Differences in the Enzymatic Digestibility of the Variable and Constant Halves of Bence-Jones Protein with Temperature*

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SUMMARY

A study of the limited peptic and tryptic fragmentation of intact type K Bence-Jones proteins into the variable and the constant halves showed a strong difference in the subsequent digestibility of the variable half and the constant half at different temperatures. The variable half was much more resistant to enzymatic digestion than the constant half at 37°, whereas the situation was reversed at 55°. This suggests that there is a larger contribution of hydrophobic bonds to the tertiary structure of the constant half than to the tertiary structure of the variable half and perhaps a greater contribution of salt linkages to the tertiary structure of the variable half.

The temperature differential permits the easy preparation and isolation of the fragments of the variable and the constant halves since only the variable-half fragment and the remaining intact Bence-Jones protein are found in the digest after digestion at 37° and only the constant-half fragment is found after digestion at 55°.

Limited enzymatic and chemical digestions of immunoglobulins and their heavy chains have been extensively studied and have greatly contributed to the understanding of the chemical structure and biological function of immunoglobulin molecules (1, 2).

The results of limited digestion of immunoglobulins and their component chains have provided evidence important in developing the domain hypothesis of immunoglobulin evolution and structure (3, 4).

Recently Solomon and McLaughlin (5) and Karlsson et al. (6) have reported that limited enzymatic digestion of Bence-Jones proteins and light chains yields \( V_L \) (variable portion) and \( C_L \) (constant portion) fragments.

We have undertaken a detailed study of the limited digestion of intact \( \kappa \) Bence-Jones proteins. Various chemical properties are known for one of the two Bence-Jones proteins studied (7-10). During an extensive examination of the various digestion conditions, we have found differences in the enzymatic digestibility of the \( V_L \) and \( C_L \) fragments with temperature, since only the \( V_L \) fragment and the remaining intact Bence-Jones protein are found in the digest of the intact Bence-Jones protein after digestion at 37°, and only the \( C_L \) fragment after digestion at 55°.

The present finding appears to be of interest in view of the evidence that the variable and constant portions of the immunoglobulin light chain molecule are each synthesized by different genes (3, 11, 12) and they are considered to each have a different function (13, 14). The present finding can be used for the easy preparation and isolation of \( V_L \) and \( C_L \) fragments.

In this connection, Plaut and Tomasi (15) reported that trypsin cleavage of human IgM at temperatures exceeding 50° results in the production of excellent yields of an intact \( Fe \) fragment, whereas no detectable \( Fe \) fragment is produced by trypsin cleavage at 37°.

MATERIALS AND METHODS

Materials—A type K Bence-Jones protein, Col,† was purified on a carboxymethylcellulose column and subsequently on a Sephadex G-100 column as described previously (7). Eighty-five per cent of this protein exists as a disulfide-linked dimer (8).

Another type K Bence-Jones protein, Buc, was isolated as an ammonium sulfate precipitate.

Pepsin was purchased from Worthington Biochemical Corp., Freehold, N. J. L-(1-Tosylamido-2-phenyl)ethyl chloroformyl ketone-treated trypsin was from Calbiochem and soybean trypsin inhibitor from Sigma. Goat antikeratin sera were prepared against a \( \kappa \) light chain from a human myeloma IgG (Eri) and against a \( \kappa \) type Bence-Jones protein, Col.

NH₂-Terminal Sequence of Protein—The amino acid sequence of the NH₂-terminal portion of Protein Buc was determined by the manual 3-stage Edman degradation as applied previously to Protein Col (7).

Limited Enzymatic Digestion. The conditions for limited enzymatic digestion with pepsin and trypsin were extensively examined by varying the enzyme to substrate ratio (1:100 to 1:1000 (w/w)), the digestion time (20 to 180 min), and temperature (37-55°). The concentration of Bence-Jones protein was 0.5 or 1.0%.

