The Specific Cleavage of Immunoglobulin Polypeptide Chains at Cysteinyl Residues

(Received for publication, November 20, 1967)

LAWRENCE I. SLOBIN and S. J. SINGER

From the Department of Biology, University of California at San Diego, La Jolla, California 92037

SUMMARY

A method has been developed for fragmenting immunoglobulin light and heavy polypeptide chains at the cysteinyl residues that participate in intrachain disulfide bridges. The method involves the quantitative blockage of positive charges associated with lysyl and arginyl residues, the reduction and subsequent aminoethylation of disulfide bridges, and the treatment of aminoethylated, blocked chains with trypsin to yield the desired blocked peptide fragments. Lysine and arginine are modified by succinic anhydride and cyclohexanedione respectively, to yield stable "trypsin-insensitive" residues. The blocked peptides of heavy and light chains are separated on the basis of size by gel filtration chromatography in either 8 M urea or 0.01 M ammonium bicarbonate.

The blocked peptides from a K-type Bence-Jones protein can be resolved into six chromatographic peaks. The peaks, in order of their elution, have been assigned loci within the known over-all structure of a Bence-Jones protein: E and D have not been characterized and probably represent partial digestion products; C and C' probably correspond to regions between the two intramolecular disulfide bridges; B contains the peptide corresponding to the "switch region" as judged by amino-terminal analysis and amino acid composition; A contains the carboxyl terminal cysteinyl residues and corresponds to peptide fragments from the amino and carboxyl-terminal regions of the starting Bence-Jones protein.

The peptide patterns obtained from whole normal mouse and human light chain are closely similar to the Bence-Jones profile and strongly suggest that the disulfide bridge pattern found for Bence-Jones proteins applies to the majority of K and X chains in the whole normal light chain population.

A comparison of the gel filtration patterns of a γ2a mouse myeloma heavy chain and whole normal mouse heavy chains also indicates that a single heavy disulfide bridge pattern is largely conserved in the normal population. The similarity found between heavy and light chain peptide profiles supports the idea that heavy chains and light chains arose from a common ancestral gene as a result of gene duplications and mutations.

An understanding of the structure of immunoglobulin polypeptide chains was greatly advanced by the discovery of Hilschmann and Craig (1) that Bence-Jones proteins (L chains) of a given antigenic class consist of an amino-terminal variable half and a carboxyl-terminal invariant half. An analogous model of the heavy polypeptide chains of immunoglobulins has recently been proposed (2, 3). The inference has been drawn that it is the variable half of antibody molecules that contains the amino acid residues responsible for antibody specificity. A consequence of this hypothesis is that any studies on the structural basis of antibody specificity must necessarily deal directly with the chemistry of these variable regions. Unfortunately, such structural studies are greatly hampered by the fact that any population of antibody molecules, even those with specificity to simple haptenic determinants, is grossly heterogeneous. This heterogeneity, translated into practical terms, usually results in a bewildering array of peptide fragments when immunoglobulin molecules or their corresponding polypeptide chains are extensively hydrolyzed by proteolytic enzymes.

In connection with structural studies on antibody-active sites (4), we sought a method which would allow us to prepare reproducibly large and characteristic peptide fragments from both the variable and constant regions of L and H chains. The inspiration for our experiments was the observation, first made by Milstein (5), that all Bence-Jones proteins of the K class possess a single disulfide bond in the variable region (as well as one disulfide bond in the constant region) and that the 2 cysteinyl residues that participate in this bond occur at characteristic positions in the linear sequence. It was hoped that, whatever the variability of the amino-terminal region, the cysteinyl residues might be used as anchor points in the polypeptide sequence.

Fig. 1 depicts a one-dimensional model of a K type Bence-
chains at the cysteinyl bonds, in the order of their increasing size. Denote the peptides expected upon block fragmentation of the and invariant halves of the chain. The letters A, B, and C type L chains, with the amino acid numbering system of Gray, Dreyer, and Hood (6). The v and i segments refer to the variable molecule (6, 7). From an examination of Fig. 1, it may be seen other forming a loop of similar size in the constant region of the which spanning about 64 residues in the variable region and the Jones protein of either human or murine origin, illustrating the Also shown are the positions of the two disulfide bridges, one of (8) that cysteinyl residues may be converted to aminoethyl- and the c-terminal (variable) peptide; (b) one class (B, Fig. 1) of about 46 amino acid residues containing the “switch region” peptide (the NH2-terminal half of which is variable, the COOH-terminal half constant); and (c) one class (C, Fig. 1) of about 60 to 65 amino acid residues containing the regions of the molecule within the two disulfide loops.

The method was based, in part, upon the finding of Lindley cysteinyl residues may be converted to aminooethylcysteinyl residues which, in turn, form trypsin-sensitive bonds. In order to produce tryptic peptides which span from 1 cysteinyl residue to the next, it was first necessary to block the ε-ammonium groups of lysine and guanidinium groups of arginine residues. Such an aminoethylated, blocked, polypeptide chain could be specifically cleaved by trypsin to yield the desired peptide fragments.

**EXPERIMENTAL PROCEDURE**

Materials

We gratefully acknowledge gifts of a κ type Bence-Jones protein from Dr. Howard Grey and a mouse γ2a H chain from Drs. Michael Parkhouse and Edwin Lennox.

