Carboxy-Terminal Structure of the α Chain of Human IgA Myeloma Proteins*

James W. Prahl,† Carlos A. Abel,‡ and Howard M. Grey‡,§

ABSTRACT: The carboxy-terminal octapeptides of several human IgA myeloma proteins have been isolated after cyanogen bromide cleavage of the α chains. Edman degradation has established the sequence as: (Met)-Ala-Glu-Val-Asp-Gly-Thr-Cys-Tyr. Identical sequences were observed in proteins of both the IgA_1 and IgA_2 subclasses. The ultimate tyrosine residue was found to be present in less than stoichiometric amounts, presumably the result of carboxypeptic activity *in vivo*. Cyanogen bromide cleavage studies on intact myeloma proteins indicate that the penultimate half-cystine, if involved in disulfide bonding of polymeric forms, must exist as asymmetric inter-heavy-chain bonds. A simpler interpretation of the data postulates the penultimate half-cystine is probably involved in an intra-heavy-chain bond.

The group of proteins known as immunoglobulins is characterized by the evolution of multiple classes differing in biological function and presumably primary structure. These classes appear to share a basic architecture, *i.e.*, two pairs of identical polypeptide chains, light and heavy, per monomeric subunit. As the light-chain components are associated with all the classes, the information which allows biological and antigenic differentiation among the classes must reside therefore in the structure of the heavy-chain components.

The IgA class of immunoglobulin is found both in the serum (Grabar *et al.*, 1956) and external secretions (Hanson, 1961; Tomasi *et al.*, 1965), as the predominant immunoglobulin in the latter case. The proteins of the IgA class have been shown to be heterogeneous by sedimentation analysis, the larger components being polymers of a 6.6S component (Vaerman *et al.*, 1965) that are stabilized by disulfide bonding. Immunochemical analysis of IgA myeloma proteins has allowed the differentiation of two subclasses, *i.e.*, IgA_1 and IgA_2 (Feinstein and Franklin, 1966; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966).

Cyanogen bromide cleavage has been of great assistance in structural investigations of human γ (Press *et al.*, 1966; Press and Hogg, 1970; Edelman *et al.*, 1969; Pink *et al.*, 1970) and μ chains (Wikler *et al.*, 1969). The present study reports the sequence of the carboxy-terminal octapeptide isolated from the cyanogen bromide cleavage products of α chains of human myeloma proteins of both IgA_1 and IgA_2 subclasses. In addition, cleavage of intact myeloma proteins has led to the conclusion that the penultimate half-cystine residue is probably not involved in disulfide bonding responsible for polymer stabilization.

Materials and Methods

Protein Isolation. Human IgA myeloma proteins were isolated by block electrophoresis of serum (Kunkel, 1954) followed by gel filtration on Sephadex G-200 in phosphate-buffered saline (pH 7.2). Polymeric and monomeric forms of the same protein were isolated separately.

Antigenic Analysis. IgA preparations were tested for purity by immunoelectrophoretic analysis using rabbit antisera to whole human serum. The isolated IgA proteins were typed as IgA_1 or IgA_2 by immunodiffusion analysis using rabbit antisera specific for the two subclasses.

Preparation of Heavy and Light Chains. IgA myeloma proteins were reduced with 0.02 M dithiothreitol (Calbiochem) and alkylated with a 10% molar excess of iodoacetic acid or iodoacetamide (K & K Laboratories, recrystallized). In some experiments iodoacetamide-*1*-^{14}C (New England Nuclear Corp.) was used to radiolabel sulfhydryl residues. Heavy and light chains were isolated by gel filtration on Sephadex G-100 equilibrated in 1 M acetic acid as described previously (Cohen, 1963). When employed, total reduction was carried out in the presence of 8 M urea using the same concentrations of reducing and alkylating agents.

For amino acid composition data, heavy and light chains were separated after reduction but without alkylation. The reduced heavy chains were oxidized with performic acid prior to acid hydrolysis (Hirs, 1967).

