Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 89-95

CHARACTERIZATION OF A VARIABLE FRAGMENT OF A HUMAN IMMUNOGLOBULIN λ CHAIN OBTAINED BY PEPTIC CLEAVAGE IN UREA

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Received January 7, 1983

SUMMARY: The digestion of a human dimeric λ Bence Jones protein with pepsin in 8 M urea without prior reduction and alkylation produced a fragment of molecular weight 11,000, designated as upV_λ, corresponding to almost the entire variable region of an immunoglobulin light chain. The C-terminal amino acid residue of fragment upV_λ was shown to correspond to leucine at position 104 of the immunoglobulin λ light chain. Fragment upV_λ was isolated in the yield of 30 % by ion exchange chromatography after gel filtration. When another λ Bence Jones protein was treated in the same way, similar results were obtained.

Bence Jones proteins are known to be identical to light polypeptide chains of immunoglobulins. Immunoglobulin light chains consist of a N-terminal half with a valiable amino acid sequence and a C-terminal half with a practically constant amino acid sequence. Each half is independently folded into a globular domain which is connected to the other by a short stretch of polypeptide, known as the switch region. Solomon et al. (1) and Karlsson et al. (2) reported that the cleavage sites of various enzymatic digestion of light chains were within the switch region. Parr et al. (3) pointed out that the products of peptic digestion of intact IgG in 8 M urea solution were entirely different from the peptic digestion products of the same IgG in the solution without urea.

We applied peptic digestion in 8 M urea solution to a dimeric λ Bence Jones protein and the main fragment was isolated and characterized.

MATERIALS AND METHODS

Bence Jones proteins: Two dimeric λ Bence Jones proteins (Fuj and Os) used as substrates were isolated from the urine of two multiple myeloma patients. These proteins were purified by 60 % ammonium sulfate precipitation followed by DEAE-cellulose ion exchange chromatography and Sephacryl S-200 gel filtration chromatography, until only one band was shown on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and disc polyacrylamide-gel electrophoresis.
Urea-pepsin digestion: Urea-pepsin digestion was carried out essentially according to the method of Parr et al. (3). The purified dimer of Bence Jones

0006-291X/83/040089-07$01.50/0

protein was concentrated to approximately 20 mg/ml by ultrafiltration using Sartorius collodion bags and then dialysed against three changes of 0.1 M sodium acetate, pH 4.3, containing 8 M urea. The pH was adjusted to 3.6 with HCl. Pepsin (from hog stomach mucosa, 2x crystallized, Sigma Chemical Co., USA) was added to give an enzyme/substrate ratio of 1:100 (w/w), and the digestion was carried out at room temperature (23° C) with continuous stirring. The digestion was stopped by the addition of 20 µl of 2 M Tris/HCl, pH 9.0, followed by 1 N NaOH to bring the pH to 9.0. The time required for preparative digestion of the dimeric λ Bence Jones protein (Fuj) was determined by a time course study on small amounts of the protein.

Separation of the digestion products: After preparative digestion, the digestion products were separated by gel filtration on a column (2.6 cm × 97 cm) of Sephadex G-50 equilibrated with 0.01 M sodium acetate, pH 5.1, containing 6 M urea. The Sephadex G-50 gel filtration had been initially performed with 0.1 M sodium acetate buffer, pH 5.0, containing 6 M urea at room temperature, but the main eluate had been precipitated while keeping it at 4° C. The solubility of the precipitate could not be recovered by heating, but it was easily redissolved either by lowering the sodium acetate concentration to 0.01 M or by dialysis against 0.01 M sodium acetate buffer, pH 5.1, containing 6 M urea. Accordingly, 0.01 M sodium acetate buffer, pH 5.1, containing 6 M urea was used for the separation of the digestion products. Further separation of one of the peaks obtained by gel filtration was achieved by ion exchange chromatography on a column (1.4 cm × 41 cm) of DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals Inc., Sweden) with a gradient consisting of 500 ml of 0.01 M sodium acetate buffer, pH 5.1, containing 6 M urea, and 500 ml of the same buffer containing 0.3 M NaCl. All column chromatography was performed at room temperature. Fractions were pooled and dialysed extensively against distilled water using cellulose tubular membrane (Spectra Por 3, molecular weight cutoff: 3,500), and lyophilized.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis: Sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis was carried out in the gel column containing 1 % SDS, 6 M urea, 4 % polyacrylamide and 0.14 % bisacrylamide according to a modification of the method of Shapiro et al. (4). Mobility of each peptide or protein was calculated relative to Malachite green as described by Weber and Osborn (5).