Human and mouse normal γ-globulin (Fraction II) were obtained from Pentex, Inc. (Kankakee, Illinois). 3H-iodoacetamide (2.67 mC per mmole) and 3H-phenylisothiocyanate (83 mC per mmole) were obtained from New England Nuclear and used without further purification. Succinic anhydride (Eastman Organic Chemicals), ethyleneimine (Matheson, Coleman and Bell), diethothreitol (Calbiochem), and cyclohexanedione (Aldrich) were all used without further purification. Guanidine hydrochloride (Matheson, Coleman and Bell) was recrystallized twice from methanol prior to use. Solutions of 8 M urea (Mallicankrodt) were passed through a column of Amberlite MonoB Resin MB-1 (Mallicankrodt) prior to use to remove cyanate ions. Sephadex G-100 was obtained from Pharmacia (Uppsala, Sweden). Analytical-grade reagents and chemicals were used for all experiments.

**Methods**

Procedure for Specific Cleavage at Cysteinyl Bonds

A satisfactory procedure was developed which involved the succession of steps outlined in Table I. In the following sections each step is given in detail.

Succinylation of L and H Chains (9)—To a chilled (ice bath) solution of L or H chain (1 to 2%) in deionized water (pH adjusted to 8.0 to 9.0 with 0.1 N NaOH) was added an amount of solid succinic anhydride equal to a 5-fold molar excess over total free (α and ε) amino groups. The solution was kept in an ice bath and the pH was either maintained in a pH state at 8.5 (with 1 N NaOH) or manually adjusted to between 8 and 9 with dilute NaOH. After 30 min, the pH remained essentially constant. In the case of the L chain, the solution was maintained at ice bath temperature for an additional 30 min and then dialyzed at 4° against several 2-liter portions of deionized water. The protein was then recovered by lyophilization.

In the case of H chain, at 30 min, an additional portion of solid succinic anhydride, equal in amount to the original portion, was added; at 60 min, the same amount of anhydride was again added. The reaction was allowed to proceed for another 30 min (total reaction time of 90 min). Any insoluble material, which was usually less than 10% of the starting H chain, was removed by filtration through a Millipore filter. The succinylated H chain was then dialyzed against several 2-liter portions of deionized water and lyophilized.

The degree of succinylation was measured by treating the derivatized protein with trinitrobenzenesulfonic acid. This reagent reacts with the residual free amino groups to give a product with characteristic absorption spectrum (10).

Complete Reduction and Aminoethylatbn (11)—To a 2% solution of succinylated L or H chain in 6 M guanidine hydro-

<table>
<thead>
<tr>
<th>Table I</th>
<th>Procedure for block fragmentation</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) NH₂ +</td>
<td>C₆H₄-C(=O)-CH₂-C≡N</td>
<td>1) 90-93%</td>
</tr>
<tr>
<td>2) -SH +</td>
<td>C₆H₄-NH₂</td>
<td>2) 100%</td>
</tr>
<tr>
<td>3) NH₂ +</td>
<td>C₆H₄-NH₂</td>
<td>3) &gt; 90%</td>
</tr>
<tr>
<td>4) NH₂ +</td>
<td>C₆H₄-NH₂</td>
<td>4) &gt; 95%</td>
</tr>
<tr>
<td>5) TRYP SINIZATION</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
chloride-0.2 M Tris-0.01 M EDTA, pH 8.6, was added enough solid\(^1\) (or freshly prepared 1 M solution in 6 M guanidine) diithiothreitol (12) to make the resulting solution of polypeptide chain 0.1 M with respect to diithiothreitol. (No attempt was made to exclude oxygen.) The solution was incubated for 2 hours at room temperature (22–23°). At 2 hours, a solution of 6 M guanidine-3 M Tris-0.01 M EDTA, pH 8.0, equal in volume to the original 2% protein solution was added. A stream of N\(_2\) was passed gently over the surface of the reaction mixture for approximately 5 min. While maintaining the solution under N\(_2\), five equal portions of ethyleneimine were added with 5-min intervals between additions. The total amount of ethyleneimine added was equal to a 10-fold molar excess over diithiothreitol. The reaction was allowed to proceed for 40 min at room temperature (total reaction time, 1 hour) at which time 1 volume of ice-cold deionized water was added to the reaction mixture. The resulting solution was quantitatively transferred to a dialysis sac and the protein was freed of salts by dialysis at 4° against 0.1 M deionized water. The reaction mixture was allowed to proceed for 40 min at room temperature. The reaction was stopped by addition of an equal portion of ethyleneimine. The reaction was allowed to proceed for 3 hours at room temperature. The solution was then neutralized with an equivalent amount of NaOH and dialyzed at 4° against three to four successive 2-liter portions of deionized water. The deamidated, aminoethylated protein was recovered by lyophilization.

The degree of aminoethylation was determined by amino acid analysis (see "Results").

Modification of Arginyl Residues with Cyclohexanedione (18)—To a solution of protein in 0.2 M NaOH was added enough of a freshly prepared 1% solution of cyclohexanedione to equal a 10-fold molar excess of cyclohexanedione over arginyl residues. The reaction was allowed to proceed for 3 hours at room temperature. The solution was then neutralized with an equivalent amount of HCl and dialyzed at 4° against several successive 2-liter portions of deionized water. The protein was recovered as a lyophilized powder. The derivative of cyclohexanedione with arginine, although stable to acid hydrolysis, emerges from the amino acid analyzer resin at the same position as aminoethylarginine. Therefore, the degree of reaction was measured on the amino acid analyzer by the disappearance of arginine.