Cyanogen Bromide Cleavage and Separation of Fragments. The α chains were dissolved in 70% formic acid at a concentration of 10–15 mg/ml. Solid cyanogen bromide (Eastman Kodak) was added (10 mg/mg of protein) and the mixture agitated until the reagent was in solution. After standing for 24 hr at room temperature in the dark, the reaction mixture was diluted tenfold with distilled water and lyophilized. The lyophilized product was extracted twice with 2-ml aliquots of 0.05 M NH_4OH, and the pH was adjusted to 9–10 by the dropwise addition of concentrated NH_4OH. Any insoluble protein was removed by centrifugation and stored in the cold. The two supernatant extracts were pooled and fractionated on a Sephadex G-50 column (210 × 1.9 cm) equilibrated with 0.05 M NH_4OH. The eluate was monitored at 215 mμ and when applicable, by scintillation counting. Appropriate fractions were pooled and concentrated by lyophilization.

Other Methods. Amino acid analyses were performed as

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† Fellow of the Arthritis Foundation. Present address: Department of Biology, California Institute of Technology, Pasadena, Calif. 91109. To whom to address correspondence.

‡ Present address: National Jewish Hospital and Research Center, Denver, Colo. 80206.

§ Established Investigator, American Heart Association.

TABLE I: Amino Acid Composition of Oxidized Human α Chains.[a]

	Bro α1	Cla α2	Hay α2[c]	Pac α1[c]	Tri α1[c]	Human γ Chains[d]
Lys	19.2	19.1	17.1	19.8	17.7	25–29
His	8.1	9.3	12.6	9.7	10.9	5–11
Arg	18.6	18.9	17.6	17.3	17.1	12–17
Asp	37.1	35.5	41.0	34.6	27.3	34–39
Thr	43.5	48.7	42.3	48.4	52.4	33–40
Ser	51.5	45.0	51.0	56.0	51.1	40–47
Glu	48.5	45.5	45.6	46.3	42.7	36–41
Pro	43.5	44.3	44.5	41.2	41.9	29–42
Gly	36.5	39.7	32.6	35.7	37.1	26–32
Ala	32.5	33.8	32.1	30.7	30.1	16–20
Val	34.2	32.9	32.0	34.1	35.8	38–41
Met[b]	4.1	5.7	4.7	4.4	3.0	4–8
Ile	6.8	5.5	7.2	6.2	6.4	6–9
Leu	45.8	50.2	48.0	44.2	46.2	30–36
Tyr	14.0	8.1	13.7	12.1	15.1	13–20
Phe	16.3	18.4	17.1	12.1	15.1	12–15
Cysteic acid	15.7	13.9	15.4	17.3	16.3	

[a] Calculated as moles of amino acid per mole of heavy chain. The molecular weight of the polypeptide portion of the α chain was taken to be 52,000. This figure was calculated on the basis of molecular weight determinations and sugar composition. [b] Determined as methionine sulfone. [c] Average of two determinations. [d] Taken from Prahl (1967).

described previously (Prahl, 1967). Before analysis for homoserine the hydrolysate was dissolved in 1 ml of pyridine acetate buffer (pH 6.5) and boiled for 1 hr to convert the lactone into homoserine (Ambler, 1965). The solvents and methods of high-voltage paper electrophoresis have been described by Crumpton and Wilkinson (1965); mobilities are expressed relative to an aspartic acid marker. Subtractive Edman degradation of peptides was carried out according to Konigsberg and Hill (1962); the composition of the resultant peptide and percentage recovery reported. Hydrazinolysis was performed by the method of Bradbury (1958); the free amino acid was detected on the amino acid analyzer. Carboxypeptidase A (Worthington) digestion was done in 0.1 M NH_4HCO_3 at an enzyme concentration of 0.2 mg/μmole of peptide. Radiolabeled peptides were counted in the scintillation fluid described by Kinard (1957).

Results

The α chains isolated from the IgA myeloma proteins were representative of both subclasses. The amino acid composition of several of these chains is given in Table I. When compared to human γ chains there were some distinctive differences in the levels of lysine, threonine, alanine, valine, and leucine. Of particular interest to this study were the methionine values which varied from 3 to 5.7 and the values for cysteic acid which varied from 14 to 17, the two α2 chains having somewhat lower values than the three α1 chains.