Disc polyacrylamide-gel electrophoresis: Disc polyacrylamide-gel electrophoresis was performed by a modification of the method of Ornstein (6) and Davis (7).

Amino acid analysis: Protein samples were analysed for their amino acid content on a Jeol model JLC-6AH amino acid analyser. Approximately 0.5 mg of each sample was hydrolysed in 1 ml of 6 N HCl for 24, 48, and 72 hr at 110° C under vacuum. The hydrolysates were dried and redissolved in 1 ml of 0.2 M sodium citrate, pH 2.20, and analysed with the standard hydrolysate system. Corrections for the variable yield of serine and proline at different hydrolysis time were made by extrapolation to 0 hr and the values for the other amino acids were the average values of three analyses.

C-terminal amino acid sequence analysis: C-terminal amino acid residues were determined essentially as described by Ambler (8). Approximately 100 nmol of peptide was dissolved in 1 ml of 0.2 M N-ethylmorphorine acetate, pH 8.5. Phenylmethylsulfonylfluoride (PMS)-treated carboxypeptidase A solution (Millipore Corp., USA) was added to give an enzyme/substrate ratio of 1:100 (mol/mol), and the mixture was incubated at 25° C. At 1/16, 1/8, 1/4, 1/2, 1, 2, 4, 8, 16, and 24 hr intervals, 9 nmol portions of the mixture were removed from the supernatant after centrifugation. Digestion was stopped by adding sufficient acetic acid to each portion to lower the pH to 2.5. The samples were dried and redissolved in 500 µl of 0.02 N HCl, and analysed on a Hitachi model 835 amino acid analyser with a lithium citrate buffer system.

N-terminal amino acid sequence analysis: N-terminal amino acid residues were determined by the manual Edman degradation technique (9). Prior carboxymethylation was carried out according to the method of Gurd (10). N-terminal amino acid residues were identified by amino acid analysis after back hydrolysis of the thiazolinone derivatives. Threonine was recovered as α-aminobutyric acid.

RESULTS

Urea-pepsin digestion of dimeric Bence Jones protein (Fuj): SDS polyacrylamide-gel electrophoresis patterns in a time course study of urea-pepsin digestion of the dimeric Bence Jones protein (Fuj) showed that it was cleaved into three bands, namely band *a*, *b*, and *c* (Figure 1). At 30 min band *a* was only slight, but band *b* and *c* were clear. After 40 min band *b* became more diffuse, and after 120 min band *b* disappeared. Therefore, a reaction time of 30 min was used for preparative urea-pepsin digestion of approximately 30 mg of the purified dimer (Fuj). We applied urea-pepsin digestion for 30 min to another dimeric λ Bence Jones protein (Os) and obtained a very similar SDS polyacrylamide-gel electrophoresis pattern of the digestion products.

Separation of the digestion products: The gel filtration profile of the digestion products is illustrated in Figure 2. SDS polyacrylamide-gel electrophoresis showed that the main peak obtained by gel filtration consisted of several components, and the major component corresponded to band *b*. This peak was separated by ion exchange chromatography as illustrated in Figure 3. The digestion products corresponding to band *b* were separated into peak A and B.