Trypsinization of Succinylated, Aminoethylated, Cyclohexanedione-treated (Blocked) L (or H) Chains—A 1% solution of protein in 0.1 M ammonium bicarbonate, pH 8.0, was digested with L-\((\text{p-toluene sulfonyl}-\text{amido}-2\text{-phenyl})\text{ethyl chloromethyl ketone}-\text{treated trypsin}\) (14), (50 parts of protein to 1 part trypsin by weight) for 6 hours at 37°.

Treatment of Blocked L (or H) Chains with Tritiated Phenylisothiocyanate—For analysis of the gel filtration experiments with the trypptic peptides in 6 M urea, it was convenient to tag the liberated \(\alpha\)-NH\(_2\) groups and the NH\(_3\) groups of the AE-Cys\(^2\) residues with \(^{3}H\)-phenylisothiocyanate.\(^3\) This was carried out as follows.

A 1% solution of a trypptic digest of L (or H) chain in coupling buffer (15.0 ml of pyridine, 1.2 ml of dimethylformamide, and 10.0 ml of water adjusted to pH 9.2 with trifluoroacetic acid) (15) was treated with an amount of \(^{3}H\)-phenylisothiocyanate equal to a 2-fold molar excess over the expected number of free amino groups. The reaction mixture was incubated for 1 hour at 40°, after which most of the excess \(^{3}H\)-phenylisothiocyanate was extracted by washing with five 3-ml portions of n-butyl acetate. The lower phase, containing the derivatized peptides, was taken to near dryness under a stream of nitrogen and then lyophilized.

Chromatography of Blocked Tryptic Peptides on Sephadex G-100, 0.01 M Ammonium Bicarbonate

The trypptic digest (10 to 25 mg) of blocked L or H chains (not treated with \(^{3}H\)-phenylisothiocyanate) was chromatographed on a column (146 x 2.5 cm) of Sephadex G-100, previously equilibrated and subsequently developed with 0.01 M ammonium bicarbonate. The column was run at a flow rate of 12 ml per hour; 30-min (6-ml) fractions were collected; peptide fractions were detected by measuring absorption at 215 and 225 nm.

Sephadex G-100, 8 M Urea—The trypptic digest (10 to 20 mg) of blocked, \(^{3}H\)-phenylisothiocyanate-treated L (or H) chain was chromatographed on a column of Sephadex G-100, 8 M urea, pH 8.0 (a solution of deionized 8 M urea was adjusted to pH 8.0 with a few drops of concentrated ammonium hydroxide). The column dimensions were 146 x 2.5 cm. Aliquots (which varied from 0.1 to 0.5 ml in different experiments, but were constant in any given experiment) from each tube were counted in 10 to 18.5 ml of ethanol-toluene scintillation fluid (0.01% 1,4-bis(2-(5-phenyloxazoyl))benzene, 0.6% 2,5-diphenyloxazole per liter of 67.5% (by volume) toluene, 32.5% absolute ethanol) at an efficiency of about 24% with the use of the Beckman LS-200B liquid scintillation system. Peptide profiles may be determined in 8 M urea solutions by optical density measurement. However, in order to measure the \(A_{280}\) (the lowest wavelength at which 8 M urea solutions may be used as a blank in the Zeiss model PMQ 11 spectrophotometer) at least 20 mg of L or H chain should be chromatographed.

Partial Reduction of Bence-Jones Protein and Alkylation with \(^{14}C\)-Iodoacetamide

For certain experiments, it was useful to tag the carboxyl-terminal cysteine residue (5) of the Bence-Jones protein before carrying out the procedure for specific cleavage at the internal cysteinyl bonds. This was carried out as follows.

To a 1.2% solution of Bence-Jones protein (55.1 mg) in 0.2 M Tris-0.01 M EDTA, pH 8.6, was added enough of a freshly prepared solution of 0.01 M diithiothreitol in 0.2 M Tris buffer, pH 8.6, to make the resulting solution of polypeptide chain could be directly subjected to the cyclization step of the Edman procedure. Unfortunately, several attempts to recover radioactive phenylthiohydantoin amino acids from isolated blocked and \(^{3}H\)-phenylisothiocyanate-treated peptides proved unsuccessful. (The yields of phenylthiohydantoin amino acids were always less than 5% of the expected value.) Nevertheless, treatment of the blocked peptides with \(^{3}H\)-phenylisothiocyanate was useful for analytical purposes.
1 × 10⁻⁶ M with respect to dithiothreitol. After 1 hour at room temperature, a solution of 14C-iodoacetamide (0.6 ml, 17 μmoles per ml) was added and the reaction mixture was incubated for 30 min at room temperature. The reaction was then diluted with 1 volume of ice-cold 5% acetic acid, followed by four successive dialyses against 4 liters of deionized water. The 14C-alkylated κ chain was recovered by lyophilization.

### Amino Acid Composition

Amino acid analyses of the L chain and its derivatives were determined by the method of Spackman, Stein, and Moore (16) and Moore, Spackman, and Stein (17) with a Beckman/Spinco amino acid analyzer model 120E. Samples of protein (2 to 5 mg) in thick walled pyrex combustion tubes were suspended in equal volumes of deionized water and concentrated hydrochloric acid and the contents of the tube were repeatedly frozen and thawed under vacuum to remove any dissolved oxygen (18).