After cyanogen bromide cleavage, the reaction mixture of

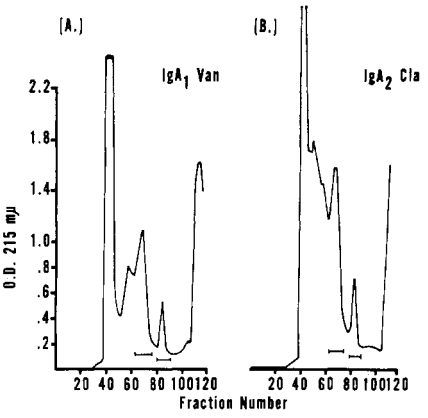

FIGURE 1: Gel filtration of cyanogen bromide reaction products of the α chains of Van (IgA_1) and Cla (IgA_2) myeloma proteins through a 210 × 1.9 cm column of Sephadex G-50 fine in 0.05 M NH_4OH. The fractions pooled are indicated by the bars.

the α chain was fractionated by gel filtration on a column of Sephadex G-50 (210 × 1.9 cm) in 0.05 M NH_4OH at room temperature. The eluate was monitored by its extinction at 215 mμ, and by radioactivity in those experiments where radionucleides had been introduced upon alkylation. The fractionation patterns obtained in the case of the Van and Cla fragments is shown in Figure 1a,b, respectively. In five of the seven α chains investigated a peptide was isolated, eluting from the column at approximately 70–77% of the total column volume. All were devoid of homoserine and released tyrosine and carboxymethylcysteine when digested with carboxypeptidase A, thus establishing them as the carboxy-terminal peptides of the α chain. The compositions of these are given in Table II. In addition, a second peptide was recovered in good yield eluting at 54–64% of the total column volume. This peptide was found to lack half-cystine or cysteine alkylation products and to contain glucosamine in addition to homoserine. No attempt was made at this time to further purify this peptide.

The recoveries of tyrosine in each carboxy-terminal peptide were less than stoichiometric (*i.e.*, less than 1 mole of tyrosine/

TABLE II: Amino Acid Compositions of the Carboxy-Terminal Octapeptide of Human α Chain.[a]

	Van α1	Tri α1	Hay α2	Cla α2	Hoa α2	Bro α1
Ala	1.03	0.96	1.02	1.00	0.97	1.10
Asp	0.96	1.00	0.99	1.15	1.03	1.05
CM-Cys	0.69	0.82	0.79	0.62	0.70	0.50
Glu	1.03	1.07	1.09	1.10	1.09	0.95
Gly	1.26	1.05	1.17	1.20	1.03	1.15
Thr	0.97	0.96	0.92	0.95	0.96	0.95
Tyr	0.56	0.66	0.67	0.35	0.37	0.65
Val	0.96	0.91	0.92	0.90	0.87	1.15
Yield (%)	82	75	93	89	91	72

[a] Compositions are reported as amino acid residues per mole of peptide.

TABLE III: Amino Acid Compositions of the Components Isolated from Hay Carboxy-Terminal Peptide upon Paper Electrophoresis at pH 6.5.[a]

	Hayter Carboxy-Terminal Peptide		
	Original	Band 1	Band 2
Ala	1.02	1.01	1.00
Asp	0.99	1.04	1.02
CM-Cys	0.79	0.73	0.85
Glu	1.09	1.25	1.10
Gly	1.17	1.29	1.14
Thr	0.92	0.88	0.95
Tyr	0.67	0.11	0.96
Val	0.92	0.87	0.95
Mobility relative to aspartic acid		−0.60	−0.52

[a] Compositions are reported as amino acid residues per mole of peptide.

TABLE IV: The Edman Degradation and Sequence of the Hay Carboxy-Terminal Peptide.[a]

	Orig	Ed 1	Ed 2	Ed 3	Ed 4	Ed 5	Ed 6
Ala	1.03	*0.15*	0.12	0.12	0.13	0.11	0.13
Asp	0.96	0.99	1.00	1.00	*0.36*	0.39	0.33
CM-Cys	0.69	0.80	0.93	0.63	0.88	0.86	0.85
Glu	1.03	1.03	*0.27*	0.25	0.28	0.30	0.25
Gly	1.26	1.02	1.12	1.11	1.10	*0.65*	0.54
Thr	0.97	0.88	0.89	0.92	0.98	0.96	*0.48*
Tyr	0.56	0.48	0.54	0.37	0.56	0.63	0.52
Val	0.96	0.88	0.82	*0.15*	0.21	0.20	0.20
Yield (%)		93	78	89	72	57	43

