Structure of Immunoglobulin A. II. Sequence around the Hinge Region and Labile Disulfide Bonds of an Immunoglobulin A2 Myeloma Protein†

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ABSTRACT: A peptide containing the inter-heavy-chain bridges ("hinge" region) of an IgA2, Am2(+) myeloma protein was isolated. It contained 3 cysteines and 8 prolines, 5 of which occurred consecutively. This peptide was similar to one obtained from an IgA1 myeloma protein although it had a gap of 12–13 residues and lacked carbohydrate. Diagonal electrophoresis showed that it is apparently bridged to two other peptides. Several other cysteine-containing peptides were isolated from the heavy chain, all but one of which showed a high degree of homology with those of IgA1.

Two subclasses of immunoglobulin A, known as IgA1 and IgA2, which differ in the structure of their heavy (α) chains, exist in man (Feinstein and Franklin, 1966; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966). Although IgA2 is present in much lower concentration than IgA1 in all body fluids, its relative concentration compared to IgA1 is greater in exocrine secretions than in serum of the same individual (Vaerman, 1970).

A genetic polymorphism was found serologically in the IgA2 subclass (Kunkel *et al.*, 1969; Vyas and Fudenberg, 1969). It was subsequently demonstrated that the heavy–light disulfide bridge characteristic of all immunoglobulins was present only in the minor allotype Am2(−), while it was absent in Am2(+) molecules (Jerry *et al.*, 1970). Amino acid sequence studies of the α-chain peptide involved in this bridge showed it to be identical in IgA1 and IgA2, Am2(−) molecules (Mihaesco *et al.*, 1971; Wolfenstein *et al.*, 1971).

In a previous study, we have characterized the peptides containing the labile disulfide bonds from an IgA1 myeloma protein (Wolfenstein *et al.*, 1971). In the present report we present similar data for an IgA2(+) myeloma protein. Special emphasis is given to the sequence of the section that includes the inter-heavy-chain disulfide bridge (hinge region) since it differs in the two types of α chains. A preliminary report of these studies has been presented (Frangione *et al.*, 1971b).

Materials and Methods

Protein Avi was a polymeric IgA2,κ,Am2(+) myeloma protein. It was isolated from serum by starch zone electrophoresis at pH 8.6 (Kunkel, 1954), followed by gel filtration on Sephadex G-200 in 0.3 M saline, and tested for purity by immunoelectrophoretic analysis using rabbit antisera to whole human serum and to γ, α, κ, and λ chains. The antisera specific for α1 and α2 determinants were the same as previously described (Wolfenstein *et al.*, 1971).

Partial Reduction and Radioactive Alkylation. This was done under the same conditions as described for immunoglobulin IgA1 (Wolfenstein *et al.*, 1971). Heavy, light, and J chains (Halpern and Koshland, 1970; Mestecky *et al.*, 1971) were separated by chromatography on Sephadex G-100 in 1 M acetic acid. Under these conditions, H chain eluted first and the J chain appeared just before the L chain (Mendez *et al.*, 1972). The pooled fractions were tested for purity by immunoelectrophoretic analysis and polyacrylamide gel electrophoresis in urea. The H chain was shown to be free of L or J chains.

Enzyme Digestion and Separation of Peptides. The labeled heavy chain (200 mg) was digested with L-(1-tosylamido-2 phenyl)ethyl chloromethyl ketone trypsin (Worthington) in 0.2 M ammonium bicarbonate (pH 8.3) for 15 hr at 37°, enzyme–substrate ratio 1:50 (w/w). The digest was freeze-dried and dissolved in 5 ml of 1 M acetic acid. The peptides were separated by chromatography on a column of Sephadex G-50 (3 × 130 cm) equilibrated in 1 M acetic acid at room temperature. Fractions of 5–6 ml were collected at a flow rate of 25 ml/hr. The eluates were monitored by measuring the radioactivity in 50-μl aliquots dissolved in a toluene-based counting solution containing 10% (v/v) Bio-Solv 3 (Beckman) using a Beckman LS-150 liquid scintillation counter. Each of the peaks was further digested with pepsin (Worthington, twice crystallized), enzyme–substrate ratio 1:50 (w/w) in 5% formic acid for 15 hr at 37°.

The whole protein (600 mg) was digested with L-(1-tosylamido-2 phenyl)ethyl chloromethyl ketone trypsin under the same conditions as the heavy chain. The digest was freeze-dried and further digested with pepsin (Worthington, twice crystallized), enzyme–substrate ratio 1:50 (w/w), in 5% formic acid for 15 hr at 37°. The freeze-dried products of digestion were fractionated on a column of Sephadex G-100 (3.5 × 180 cm) in 1 M acetic acid at room temperature. Fractions of 5 ml were collected at a flow rate of 25 ml/hr. The eluate was monitored at 280 mμ.