Duplicate samples of Bence-Jones protein (unmodified) were hydrolyzed for 20, 44, and 68 hours at 110 °C in an oil bath; thawed under vacuum to remove any dissolved oxygen (18). The amino acid recoveries from modified Bence-Jones protein were normalized to a glycine content of 12.0 residues. Aminoethylcysteine was determined by the method of Koshland, Karkhanis, and Latham (25) or the method of Spies and Chambers (26). Both methods gave comparable results.

### TABLE II

**Amino acid composition of Bence-Jones protein and derivatives**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Bence-Jones protein</th>
<th>Succinylated, aminoethylated</th>
<th>Fully blocked Bence-Jones protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>8.7</td>
<td>16.9</td>
<td>17.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.4</td>
<td>10.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.3</td>
<td>26.6</td>
<td>26.7</td>
</tr>
<tr>
<td>Aminomethylcysteine</td>
<td>4.4</td>
<td>11.6</td>
<td>11.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>17.0</td>
<td>16.9</td>
<td>17.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>10.0</td>
<td>10.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Serine</td>
<td>39.4</td>
<td>35.8</td>
<td>33.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>25.1</td>
<td>26.6</td>
<td>26.7</td>
</tr>
<tr>
<td>Proline</td>
<td>12.0</td>
<td>11.6</td>
<td>11.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>11.8</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>13.1</td>
<td>13.2</td>
<td>13.3</td>
</tr>
<tr>
<td>Valine</td>
<td>11.0</td>
<td>10.8</td>
<td>11.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.3</td>
<td>4.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>16.7</td>
<td>16.6</td>
<td>17.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.8</td>
<td>8.4</td>
<td>9.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.2</td>
<td>7.3</td>
<td>7.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.7</td>
<td>2.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Expressed in number of residues per monomer.

### Results

The method developed depends on quantitative reactions at each of the successive steps given in Table I. (a) It was found that succinylation modified between 90 and 93% of the lysyl residues, according to the trinitrobenzenesulfonyl acid assay (10). (Succinylation also served to solubilize the H chains for further modification (9).) (b) That the reduction of the S-S bridges of the succinylated proteins was nearly complete and that (c) the subsequent reaction of the liberated —SH groups with ethylenediamine was exhaustive were simultaneously demonstrated by direct measurement of the number of AE-Cys residues formed. If all of the cysteiny1 residues, including the carboxyl-terminal cysteine (5) were reacted, the expected number of AE-Cys residues per mole of k chain is 5.0. The value of 4.4 found (Table II) is in good agreement with this theoretical limit. (d) The modification of the arginyl residues was accomplished by treatment of the succinylated, aminoethylated protein with cyclohexanedianeine, according to the procedure of Toi et al. (13). Although the arginyl derivative formed (Table I) is stable to acid hydrolysis, it emerges from the 15-cm short column of the amino acid analyzer in the same position as AE-Cys. Therefore, it was not possible to measure the degree of modification directly. However, the complete disappearance of arginine from hydrolysates of cyclohexanedianeine-treated protein indicated that the reaction was close to 100% complete. Amino acid analysis (Table II) indicates that, with the possible...
exception of serine, no other significant alteration in the amino acid composition of the protein has occurred upon chemical modification. The low value of serine obtained may reflect a more rapid destruction of this residue in the fully blocked protein compared with the starting $\kappa$ chain.

Analysis of a $\gamma{2a}$ myeloma H chain gave nearly identical results for the degree of succinylation and modification of arginy1 residues. The number of AE-Cys residues per mole of H chain was not determined.

**Action of Trypsin on Blocked Chains**

The effectiveness of the blocking procedures could be tested directly. If the action of trypsin on blocked L chain was limited to the hydrolysis of peptide bonds involving AE-Cys, then one would expect to find, after tryptic digestion, new amino-terminal residues corresponding to those amino acids that were originally linked to the carboxyl group of AE-Cys. An examination of available sequences of human $\kappa$ type Bence-Jones proteins revealed that the predominant amino acids carboxyl-terminal to cysteinyl residues are glutamine (or glutamic acid) and leucine. To identify the new amino-terminal residues released, a 6-hour tryptic digest of blocked Bence-Jones protein was subjected to a one-step Edman degradation. As expected, only PTH-Gln and PTH-Leu (or Ile) were found. A one-step Edman degradation was also carried out on a tryptic digest of blocked whole human L chains. In addition to PTH-Gln and PTH-Leu (or Ile), only PTH-Asn and PTH-Ala were found in significant amounts. It is of interest that the recently reported amino acid sequences of several human $\lambda$ type Bence-Jones proteins reveal the presence of Ala and Asn among the amino acids carboxyl-terminal to cysteinyl residues at position 88 in the chains. In the constant half of $\lambda$ chains, the amino acids carboxyl-linked to cysteine are the same as those in the $\kappa$ chains. Since whole normal human L chains contain both $\lambda$ and $\kappa$ types, these results support the notion that tryptic hydrolysis occurs specifically at AE-Cys residues. In another experiment, the liberated —SH groups of succinylated fully reduced human L chains were alkylated with iodoacetamide instead of ethyleylenimine and then subjected to reaction with cyclohexanedicione. Treatment of this blocked protein with trypsin did not result in any significant number of new amino-terminal residues, as judged by the Edman procedure.