(Met)-Ala-Glu-Val-Asp-Gly-Thr-CM-Cys-Tyr

[a] The amino acid presumed to be removed at each step has its content printed in italic type. Compositions are reported as amino acid residues per mole of peptide.

mole of peptide) despite all efforts to protect tyrosine from destruction during hydrolysis. The reason for this discrepancy became clear when it was observed that the apparent octapeptide could be further resolved by paper electrophoresis at pH 6.5 into two components, a hepta- and octapeptide, which differed only in the absence and presence of the ultimate tyrosine residue, respectively. The mobilities and compositions of the components isolated from the Hay carboxy-terminal peptide are given in Table III, in which case the cysteine derivative was carboxyamidomethylcysteine. Staining for tryptophan was negative in all the peptides so examined.

The α chains of two myeloma proteins were encountered which did not release significant amounts of tyrosine or carboxymethylcysteine upon digestion with carboxypeptidase A-Bro and Dre, both IgA_1 proteins. The α chain of Bro IgA myeloma was isolated after mild reduction and alkylation with iodoacetamide-1-^{14}C. When the cyanogen bromide fragments of this polypeptide chain were fractionated on Sephadex G-50 in 0.05 M NH_4OH, no peptide material was found in the range of 70–80% of the total column volume. However, radioactivity was detected in the earlier carbohydrate-containing peptide fractions which was resolved by rechromatography on the Sephadex G-50 column. The composition of the radiolabeled peptide, given in Table II, and its electrophoretic behavior were characteristic of the homologous carboxy-terminal peptides isolated from the other α chains. The reason for its association with the carbohydrate-containing fragment is not understood at this time. Fractionation of the cyanogen bromide fragments of the α chain of the Dre myeloma also did not reveal a carboxy-terminal peptide in the expected position. Unfortunately, the possibility of anomalous association with the carbohydrate-containing fragment had not been appreciated at the time the protein was investigated, and insufficient material was available to repeat the experiment.

The carboxy-terminal peptides of several of the α chains were subjected to subtractive Edman degradation resulting in the sequence shown in Table IV. Also given in the same table is the data obtained on the peptide isolated from the Hay α chain. The electrophoretic mobility at pH 6.5 of the residual peptide decreased to −0.33 after the second Edman degradation and to 0.0 after the fourth, establishing the residues as glutamic and aspartic residues, respectively. These observations were in agreement with those predicted by the data of Offord (1966).

The nature of the disulfide bonding of the penultimate half-cystine of the α chain was investigated by subjecting the intact IgA myeloma protein to cleavage with cyanogen bromide, and examining the fragments formed by gel filtration. Under the acidic conditions of the cyanogen bromide cleavage no detectable disulfide interchange has been reported by other workers (Press *et al.*, 1966; Edelman *et al.*, 1969), and the fragments isolated have permitted some conclusions as to the disulfide arrangement in the original proteins. Since in this work subsequent fractionation of the fragments was obtained by gel filtration in 0.05 M NH_4OH at a pH of 9.5–10, although the fractionation was usually achieved in less than 24 hr at room temperature, the possibility of disulfide interchange had to be considered once again. The reproducibility of the results observed with several unrelated myeloma IgA proteins, and the absence of partial yield of any of the peptides sought argue strongly against the interchange reaction as a significant event in these studies.

The monomeric and polymeric forms of both Tri IgA_1, and Hoa IgA_2 proteins were isolated and examined separately. Each protein was dissolved in 70% (v/v) formic acid, cleaved by cyanogen bromide, and lyophilized as described earlier for the α chains. Immediately before fractionation, the appropriate reaction mixture was extracted twice with 2 ml of 0.05 M NH_4OH, adjusting the pH to 8.5–9.0 (phenol red indicator) by the judicious addition of concentrated NH_4OH. Any insoluble material was removed by centrifugation and stored at −20°. The supernatants were pooled and applied to the Sephadex G-50 column (210 × 1.9 cm) equilibrated in 0.05 M NH_4OH, again monitoring the extinction of the eluate at 215 mμ. The chromatographic pattern obtained upon fractionation of the cyanogen bromide fragments of the polymeric Tri IgA myeloma protein is shown in Figure 2A. Pools of the fractions were made as indicated; from 35 to 48, 50 to 62, and 64 to 96% of the total column volumes. No significant peptide material was found in the latter pool. The intermediate pool