Amino acid compositions: Disc polyacrylamide-gel electrophoresis of peak A and that of peak B demonstrated almost the same migration distance, but the results of amino acid analyses suggested that the digestion product A was a further degradation product of product B as shown in Table 1. When the amino acid composition of product B was subtracted from that of Bence Jones protein (Fuj), the amino acid composition of that part of Bence Jones protein (Fuj) which was not present in product B was obtained. The amino acid composition of this part was identical to those of the constant region of λ Bence Jones proteins (New, X, Vil, Sh, Bo, Kern, Newm, Mcg, and Hil) whose sequences had already been known (11). These results led us to conclude that product B corresponds to the variable region of Bence Jones protein (Fuj), and product B was designated as fragment upV_{λ}. It was observed in SDS polyacrylamide-gel

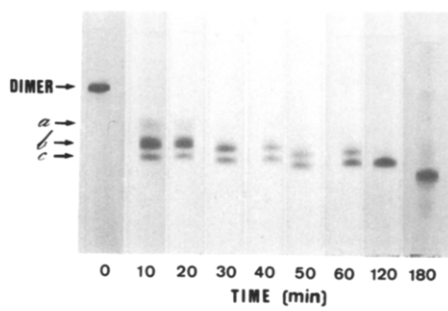

Figure 1. SDS polyacrylamide-gel electrophoresis patterns in a time course study of urea-pepsin digestion of dimeric λ Bence Jones protein (Fuj). It is shown that the dimer was cleaved into three bands, namely *a*, *b*, and *c*.

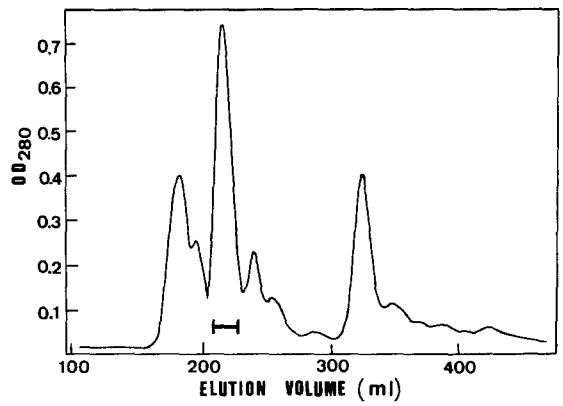

Figure 2. Sephadex G-50 separation of the 30 min urea-pepsin digestion products of approximately 30 mg of the purified dimeric λ Bence Jones protein (Fuj). The column (2.6 cm × 97 cm) was equilibrated with 0.01 M sodium acetate, pH 5.1, containing 6 M urea. The flow rate was 7.2 ml/hr, and 3.8 ml fractions were collected. The main peak indicated by the solid bar consisted mainly of the fragments corresponding to band *b*.

electrophoresis that fragment upV_λ was not cleaved into smaller fragments after reduction of the intrachain disulfide bonds with 2-mercaptoethanol. It means that there is no small peptide lost within the intrachain disulfide loop of fragment upV_λ.

Sequence studies: The C-terminal amino acid sequence of fragment upV_λ was determined to be -Thr-Lys-Leu by the time course of the release of free amino acids from fragment upV_λ by digestion with PMS-carboxypeptidase A (Figure 4). The sequence of the λ light chains (Vil, Sh, Bo, Kern, Newm, and Hill) for residues at positions 102-104 is known to be -Thr-Lys-Leu, and no

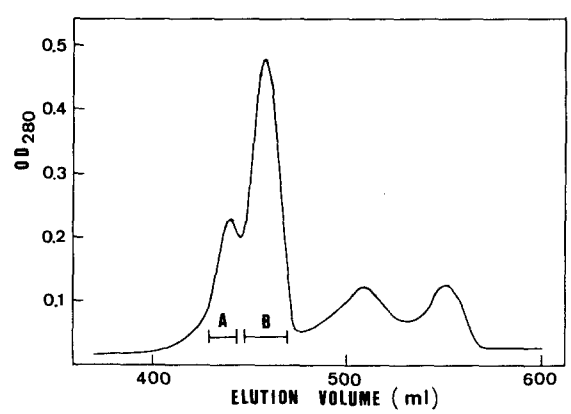

Figure 3. DEAE-Sepharose CL-6B ion exchange separation of the main peak indicated in Figure 2. The column (1.4 cm × 41 cm) was equilibrated with 0.01 M sodium acetate, pH 5.1, containing 6 M urea and eluted with a gradient consisting of 500 ml of the starting buffer and 500 ml of the same buffer containing 0.3 M NaCl. 2.5 ml fractions were collected at the flow rate of 8.8 ml/hr. The peaks A and B indicated by the solid bars were pooled. Both products A and B corresponded to band *b*. Product B was designated as fragment upV_λ.