**Gel Filtration Chromatography of Blocked Tryptic Peptides**

In order to separate the blocked peptides of L chain, it was decided to take advantage of their size differences (see Fig. 1) and chromatograph the peptide mixture on Sephadex. Although the maximum molecular weight of the expected peptides was only about 6500, it was found that chromatography on Sephadex G-100 was necessary before any separation occurred. This is probably due to a considerable increase in the radii of gyration of the succinylated peptides compared with unmodified peptides (31). Succinylated proteins also display large increases in $R_f$ values upon Sephadex chromatography (9, 32). At first it was thought desirable to perform the chromatography in 8 M urea in order to minimize any noncovalent interactions among peptides. Later it was found that, in most cases, chromatography in a low ionic strength medium, such as 0.01 M ammonium bicarbonate, was also effective.

When the $3H$-phenylisothiocyanate-treated blocked tryptic peptides of $\kappa$ type Bence-Jones protein were chromatographed on Sephadex G-100 in 8 M urea, six peaks of radioactivity were found (Fig. 2a). The profile was interpreted in the following manner. Peaks A, B, and C were associated with the regions designated A, B, and C in Fig. 1. Since complete tryptic digestion should have released three or at most four classes of peptides (Fig. 1), very probably peaks E and D (and possibly C') represent incomplete digestion products (see "Discussion").

Before attempting to characterize the material under the various peaks, it seemed desirable to subject blocked tryptic peptides from mouse and human normal L chains to the same chromatographic procedure. When this was done (Fig. 2b, c), it was found that both human and mouse L chains gave chromatographic profiles similar to the Bence-Jones protein. The only significant difference was that the area represented by Peaks C and C' in the Bence-Jones proteins had fused into one peak for the human chains. (The possible significance of

![Fig. 2. Chromatography of blocked tryptic peptides of L chains on Sephadex G-100 in 8 M urea, pH 8.0: a, a human $\kappa$ type Bence-Jones protein; b, normal human L chains; c, normal mouse L chains. The column dimensions were 146 X 2.5 cm. The flow rate was 7 g of eluate per hour; 5-g fractions were collected. Before chromatography of the tryptic digest (from 10 to 25 mg of L chain), the free $\alpha$-amino groups and the free amino groups of the aminoethylcysteine residues were reacted with 3H-phenylisothiocyanate. Aliquots (0.1 or 0.2 ml) of each fraction were counted in ethanol-toluene scintillation fluid. The arrows refer to the front (determined with blocked 3H-phenylisothiocynate-treated whole L chain) and back (determined with 3H-phenylisothiocyanate) of the column. The letters above the peaks a correspond to the letters in Fig. 1 (also, see text).]
this result is discussed below.) This result is entirely consistent with the idea that a single disulfide bridge pattern has been conserved during the evolution of L chains despite extensive sequence variations elsewhere in the molecules (see "Discussion").

**Gel Filtration of Blocked Peptides from Partially Reduced, $^{14}$C Alkylated, Bence Jones Protein**

If our interpretation of the Sephadex chromatographic pattern is correct, then the peptide containing the carboxyl-terminal cysteine residue of $\kappa$ chains should be contained under Peak A. To test this conclusion, this cysteine residue of the Bence-Jones protein was reacted with $^{14}$C-iodoacetamide, as indicated under "Methods." The alkylated protein was then blocked in the usual fashion and subjected to tryptic digestion. The blocked tryptic peptides were then chromatographed on Sephadex G-100 in 0.01 M ammonium bicarbonate.

Although the peptide profile in this case was obtained by measurement of absorbance at 215 $\mu$m (and therefore not strictly comparable to the profile obtained by measurement of triitated phenylthiocarbamyl groups), the three peaks of interest (A, B, and C) emerge from the column at similar positions in both chromatographic systems. As can be seen in Fig. 3, the position of the major radioactive peak, containing more than 65% of the counts applied to the column, coincided with the peak of optical density which corresponds to Fraction A. The only other peaks that contain appreciable amounts of radioactivity correspond to fractions which are thought to represent partial digestion products.

**Characterization of Fraction B**

The only chromatographic peak that should contain a single peptide species corresponds to B in Fig. 2a. This blocked peptide (see Fig. 1) represents a sequence of some 45 amino acids and contains the so-called "switch" point (1). Thereafter, in order to define further the Sephadex chromatographic pattern, it was decided to characterize Peak B. After chromatography on Sephadex G-100 in 0.01 M ammonium bicarbonate, the fractions corresponding to Peak B were pooled and the peptide was obtained as a lyophilized powder. The peptide material was then subjected to rechromatography on Sephadex G-100 under conditions identical with the first chromatographic run (Fig. 4). The fractions between the vertical lines were pooled for isolation of purified Fraction B.

A portion of purified Fraction B was subjected to amino acid analysis. Although the detailed amino acid sequence of the Bence-Jones protein that we have used has not been determined, it was nevertheless felt that amino acid analysis might be useful in identifying Fraction B because presumably more than half the sequence of this peptide (i.e., residues 109 to 134) is common to all $\kappa$ type Bence-Jones proteins. The results are given in Table III, along with the calculated composition of the corresponding fragment in Bence-Jones protein Ag (7). Special features of the amino acid composition strongly suggest that the purified peptide represents the stretch of sequence between residues 89 and 134. First, the amount of tryptophan present in a 0.3-mg sample of peptide was below the limits of detection (approximately 5 nmoles of tryptophan) and was calculated to correspond to less than 0.3 residue per residue of isoleucine. Tryptophan does not occur in Fragment B in any human $\kappa$ type Bence-Jones protein so far studied (1, 7, 28, 29).