Pepsin digestion was carried out in 0.025 M or 0.1 M sodium acetate adjusted to pH 4.5 with acetic acid and the digestion stopped by raising the pH to 7.5 to 8.0 by the addition of 1 M Tris-HCl buffer (pH 8.0) and 0.1 N or 1 N NaOH.

† Col, Buc, and Eri designate myeloma proteins obtained from patients at Roswell Park Memorial Institute.
Trypsin digestion was carried out in 1% \( \text{NH}_4\text{HCO}_3 \) (pH 8) and stopped by the addition of soybean trypsin inhibitor in an amount equal to the trypsin on a weight basis.

Immunoelectrophoresis—The progress of the digestion was followed by immunoelectrophoresis (pH 8.6) using homologous (anti-Col) and heterologous (anti-Eri) goat anti-\( \kappa \) sera for the digests of Protein Col, or heterologous (anti-Col and anti-Eri) goat anti-\( \kappa \) sera for the digests of Protein But. In every immunoelectrophoresis slide, control intact protein was also run. The amount of control protein applied was one-half to one-seventh of the amount of total proteins of the digest applied in the other well. The apparatus used was LKB type 3290B.

RESULTS

Peptic Digestion of Protein Col—Peptic digestion was carried out at several temperatures, i.e. 37°, 45°, 50°, and 55°. The digests were examined by immunoelectrophoresis using homologous anti-\( \kappa \) serum (anti-Col). The results obtained at 37° for 20 or 90 min at an enzyme to substrate ratio of 1:200 or 1:1000 (w/w) and a substrate concentration of 1% are shown in Fig. 1. A at a pepsin to substrate ratio of 1:200, a VL fragment arc was found in addition to the remaining intact Protein Col arc but no CL arc was apparent. With digestion at a lower pepsin to substrate ratio (1:1000), the arcs due to VL fragment and intact Protein Col were present and, in addition, a weak arc due to C\( _L \) fragment was observed. These results indicate that at 37° the C\( _L \) fragment is more susceptible to peptic digestion than is the VL fragment. The positions of the precipitin arcs of C\( _L \) and VL fragments relative to that of intact Protein Col shown in Fig. 1 are consistent with those reported by Solomon and McLaughlin (5) for digests of other \( \kappa \) type Bence-Jones proteins. With heterologous anti-\( \kappa \) serum (anti-Eri) no VL were present, but CL and intact protein arcs did appear (Fig. 1).

The results obtained with peptic digestion at 45°, 50°, and 55° are shown in Fig. 2. The digests at 45° and 50° show both CL and VL fragment arcs in addition to the remaining intact Bence-Jones protein arc, but the digests at 55° show only CL fragment arc. Thus, in contrast to the results at 37° (Fig. 1) where VL fragments were more resistant to peptic digestion than was CL fragment, CL fragment is more resistant at 55° (Fig. 2). At intermediate temperatures both VL and CL fragments are obtained.

The great difference in the digestibility of the VL and CL fragments at 55° is illustrated in Fig. 3 by a time course experiment of peptic digestion at 55°. The digests for 10 and 20 min both show a very weak VL fragment arc and a strong CL fragment arc in addition to the arc of the remaining intact Protein Col. The digests for 30 and 45 min show only a CL fragment arc accompanied by a weak intact Protein Col arc, and after 60, 90, and 120 min the digests show only a CL fragment arc.

Tryptic Digestion of Protein Col—The results with trypsin digestion were consistent with the results with peptic digestion and are shown in Fig. 4. The digests at 37° showed strong precipitin lines due to intact protein and VL fragment and a much weaker line due to CL fragment. The digest at 55° showed only CL fragment.