Table 1. Amino acid compositions of Bence Jones protein (Fuj), its urea-pepsin digestion product A and product B.

amino acid	BJ (Fuj)*	product A**	product B**	product B minus product A	BJ (Fuj) minus product B	C_λ*** at positions 108-215
Asx	14.5	8.7	9.7	1.0	4.8	5
Thr	21.1	8.8	10.2	1.4	10.9	11
Ser	30.4	13.0	14.8	1.8	15.6	16
Glx	25.2	10.4	12.0	1.6	13.2	12
Pro	13.3	5.2	5.6	0.4	7.7	9
Gly	14.1	10.4	12.4	2.0	1.7	3
Ala	17.7	3.6	4.7	1.1	13.0	11
Cys	4.5	1.7	1.7	0.0	2.8	3
Val	16.2	5.5	5.9	0.4	10.3	9
Met	1.0	1.0	1.0	0.0	0.0	-
Ile	4.8	3.8	4.1	0.3	0.7	1
Leu	13.7	5.0	5.3	0.3	8.4	6
Tyr	8.5	4.7	4.7	0.0	3.8	4
Phe	4.9	2.9	3.3	0.4	1.6	2
His	4.1	-	1.4	1.4	2.7	2
Lys	14.8	0.8	4.3	3.5	10.5	8
Arg	3.2	1.9	2.1	0.2	1.1	1
total	212.0	87.4	103.2	15.8	108.8	103

* Calculation of the number of residues of Bence Jones protein (Fuj) was based on 212 residues per polypeptide chain.

** Amino acid compositions of product A and product B were calculated on the basis of the presence of one methionyl residue per molecule of Bence Jones protein (Fuj).

*** Amino acid composition of the constant region of λ chains (New, X, Vil, Sh, and Kern), at position 108-215.

other comparable sequence is not known in the switch region and the constant region. The C-terminal sequence of fragment upV_λ was therefore concluded to correspond to -Thr-Lys-Leu at positions 102-104. The numbering system for immunoglobulin light chains used in this report was according to Kabat et al. (11).

The N-terminal sequence of Bence Jones protein (Fuj) was found to be Lys-Glx-Leu-Thr-Glx- beginning at position 2 by the manual Edman degradation

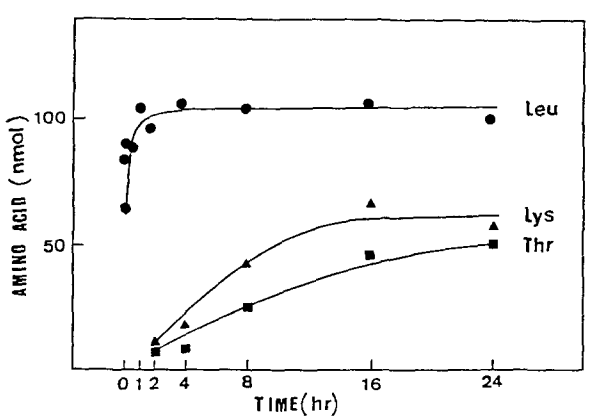

Figure 4. The time course of the release of free amino acids from approximately 100 nmol of fragment upV_λ by digestion with PMS-carboxypeptidase A. Only leucine was released by the digestion time of 1 hr. After 8 hr digestion, 104 nmol of leucine, 46 nmol of lysine and 26 nmol of threonine were released.