Special features of the amino acid composition strongly suggest that the purified peptide represents the stretch of sequence between residues 89 and 134. First, the amount of tryptophan present in a 0.3-mg sample of peptide was below the limits of detection (approximately 5 nmoles of tryptophan) and was calculated to correspond to less than 0.3 residue per residue of isoleucine. Tryptophan does not occur in Fragment B in any human $\kappa$ type Bence-Jones protein so far studied (1, 7, 28, 29).

On the other hand, when Peak C was rechromatographed the tryptophan content of the purified fraction was found to be 1.8 moles/25,000 g of peptide. This value is approximately equal to the tryptophan content of the intact protein. Peak A was also analyzed and found to contain no detectable tryptophan.

Tryptophan has not been found to occur in human Bence-Jones proteins in regions corresponding to the A peptides in Fig. 1.

---

**Fig. 3.** Chromatography of blocked tryptic peptides of a human $\kappa$ type Bence-Jones protein on Sephadex G-100 in 0.1 M ammonium bicarbonate. Before blocking the Bence-Jones protein, it was partially reduced and treated with $^{14}$C-iodoacetamide (see text). The tryptic digest (1.5 ml) was applied to the column in 0.1 M ammonium bicarbonate. The column dimensions were 145 X 2.5 cm; the flow rate was 11.2 ml per hour. Thirty-minute (5.6 ml) fractions were collected. Aliquots of each fraction (0.5 ml) were counted in ethanol-toluene scintillation fluid. The arrows refer to the front (determined with blocked L chain) and back (determined with 0.1 M ammonium bicarbonate) of the column.

**Fig. 4.** Rechromatography of tryptic Fraction B on Sephadex G-100 in 0.01 M NH$_4$HCO$_3$. Conditions for chromatography were the same as for Fig. 3. The amount of crude Fraction B applied to the column corresponded to approximately 85 mg of starting blocked Bence-Jones protein. The fractions between the vertical lines were pooled for isolation of purified Fraction B.

---

4 All of the peaks when rechromatographed, in either 8 M urea or 0.01 M ammonium bicarbonate, emerged at elution volumes identical with those of the initial chromatographic run.

7 Unfortunately, the presence of cyclohexanedione-arginine prevented an estimation of both the AE-Cys and histidine content of this fraction.
Methionine was also found to be absent from purified Fraction B, a result which is in accord with available sequence information. Fraction B was also characterized by a low tyrosine content (compared with, for example, Fraction C). In only one Bence-Jones protein so far studied (5) has there been more than 1 tyrosine residue found in blocked Peptide B.

In order to characterize Fraction B further, an amount of purified peptide corresponding to approximately 2 μmoles of starting κ chain was subjected to the Edman degradation with the three-cycle modification. The only phenylthiohydantoin amino acid detected at the amino-terminal position was glutamine which gave a characteristic striking yellow color in Solvent E. No trace of phenylthiohydantoin leucine, phenylthiohydantoin isoleucine, or any other phenylthiohydantoin amino acid was observed. Glutamine occurs at position 89 in all human κ Bence-Jones proteins so far examined. Leucine (or isoleucine), which was found as a product of the Edman degradation of the unfractionated tryptic digest of blocked Bence-Jones protein, always occurs at position 135. Phenylthiohydantoin leucine was detected, along with phenylthiohydantoin glutamine, when rechromatographed Peak C was analyzed for NH$_2$-terminal amino acids.

**Gel Filtration Chromatography of Blocked Tryptic Peptides of Mouse H Chains**

The H chains of a mouse myeloma and the whole mouse normal IgG were subjected to the same block fragmentation procedure described for L chains. The results are shown in Fig. 5. Although the fragments have not been characterized in any detail, it is clear that the gel filtration pattern obtained with the homogeneous myeloma H chain and the heterogeneous mixture of normal H chains are very similar. Furthermore, there is a strong resemblance of these H chain chromatographic profiles to those of L chains, in that blocked peptides of similar size classes are produced. A quantitative difference between the H and L chain profiles appears in the ratio of amount of fragments of about 20 to 25 amino acids to those of about 45 amino acids. The ratio is apparently smaller for H chains than for L chains.

**TABLE III**

<table>
<thead>
<tr>
<th>Amino acid composition of rechromatographed Fraction B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Threonine*</td>
</tr>
<tr>
<td>Serine*</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Half-cystine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Tryptophan*</td>
</tr>
<tr>
<td>Σ residues</td>
</tr>
</tbody>
</table>

* Values were uncorrected for decomposition.

* Determined by the method of Spies and Chambers (20).

* Data were calculated from Titani, Whitley, and Putnam (28).

**DISCUSSION**

Specificity of Peptide Cleavage—In the previous section, it was shown that each of the chemical modification steps occurred specifically and exhaustively as designed (Table I). Furthermore, the evidence is good that tryptic cleavage occurred predominantly, if not exclusively, at only the introduced aminoethyllysineyl bonds. Only the expected new amino-terminal residues were released upon trypsinization and, if the aminoethylolysisolation step was replaced by alkylation with iodoacetamide, no new amino-terminal groups were detected after trypsinization. The strong similarity between Bence-Jones protein and normal L chains in gel filtration patterns also argues against extensive nonspecific cleavage in normal L chains. Nevertheless, the possibility may have to be considered that in special cases some "nonspecific" tryptic bond cleavages could occur. It is known that trypsin, even when it is treated with L-[(1-p-toluenesulfonylamido-2-phenyl)ethyl chloromethyl ketone, possesses an inherent chymotryptic-like activity (33). Such activity occasionally produces nonspecific tryptic cleavages. In a study of the action of L-[(1-p-toluenesulfonylamido-2-phenyl)ethyl chloromethyl ketone-trypsin on S-aminoethylated ribonuclease, it was found that at least four non-trypsin-sensitive bonds were cleaved with yields of 7 to 33% (34). The conditions reported for the tryptic digestion of ribonuclease were similar to the ones used in this study.