Cystine Diagonal "Maps." These were carried out at pH 3.5 by the method of Brown and Hartley (1966).

Purification of Radioactive Peptides, Amino Acid Analysis, and Determination of Amino Acid Sequences. This was done as previously described (Wolfenstein *et al.*, 1971). The results of the dansyl-Edman procedure are shown with arrows under the peptide. Mobilities at pH 6.5 are expressed as fractions of the distance between ϵ-DNP-lysine and aspartic acid (Offord, 1966).

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TABLE I: Amino Acid Composition of Peptic–TrypticCa rboxymethylcysteine Peptides Obtained after Partial Reduction and Alkylation of Protein Avi (IgA_2 Am2(+)).[a]

Peak:	I	II			III	
Peptide[b]	TPα1[c]	TPα2[e]	TPα5	TPα6	TPα3	TPα4
Lys						0.98
His	0.85	0.90				0.91
Arg		1.10				
CMCys	0.71	2.20	0.71	0.62	1.46	0.60
Asp	0.94			0.80	1.00	
Thr	1.00	0.70	1.82	1.05		1.57
Glu	2.14			1.11		1.06
Pro	1.85	8.30				1.00
Gly	2.14			1.00	1.22	
Ala	1.10			1.11		2.02
Val		2.50		0.95		
Leu			1.00		0.82	1.09
Mobilities at pH 6.5	0.2	0.2	0.45	0.85	1.0	0
N-Terminal[d] amino acid	Pro	Val	Thr	Ala	Asp	Thr

[a] Compositions are reported as moles of amino acid per mole of peptide. [b] Hydrolysis for 20 hr. No corrections were made for destruction during hydrolysis. [c] TP = trypsin–pepsin. [d] The presence of a single N-terminal residue excludes significant contamination of these peptides. [e] This composition is in agreement with that reported by Abel and Grey (1971).

Results

Figure 1 shows the separation of the tryptic peptides obtained from the heavy chain (α) on a Sephadex G-50 column. The column fractions were combined as shown in the figure and the three pools were further digested with pepsin. The amino acid compositions, mobilities, and N-terminal amino acids of the major radioactive peptides isolated from each of the peaks are shown in Table I. The peptides were characterized as follows. Peptide TPα2 has a high content of proline and cysteine and shows similarity to the hinge region of γA1. The dansyl-Edman procedure gave its N-terminal sequence, and digestion with carboxypeptidase B yielded Arg as the C-terminal residue. The partial amino acid sequence of this peptide is

Val-Thr-Val-Pro-Cys-Pro-Val-Pro-Pro-Pro-Pro-Pro-→→→→→→→→→→→→→

(Cys$_2$,Pro,His)Arg

The peptide was submitted to paper electrophoresis at pH 6.5 after each step up to the twelfth. The only significant

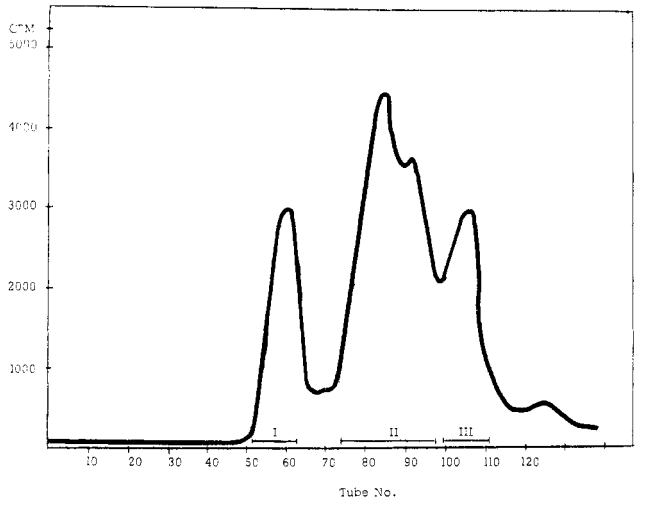

FIGURE 1: Gel filtration of a tryptic digest of partially reduced and ^{14}C-carboxymethylated heavy-chain protein (Avi, IgA2, Am2 (+)) on Sephadex G-50. Peptides from 200 mg of protein were dissolved in 5 ml of 1 M acetic acid and added to the column (3 × 130 cm) equilibrated in the same solvent. Fractions of 5–6 ml were collected at a flow rate of 25 ml/hr. Radioactivity was measured on 50-μl aliquots. The pooled fractions are indicated on the figure.