NH\(_2\)-Terminal Amino Acid Sequence of Protein But—To determine the subgroup of Protein But, the 3-stage Edman degradation procedure (7, 17) was directly applied to Protein But. The only sequence found was Glu-Val-Val-Leu-. This showed that Protein But is of Subgroup II as is Protein Col (7, 18). However, since Bence-Jones proteins are apt to precipitate under mild heating at acidic pH (16), control experiments for heat precipitation of Protein Col was carried out under the same conditions as those in peptic digestion, except that pepsin was absent. Protein Col did not show any appreciable amount of turbidity or precipitation up to 55° and for 2 hours. In the case of many digests precipitation appeared but they dissolved almost completely on neutralization to stop the digestion as described under “Materials and Methods.” When the precipitate and the supernatant were investigated separately by immunoelectrophoresis using a heterologous anti-\( \kappa \) serum, most of the constant-half fragment was found in the supernatant.
Fro. 3 (left). Effect of time on peptic digestion of Protein Col at 55°C. Digestion and immunoelectrophoresis were carried out as described in Fig. 2.

Fro. 4 (upper right). Tryptic digests of Protein Col (Vα12). The digestion was carried out at 37°C and 55°C for 90 min at an enzyme to substrate ratio of 1:1000 (w/w). Each digest was examined with both homologous and heterologous anti-α sera as in Fig. 1; only the results with homologous anti-α serum (anti-Col) are shown here. Intact Protein Col was used as a control as in Fig. 1.

ever, valine at position 2 has not been reported in any κ Bence-Jones protein of Subgroup II and has been reported for only one κ Bence-Jones protein of Subgroup I (12, 19, 20). The rest of this NH₂-terminal sequence, however, clearly indicates that Protein Buc is of Subgroup II (12, 20).

Peptic Digestion of Protein Buc—Peptic digestions at 37°C, 45°C, 50°C, and 55°C and control experiments for heat precipitation were carried out as described for Protein Col. Since Protein Buc was insoluble in 0.025 M sodium acetate (pH 4.5), all of the experiments for peptic digestions and control experiments of Protein Buc were done in 0.10 M sodium acetate at the same pH. Protein Buc was much less soluble than Protein Col and showed precipitation even at 45°C after 1-hour incubation. Accordingly, many of the digests at 50°C and 55°C showed precipitates, but most of the precipitate dissolved on neutralization. No homologous anti-Buc serum was available. Therefore, the digests were examined using the heterologous anti-κ sera, anti-Col, and anti-Eri, and both antisera showed the identical results. In the lower well of each plate, intact Protein Buc was applied as a control. Intact Protein Buc, which was an ammonium sulfate precipitate and not purified further, showed a weak, extra precipitin line in the extreme anodic position, although it was found to have only a single NH₂-terminal sequence.

The immunoelectrophoretic patterns after 90-min digestion at several temperatures at 0.5% Protein Buc are shown in Fig. 5.3 The results of this experiment parallel those for the peptic digestions.
digestion of Protein Col, i.e., Protein Col is cleaved at all temperatures and \( C_L \) is digested at lower temperatures (37\(^\circ\)), but is stable at higher temperatures (55\(^\circ\)), whereas \( V_L \) is digested at the higher temperature but is stable at the lower temperature.

In the 37\(^\circ\) digest, a substantially decreased amount of intact Protein Buc, but no \( C_L \) fragment was found and the digests at 45\(^\circ\) and 50\(^\circ\) showed neither intact protein nor \( C_L \) fragment. The digest at 55\(^\circ\) showed a strong \( C_L \) fragment and only a weak intact protein arc. By increasing the amount of enzyme from \( \frac{1}{10} \) to \( \frac{1}{10} \), intact protein could be completely eliminated and only the \( C_L \) fragment was found.

The absence of \( C_L \) fragment in the digests of 45\(^\circ\) and 50\(^\circ\) is different from the results of Protein Col where \( C_L \) fragment was observed (Fig. 2). Digestion of Protein Buc for only 30 min instead of 90 min still showed no \( C_L \) fragment and left only a weak precipitin line of intact protein, while no precipitin arc could be detected at all after the 30-min digestion if the amount of enzyme was increased from \( \frac{1}{10} \) to \( \frac{1}{10} \). The above difference might be explained if Protein Buc does not contain any significant amount of disulfide-linked dimer. The disulfide bond between monomers exists at the COOH terminus (in K type Bence-Jones proteins) (21) and, therefore, some difference between the conformation of \( C_L \) fragments from monomers and from disulfide-linked dimers probably exists.