**Blocked Peptide Patterns of a K Type Bence-Jones Protein—Chromatography of 3H-phenylsulfonylcyanoate-treated blocked tryptic peptides of Bence-Jones protein on Sephadex G-100 in 8 M urea consistently gave six peaks of radioactivity (Fig. 2a). An analysis of the known structures of Bence-Jones proteins led to the prediction that three, or at most four, size classes of peptides should be produced by the action of trypsin, each class...**
differing from next larger class by approximately 15 to 20 amino acid residues (See Fig. 1). Therefore, in order to interpret the observed chromatographic patterns, it was necessary to establish the identity of some of the peptide peaks.

After partial reduction and alkylation of Bence-Jones protein with \(^{35} \text{C}-\text{iodosacetamide, it was found that blocked Peptide A contained the bulk of the radioactivity. This result confirms the prediction that the last peptide to be eluted from the Sephadex column consists of the carboxy-terminal region (residues 194 to 214) of Bence-Jones protein. Peak A in Fig. 2 presumably also contains the 23-residue blocked peptide from the amino-terminal region of the \( \kappa \) chain. Peak B (Fig. 2) presumably contains the switch region as judged by the recovery of only phenylthiohydantoin glutamine after \( \text{NH}_2 \)-terminal analysis, and the absence of tryptophan, and an amino acid composition which is consistent with this assignment (Table III). Peaks C and C' possess glutamine (or glutamic acid) and leucine (or isoleucine) as amino-terminal residues and contained a relatively high percentage of tryptophan. Although not fully characterized, they probably contain the peptides of 60 to 65 amino acids (Fig. 1) which may differ enough in either size or shape in a particular Bence-Jones protein (6) to produce the slight separation observed (see below). Peaks D and E probably represent partially digested fragments of still larger sizes.

Plapp, Raftery, and Cole (34), in their study of the tryptic digestion of S-aminoethylated ribonuclease, found that arginyl and lysyl bonds were cleaved to an estimated 83% while the corresponding cleavage of aminoethylstearyl bonds averaged 56%. Furthermore, the extent of cleavage at AE-Cys bonds appeared to show greater variability than at arginyl and lysyl bonds. Preliminary studies on the extent of blocked L chain digestion, with the use of trinitrobenzenesulfonic acid to measure new end groups released, are consistent with the ribonuclease results. They showed that approximately 50% of digestion had occurred. (On the other hand, almost complete release of the carboxy-terminal A peptide occurred, according to the results in Fig. 3.) Partial digestion of a molecule of blocked L chain would necessarily generate peptide fragments larger than 60 to 65 residues in length; such peptides would be expected to emerge prior to blocked peptides C or C' after Sephadex chromatography.

The distribution of \(^{3} \text{H} \) counts due to labeling with \(^{3} \text{H}-\text{phenylisothiocyanate is of interest in an analysis of the peptide patterns. From the number of reactive amino groups in each blocked peptide (all have two, one amino-terminal and one from AE-Cys, except for NH\(_2\)-terminal Peptide A which has only one from AE-Cys) and the theoretical number of peptides present in each peak, one may calculate that the areas under Peaks A, B, and C in Fig. 2a should be in the ratio of 3:2:4, assuming that each AE-Cys bond is hydrolyzed to the same extent by trypsin and that the free amino groups are equivalent in their reactivity toward \(^{3} \text{H}-\text{phenylisothiocyanate. The ratios of areas A-B-C was found to be 3:2:4:2, values in good agreement with the predictions made. In general, the shape of the peptide profile has been reproducible and quite characteristic for a given type of chain preparation.}

As a precaution against possible aggregation of the blocked peptides all of our initial analytical work was performed with 8 \( \times \) urea as the solvent for development of the Sephadex columns. After gaining some experience with the urea chromatographic system, it was decided to test dilute aqueous buffer solutions (\( \text{e.g. } \text{0.01 M ammonium bicarbonate} \)) for their ability to separate blocked peptides. The results (Fig. 3) show that the desired separation can be achieved, at least in the case of a Bence-Jones protein. However, recent experiments8 with affinity-labeled mouse antibody L chains indicate that dilute aqueous buffer is not always effective in separating blocked peptides. Therefore, it appears advisable to use the gel filtration pattern obtained in 8 \( \times \) urea (or in some other dissociating solvent) as a guide to the effectiveness of any other elution media.

### Blocked Peptide Patterns and the Evolution of Immunoglobulin Chains

At the outset of this work, the amount of amino acid sequence data for Bence-Jones proteins was small. On the basis of that data, however, the prediction was made that the intra-chain disulfide bridges might be a feature of immunoglobulin chains that was conserved during evolution. During the time that this work was in progress, additional sequence data for human and mouse Bence-Jones proteins have amply demonstrated that this hypothesis is correct (5-7). The similarity between the blocked peptide patterns of Bence-Jones and whole normal L chains (Fig. 2, a, b, and c) is entirely consistent with this view. The only significant difference between the profiles was a merging of Peaks C and C' in the normal L chain peptide patterns. This probably reflects a size heterogeneity of "variable" Fragment C (Fig. 1) resulting from dilutions and insertions that are known to occur in this region (6).