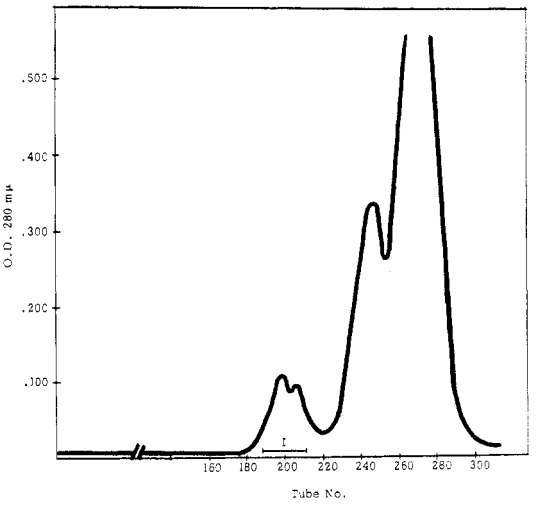

FIGURE 2: Gel filtration of a tryptic–peptic digest (600 mg) of protein Avi (IgA2, Am2(+)) on Sephadex G-100 (3.5 × 180 cm) in 1 M acetic acid. Fractions of 5 ml were collected at a flow rate of 25 ml/hr. The pooled fractions are indicated on the figure.

TABLE II: Amino Acid Analysis of Peptide TPα2 after Several Steps of the Edman Degradation.[a]

Amino Acid[b]	His	Arg	CMCys	Thr	Pro	Val
Initial	0.9 (1)	1.1 (1)	2.2 (3)	0.7 (1)	8.3 (8)	2.5 (3)
Edman eighth step	0.8 (1)	1.0 (1)	1.0 (2)	0 (0)	5.4 (5)	0.2 (0)
Edman tenth step	0.8 (1)	0.8 (1)	0.8 (2)	0.(0)	3.3 (3)	0.2 (0)
Edman twelfth step	0.7 (1)	0.8 (1)	1.0 (2)	0 (0)	1.8 (1)	0 (0)

[a] Residual peptides were hydrolyzed without further purification. The theoretical integral value is given in parentheses. [b] Hydrolysis for 20 hr. Compositions are reported as moles of amino acid per mole of peptide.

change of mobility was observed when the fifth amino acid was removed. The mobility changed from 0.2 to neutrality, and remained neutral from then on. Unlike the corresponding peptide from an IgA1 protein, this peptide does not contain carbohydrate, detectable in the amino acid analyzer. In order to elucidate its sequence, which is difficult due to the high content of proline, the residual peptide was hydrolyzed at certain steps of the Edman degradation, without further purification. As shown in Table II, the results of these analyses support the results of the Edman degradation except for the values of CMCys, which were low due to destruction during hydrolysis.

The hinge peptide was then identified in a digest of the whole molecule and isolated from a diagonal map. Figure 2 shows the elution pattern obtained by fractionation of a peptic–tryptic digest of the whole protein on a column of Sephadex G-100. The first peak, which contained the hinge peptide, was further digested with subtilisin (Sigma, Type VII, crystallized and lyophilized), enzyme–substrate ratio 1:20 (w/w) in 0.2 M ammonium bicarbonate (pH 8.3) for 15 hr at 37°. The cysteic acid peptides of this peak, identified on a diagonal map at pH 3.5, are shown in Figure 3. These peptides were isolated and purified by electrophoresis at pH 6.5 and 2.1, and their amino acid composition, N-terminal residue and electrophoretic mobility are shown in Table III. Peptide TPS2 had the same composition, mobility and N-terminal residue as peptide TPα2. The presence of cysteic acid rather than carboxymethylcysteine allowed a more accurate estimation of the content of this amino acid and confirmed the existence of three residues in this peptide. By this procedure, the peptide was obtained in good yield, and it was used to elucidate its sequence completely. As expected, after 11 steps of Edman degradation, the peptide was ninhydrin negative (proline N terminal). After the twelfth step, it became ninhydrin positive. The remaining pentapeptide (Cys_2, His,Pro,Arg) had a mobility at pH 6.5 of 0.15, which is in agreement with its slightly acidic character due to the partial ionization of His at this pH. The mobility changed to −0.15 after the next step, due to the loss of an acidic residue which left the slightly basic peptide (Cys,His,Pro,Arg). After the following step, there was another change in mobility to −0.6, showing the loss of another cysteic acid residue, and leaving the peptide (His,Pro,Arg). After the next step of the degradation, a peptide (Pro,Arg) with a mobility of −0.7 was obtained which was ninhydrin negative (proline N terminal) but could be detected by chlorine stain (Reindell and Hoppe, 1954). Finally after another step, only arginine remained.