**DISCUSSION**

Our results in the present work may be considered from two different aspects. One is concerned with the preparation of \( V_L \) and \( C_L \) fragments, and the other one is concerned with the structural basis for the difference in digestibility of the \( V_L \) and \( C_L \) fragments at different temperatures. Our results have shown that it is possible to isolate \( V_L \) by digestion at 37\(^\circ\) and \( C_L \) by digestion at 55\(^\circ\). The digestions were carried out without any prior reduction. Karlsson et al. (6) reported that disulfide-linked dimers of Bence-Jones protein are relatively resistant to proteolytic digestion so that in all of their experiments partial reduction and alkylation was done prior to limited digestion. Solomon and McLachlin (5) also used prior reduction and alkylation for some Bence-Jones proteins which they stated to be resistant to limited digestion. In the latter case the properties of these proteins were not specified.

We preferred to avoid prior chemical treatment such as reduction and alkylation, since we are interested in determining the reactivity to iodination of residues in the fragments, and even selective partial reduction of immunoglobulin usually causes partial cleavage of the intrachain disulfide bonds (22, 23) and carboxymethylation can occur on histidine, lysine, and methionine in addition to cysteine (24, 25). Furthermore, it is possible that even mild reduction and alkylation of Bence-Jones protein induces minor conformational change. One of the proteins, Col, has been well characterized. It consists of disulfide-linked dimer to the extent of 85% and 15% of noncovalent dimer in neutral aqueous solution (8). The reactivities toward iodination of all of the tyrosines and histidines of this protein have been determined (7, 9). This protein was shown to be of Subgroup II of \( \kappa \) type and of InV (3) allotype (7, 9). The NH2-terminal sequence study of the other \( \kappa \) type Bence-Jones protein, Protein Buc as reported here, showed that it is also of Subgroup II. However, the content of disulfide-linked dimer of Protein Buc is not known. For both Protein Col and Protein Buc, we could demonstrate the formation of fragments by careful enzymatic digestion without any prior treatment such as reduction and alkylation.

Higher temperature can influence enzymatic reactions in several ways such as by affecting the stability of the enzyme, the rate of conversion of the enzyme-substrate complex and the enzyme-substrate affinity (26). Also, the conformation of substrate protein might change with temperature and thereby change the exposure of susceptible bonds so that the enzymatic digestibility varies with temperature. Rupley and Scheraga (27) and Ooi et al. (28) have demonstrated that the chymotryptic and tryptic digestibilities of ribonuclease at various temperatures depend on the degree of the thermally induced unfolding of the ribonuclease molecule to expose bonds susceptible to these enzymes. The use of pepsin as in our work here would be a more sensitive indicator of change of conformation, since so many more types of peptide bonds are susceptible to pepsin.

The \( C_L \) fragment is more easily digested at 37\(^\circ\) than \( V_L \) fragment, but much less digested at 55\(^\circ\). This reflects differences in their tertiary structures. Our finding suggests a larger contribution of hydrophobic bonds to the tertiary structure of the \( C_L \) fragment than to the tertiary structure of \( V_L \) fragment, since hydrophobic bonds in general are more stable at high temperatures (29, 30).

**Note Added in Proof**—We have recently prepared a goat anti-Protein Buc antiserum. Using this antiserum, we have found that the peptic digestibility of the \( V_L \) fragment of Protein Buc at 37\(^\circ\), 45\(^\circ\), 50\(^\circ\), and 55\(^\circ\) is very similar to that of the \( V_L \) fragment of Protein Col at each corresponding temperature.

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