Furthermore, it has been proposed (2, 3, 35) that both L and H chains arose from a common ancestral gene through gene duplications and mutations. This ancestral gene is assumed to have coded for a polypeptide chain of about 100 amino acids containing one intrachain disulfide bridge (3, 35, 36). In its most simple form, this hypothesis predicts that an H chain consists of two L-like fragments attached head to tail and contains four intrachain disulfide bridges, whereas an L chain contains two. Amino acid sequence data for H chains are still sparse, particularly for the variable regions of H chains. However, the similarity of the peptide profiles from a mouse myeloma H chain and whole normal mouse H chain (Fig. 5) again suggests that the disulfide bridge pattern found for myeloma H chains is conserved throughout most of the whole normal H chain population. Furthermore, the remarkable similarity between H and L chain blocked peptide profiles supports the notion that H chains originated as a result of a gene duplication of an ancestral L chain gene. Although the incompleteness of the tryptic digestion8 precludes quantitative analysis, a comparison of the observed profiles of H and L blocked peptides indicates qualitative agreement with this picture; not only are fragments of similar sizes released, but the ratio of the areas under Peak B to Peak A is consistently greater in the case of the H chain as compared to the L chain as would be expected from the model. We may expect that future sequence studies will directly show any further structural homologies between L and H chains, including the positions of the intramolecular disulfide bridges within the H chain.9

---

* L. I. Slobin and S. J. Singer, unpublished data.
* The extent of tryptic digestion of the H chain appears to be comparable to that of L chain (approximately 50%), as judged by the amount of peptide material eluted prior to fragments of about 69 residues (see Fig. 5).
* As this paper was being submitted, we learned of studies of Pink and Milstein (37) on the amino acid sequences surrounding...
Recently, we have examined the blocked peptide profiles of normal rabbit L chains and found them to be strikingly different from the ones reported here for human and mouse L chains (4). The marked decrease in Peak C and the increase in Peak B in the rabbit profile is consistent with analytical data of Crumpton and Wilkinson (38) and of Koshold, Engelberger, and Shapanks (39) which indicate that there is an additional intrachain disulfide bond in rabbit L chains as compared to human or mouse L chains. Examination of the blocked peptide profiles of L or H chains from more primitive species may contribute further to an understanding of the structural evolution of immunoglobulins.

Use of Specific Cleavage Procedure—The separation of immunoglobulin chains into defined peptide fragments may prove useful in comparative sequence studies of immunoglobulin chains and in the isolation of large labeled fragments from the chains of affinity-labeled antibodies (4). The fragmentation procedure could also be useful in the determination of regions in the chains which bear antigenic or allotypic markers. In addition, amino acid analysis of purified blocked peptides might help to localize within a polypeptide chain those amino acid differences which appear to be associated with antibodies of differing specificities (39). In order to carry such studies to their conclusion, further separation and isolation of the blocked tryptic fragments would have to be carried out. While the Fragment B of L chains appears to be relatively free of contaminants after one further chromatographic step, Peaks A and C must each contain peptide fragments of at least two types, one from the variable and one from the constant region of the molecule. It should be possible to resolve these fragments by ion exchange chromatography, although no attempt has been made to do this in the present study. For more detailed studies with the blocked tryptic fragments from H chains, it would probably be desirable first to prepare the Fe and Fd fragments of the H chains and then subject these to the block fragmentation procedure.

A useful modification of the present procedure might be to substitute maleic anhydride (40) for succinic anhydride as the blocking agent for the e-ammonium groups of the lysyl residues. Whereas the succinamide bonds are as stable as ordinary peptide bonds, the maleamide bonds can be hydrolyzed under mild conditions to recover the unblocked lysyl residues (40). This would allow further tryptic cleavages to be made after isolation of the blocked tryptic fragments.

Among all of the amino acids found in proteins, the half-cystine residue appears to be the least subject to evolutionary change (41). Among some recent examples of the conservation of half-cystine residues in evolutionarily related proteins are the observed homologies in disulfide bridge structure of trypsin, chymotrypsin and elastase (42), and renin and pepstein (43). The importance of these structures and their infrequent occurrence in proteins would make them serve as ideal anchor points in the preparation of large and characteristic peptide fragments. In this study we have developed a method for the specific cleavage of proteins at cysteinyl bonds, which should permit the preparation of characteristic peptide fragments from immunoglobulin L and H chains. Although the procedure involves the formation of derivatives of 3 different residues, all of the derivatives are chemically stable and the overall procedure involves no more than a few hours of working time. It is hoped, therefore, that the method will prove generally useful to protein structural studies, particularly those that involve heterogeneous families of proteins or proteins that are thought to be related through evolution.

Acknowledgment—The authors wish to thank Dr. R. F. Doolittle for his advice and for his generous help with the Edman procedure and amino acid analyses.

REFERENCES

33. SINGER, S. J., and DOOLITTLE, R. F., in N. DAVIDSON and
1786 XpeciJ;c Cleavage of Polypeptides at Cysteinyl Residues Vol. 243, No. 8

A. Rich (Editors), Structural chemistry and molecular biology, Freeman, San Francisco, in press.

The Specific Cleavage of Immunoglobulin Polypeptide Chains at Cysteinyl Residues
Lawrence I. Slobin and S. J. Singer


Access the most updated version of this article at http://www.jbc.org/content/243/8/1777

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/243/8/1777.full.html#ref-list-1