contained the carbohydrate peptide. The presence of the carboxy-terminal peptide either by noncovalent association or by dimerization in this pool was ruled out by the absence of cysteic acid after performic acid oxidation; nor could any peptide be identified by paper electrophoresis at pH 6.5 of either the first or intermediate pools to account for the carboxy-terminal fragment. The same results were obtained upon fractionation of the cyanogen bromide cleavage products of Hoa polymeric and Tri and Hoa monomeric IgA myeloma proteins. Thus it appears unlikely that the penultimate half-cystine residue participates in any symmetrical inter-heavy-chain bond (see Discussion).

The carboxy-terminal peptide was isolated from the larger cyanogen bromide fragments after total reduction and alkylation. The first pool, *i.e.*, that comprising 35–48% of the total column volume, was lyophilized. Any insoluble protein remaining after the earlier extractions was added back to the appropriate pool before lyophilization. The product was dissolved in 2 ml of 8 M urea–1.0 M Tris-HCl (pH 8.2), reduced with 0.02 M dithiothreitol, and alkylated with iodoacetamide-*1*-^{14}C, and resubjected to gel filtration on the Sephadex G-50 column (210 × 1.9 cm) in 0.05 M NH_4OH; radioactivity and extinction at 215 mμ of the eluate were monitored. The elution pattern obtained with the larger cyanogen bromide fragments of the Tri polymer after total reduction and alkylation is shown in Figure 2B. This was characteristic of the results obtained with the homologous fractions of the Hoa polymer and Tri and Hoa monomers. The carboxy-terminal peptide, here radiolabeled, was again found at 71–81% of the total column volume. A control experiment, in which the reduction was omitted, did not release the carboxy-terminal peptide, thus establishing it as covalently linked by a disulfide bond to a larger cyanogen bromide fragment.

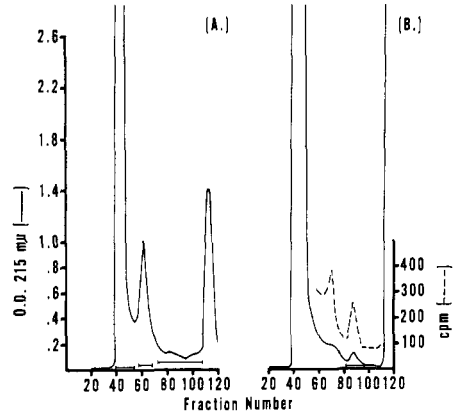

FIGURE 2: Gel filtration of the cyanogen bromide reaction product of (A) the monomeric form of intact Tri IgA, myeloma protein; and (B) the same after total reduction and alkylation with iodoacetamide-^{14}C. (———) OD_{215} and (· · · · · ·) radioactivity. The fractions pooled are indicated by the bars.

Discussion

The marked homology between the carboxy terminus of the μ chain recently reported by Wikler *et al.* (1969) and the α chain reported here is immediately obvious (Table V), as is the lack of homology with the γ chain. In the latter case, attempts to accentuate homology by aligning either the ultimate methionyl (no. 19 in the γ chain as opposed to 9 in the α or μ chains, numbering from carboxy terminus) or the terminal residues were not convincing. Indeed the homology of the carboxy termini of the α and κ or λ light chains was more impressive. Single base changes are sufficient to account for the amino acid replacements seen in the μ- to α-chain transition, with the exception of the threonine for valine substitution which requires a double base change (Morgan *et al.*, 1966). In these studies, which included both IgA_1 and IgA_2 myeloma proteins, no subclass related sequence variation in the carboxy-terminal octapeptide was seen.

The ability to further fractionate the apparent octapeptide into a heptapeptide lacking tyrosine and an octapeptide containing tyrosine resolved some difficulties surrounding the carboxy terminus of the μ and α chains. Doolittle *et al.* (1966) reported a sequence of Cys-Tyr or Tyr-Cys based upon the carboxypeptidase A digest of a μ chain. By utilizing a combination of kinetic studies of the carboxypeptidase digestion and hydrazinolysis, Abel and Grey (1967) established the sequence of the α chain as Thr-Cys-Tyr, although they too reported a disturbingly low recovery of tyrosine relative to that of carboxyamidomethylcysteine and threonine after prolonged digestions. It is now apparent that the α chains of the myeloma proteins, and presumably the μ chains by analogy, possess a variability in the ultimate residue. As the same variability in this ultimate tyrosine was observed in the carboxy-terminal octapeptide isolated from both the polymeric and monomeric forms of an individual myeloma, it appears unlikely that the residue plays a functional role in the polymerization process. A carboxypeptidase-like enzyme active *in vivo* either intra- or extracellularly could be responsible for such an observation, or more remotely it could be the result of a termination error at a transcriptional level in the neoplastic cell.