technique. On the other hand, several attempts of N-terminal determination of fragment upV_λ not only by the manual Edman degradation technique but also by the dansyl chloride method were unsuccessful. Then the aqueous phase of an ethyl acetate/water mixture at the first cycle of the Edman degradation of fragment upV_λ was subjected to amino acid composition analysis, resulting in practically the same amino acid composition as that of fragment upV_λ. These findings led us to conclude that the N-terminal residue of fragment upV_λ was blocked. The exact position of the N-terminus of fragment upV_λ in the λ chain numbering system could not be determined, but it must be situated at the position near the N-terminus of the intact Bence Jones protein (Fuj), because fragment upV_λ, whose sequence ends at position 104, consists of approximately 100 residues in the estimation of its molecular weight and amino acid composition analysis. The N-terminal residue of fragment upV_λ might be glutamine, for the glutaminyl peptides are quite labile and easily converted into pyroglutamyl peptide when they are present in the digest (12). As our sequence data concerning Bence Jones protein (Fuj) was obtained from amino acid analysis after back hydrolysis of the thiazolinone derivative, the difference between

DISCUSSION

Fragment upV_λ was characterized by the amino acid composition and the terminal sequence as a fragment which begins with a blocked terminus at the position near the N-terminal residue of intact Bence Jones protein (Fuj), glutamic acid and glutamine, either of which is situated at position 3 or 6, is indistinguishable. Many of the λ chain sequence data (11) suggest that the residue at position 3 is glutamic acid, and the residue at position 6 is glutamine. Therefore, it is possible that the N-terminus of fragment upV_λ is at position 6.

Molecular weight of fragment upV_λ: The mobility of fragment upV_λ on SDS polyacrylamide-gel electrophoresis was 0.57. The mobilities of Bence Jones (Fuj) tetramer, dimer and monomer used as standard molecular weight markers were 0.22, 0.34, and 0.42 respectively. The molecular weight was estimated to be 11,000 by linear extrapolation of the plot of the logarithms of the molecular weights against the electrophoretic mobilities.

Yield of fragment upV_λ: The extinction coefficient ($E^{1\,\%}_{1\,cm}$) of fragment upV_λ was calculated to be 10.9 by measuring the molar concentration of fragment upV_λ solution by amino acid analysis whose absorption at 280 nm had previously determined in a 1 cm cell. By using this extinction coefficient at 280 nm the recovery of fragment upV_λ from the digestion mixture after gel filtration and ion exchange chromatography was found to be 30 %.

possibly at position 6, and ends in the switch region at position 104, as shown in Figure 5. Karlsson et al. (13) reported that the peptic digestion in the solution without urea of a dimeric λ Bence Jones protein (A.J.) after

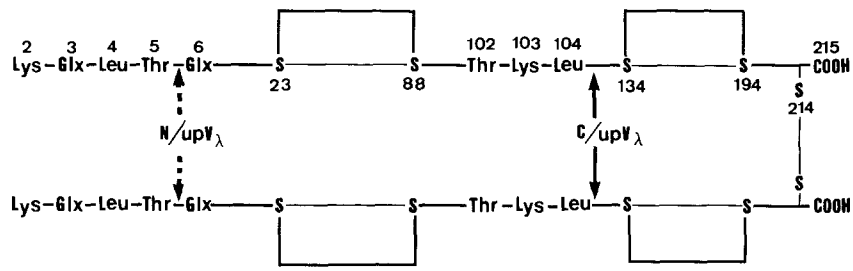

Figure 5. Schematic representation of the terminal sites of fragment upV$_\lambda$ in a disulfide-linked dimeric Bence Jones protein (Fuj). The continuous arrows indicate the C-terminus (C/upV$_\lambda$). As the N-terminus of fragment upV$_\lambda$ was found to be blocked, a possible N-terminus (N/upV$_\lambda$) is indicated by the broken arrows.

prior reduction and alkylation produced a constant fragment, pepC$_\lambda$, begining with threonine at position 105, but no variable fragment could be detected. Comparison of the C-terminal residue of upV$_\lambda$ (Fuj) with the N-terminal residue of pepC$_\lambda$ (A.J.) indicates that the cleavage between leucine at position 104 and threonine at position 105 in the λ chain is caused by peptic digestion in 8 M urea solution as well as in the absence of urea, independently of prior reduction and alkylation. It may be advantageous in preparation of variable fragments by urea-pepsin digestion of λ Bence Jones proteins that many Bence Jones proteins, which sometimes have solubility problems, can be dissolved in 8 M urea solution, and the denaturation caused by urea is reversible (14).

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