heterogeneity on $NaDodSO_4$ gels might be due to aggregation. Second, antibody 3T74 binds a pneumococcal polysaccharide and is known to bind an octasaccharide with greater affinity than a hexasaccharide (Haber et al., 1977). Therefore, it is probable that at least seven or eight saccharide units are encompassed by the binding site. Thus, a change in the conformation of antibody 3T74 V_H may be more easily detectable than changes in myeloma proteins binding small haptens [which were used in the work of Hochman et al. (1973) and Sharon & Givol (1976)]. Third, it is possible that the conformation of V_H is not totally independent of the constant domains. This is consistent with the dependence of several biologic functions which rely on domain–domain interactions such as activation of complement.

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Metal Binding to Modified Bleomycins. Zinc and Ferrous Complexes with an Acetylated Bleomycin†

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ABSTRACT: We have studied the DNA- and metal-binding properties of a bleomycin A2 derivative in which the α-amino group of the β-aminoalanine moiety has been N-acetylated. The modified antibiotic has been shown to be without activity in mediating the in vitro release of [^{3}H]thymine from PM-2 DNA. Fluorescence experiments indicate that the degree of quenching by DNA of the bithiazole fluorescence is unaffected by N-acetylation of bleomycin. Furthermore, ^{1}H NMR experiments demonstrate that N-acetylation does not alter the stoichiometry of metal binding. The Fe(II)–Ac-bleomycin A2 complex, however, has been found to be stable in the presence of both O_2 and CO, and thus inactivation appears to be accounted for by the loss of the ability to bind and/or reduce O_2. Comparison of the ^{1}H NMR spectra of the Fe(II)–bleomycin and Fe(II)–Ac-bleomycin A2 complexes indicates that either a drastic reorganization of the ligands with respect to the central iron atom has occurred or that an altered spin state is stabilized. These experiments establish that the ability of bleomycin to cause DNA damage is sensitive to even minor structural alterations within the antibiotic.

The bleomycins are a family of structurally related antibiotics isolated from *Streptomyces verticillus* by Umezawa et al. (1966). In addition to their antibacterial activity, the bleomycins inhibit the growth of transformed mammalian cells both in culture and in experimental animals (Remers, 1979). Significantly, a mixture consisting primarily of bleomycins A2 and B2 has been used successfully in the clinical treatment of certain types of cancers, such as squamous cell carcinomas and malignant lymphomas (Umezawa, 1979). Bleomycin is believed to function by strand scission of DNA in the affected cells, a process which seems to be mediated via a metal chelate of bleomycin that is capable of generating a reduced form of oxygen in proximity to a susceptible site(s) on the DNA (Hecht, 1979).

Although the structure shown in Figure 1 has been proposed for bleomycin (Takita et al., 1978a) and many of the structural components have been verified by unambiguous synthesis [see, e.g., Hecht et al. (1979)], the detailed structures of the chelates that bleomycin forms with several metals are less clear. For example, P-3A (a putative biosynthetic intermediate structurally related to bleomycin) has been crystallized as its Cu(II) chelate and the structure has been determined by X-ray crystallographic analysis (Iitaka et al., 1978). While simple

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