Based upon the known role of disulfides in stabilizing the IgM 19S structure (Miller and Metzger, 1965, 1966) and the homology in the carboxy-terminal structure of the light and μ chains, it has been postulated the penultimate half-cystine residue in the heavy chain might play a role in the disulfide binding of the monomeric subunits to form the polymer (Singer and Doolittle, 1966; Doolittle *et al.*, 1966). With the elucidation of the α-chain carboxy-terminal structure, this argument was extended to the IgA polymers (Doolittle *et al.*, 1966; Abel and Grey, 1967). The possible structural variations which might be envisioned involving the penultimate half-cystine residue are illustrated in Figure 3; these include inter-heavy symmetric intrasubunit (3a) and intersubunit (3b), inter-heavy asymmetric intrasubunit (3c) and intersubunit (3d), and intra-heavy (3e).

The inter-heavy symmetric models predict the formation of a hexadecapeptide formed by a symmetric dimer of the carboxy-terminal peptide upon cyanogen bromide cleavage of intact IgA myeloma. Our inability to isolate such a hexadecapeptide from either the monomeric or polymeric forms of either of the two proteins investigated mitigates against this structure. In addition, the absence of free carboxy-terminal peptide after cleavage also rules out a free cysteine residue or

TABLE V

κ	Val-Thr-Lys-Ser-Phe-Asn-Arg-Gly-Glu-Cys
λ	Val-Glu-Lys-Thr-Val-Ala-Pro-Thr-Glu-Cys-Ser
α	Met-Ala-Glu-Val-Asp-Gly-Thr-Cys-Tyr
μ	Met-Ser-Asx-Thr-Ala-Gly-Thr-Cys-Tyr
γ	Met-His-Glu-Ala-Leu-His-Asn-His-Tyr
	Thr-Glu-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly

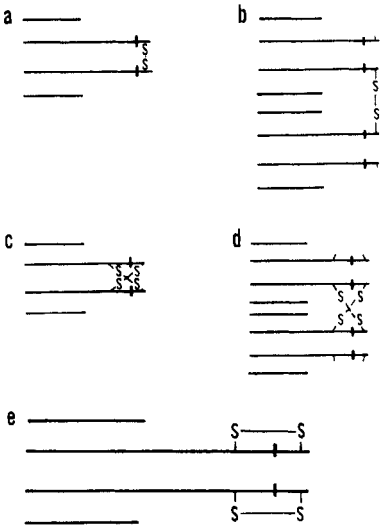

FIGURE 3: Hypothetical models showing the possible role of the penultimate half-cystine in disulfide bonding of monomeric and polymeric forms of IgA. Inter-heavy symmetric (a) intrasubunit and (b) intersubunit; inter-heavy asymmetric (c) intrasubunit and (d) intersubunit; and intra-heavy (e).

bonding to a smaller prosthetic group, such as glutathione. Rather, the isolation of the peptide only after total reduction and alkylation of the larger cyanogen bromide fragments is in agreement with an inter-heavy asymmetric or intra-heavy model, although it obviously does not allow a distinction between the two alternatives. In view of the identical observations in both the monomeric and polymeric forms, if the penultimate half-cystine residue is involved in a bond of the inter-heavy asymmetric intersubunit type in the polymer (Figure 3d), it must arise from disulfide exchange of the intrasubunit species in the monomer (Figure 3c). That the exchange is not random, as indicated by the absence of hexadecapeptide, predicates the additional restriction of stereospecificity on the model. A simpler interpretation consistent with the data presented here and with the disulfide bonding seen in the γ and light chains involves the penultimate half-cystine in an intra-heavy-chain bond which is labile to mild reduction, with polymer formation due to a different cystine residue.

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