TABLE III: Amino Acid Composition of Cysteic Acid Peptides Derived by Subtilisin Digestion from Peak I of a Peptic–Tryptic Digest of Protein Avi (IgA2).[a]

Peptide[b]	TPS 1[c]	TPS 1a	TPS 2	TPS 2a	TPS 3	TPS 3a
Lys	1.18	1.00				
His			0.85	0.72		
Arg			0.88	0.88		
Cya	1.04	1.00	2.95	3.02	1.89	1.00
Asp	1.00	0.96		1.10	0.96	0.96
Thr			0.90	0.97		
Ser					0.84	
Glu		1.07				
Pro	0.93	1.08	8.20	8.00		
Gly	1.11	1.06			1.11	1.11
Val			3.00	3.21		
Leu					1.00	0.85
Tyr					0.63	
Mobilities at pH 6.5	0.5	0.4–0.75	0.19	0.37	0.9	0.9
N-Terminal amino acid	Cya	Glu	Val	Asp	Asp	Asp

[a] Compositions are reported as moles of amino acid per mole of peptide. [b] Hydrolysis for 20 hr. [c] TPS = trypsin–pepsin–subtilisin.

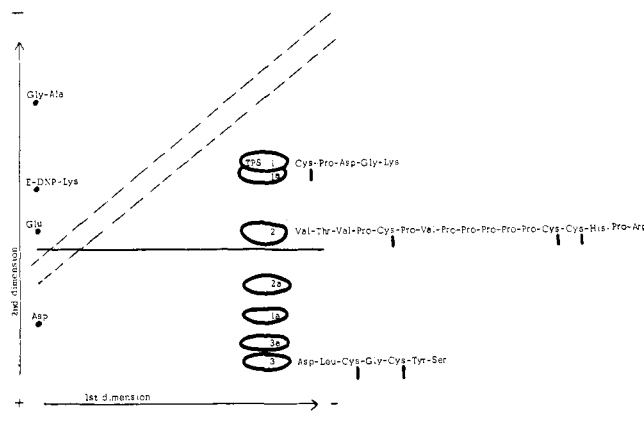

FIGURE 3: Diagram of the diagonal electrophoresis of a subtilisin digest of peak I (Figure 2) obtained after peptic–tryptic digestion of protein Avi (IgA2, Am2(+)). Electrophoresis in both dimensions was at pH 3.5. The sequence of the relevant peptides is shown; for the rest, see Table III. TPS = trypsin–pepsin–subtilisin.

TABLE IV: Comparison of the Peptic–Tryptic Carboxymethylcysteine Peptides Obtained after Partial Reduction and Alkylation of an IgA1[a] and IgA2, Am2(+) Myeloma Protein.

IgA1 (TPα1)	Ser-Leu-Cys-Ser-Thr-Glx-Pro-Asx-Gly-Asx
IgA2 (TPα1)	Pro(Asp,Cys,Thr,Glu_2,Pro,Gly_2,Ala,His)
	CHO
IgA1 (TPα2)	Val-Thr-Val-Pro-Cys-Pro-Val-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr(Ser_2,Thr_1,Pro_4,Cys_2,His_1)Arg
IgA2 (TPα2)	Val-Thr-Val-Pro-Cys-Pro-Val-Pro GAP Pro-Pro-Pro-Pro-Cys-Cys-His-Pro-Arg
IgA1 (TPα3)	Asp-Leu-Cys-Gly-Cys
IgA2 (TPα3)	Asp(Leu,Cys_2,Gly,Cys)
IgA1 (Tα4)	Thr-Cys-Thr-Ala-Ala-Tyr-Pro-Glu-Ser-Lys
IgA2 (TPα4)	Thr(Cys,Thr,Ala,Ala_2,His_1,Pro_1,Glu_1,Leu_1)Lys
IgA1 (TPα5)	Thr-Cys-Thr-Leu
IgA2 (TPα5)	Thr(Cys,Thr,Leu)
IgA1 (TPα6)	Ala-Glu-Val-Asp-Gly-Thr-Cys
IgA2 (TPα6)	Ala(Glu,Val,Asp,Gly,Thr,Cys)

[a] Wolfenstein *et al.* (1971). Broken-box areas indicate homology between IgA1 and IgA2, Am2(+).

Therefore, the complete sequence of this peptide was found to be

Val-Thr-Val-Pro-Cys-Pro-Val-Pro-Pro-Pro-Pro-Pro
1 2 3 4 5 6 7 8 9 10 11 12

Cys-Cys-His-Pro-Arg
13 14 15 16 17

Another peptide (TPS2a) was isolated in low yield, which had the same amino acid composition as TPS2, plus Asp. Since the N-terminal amino acid was Asp, and the next residue after Edman degradation was Val, it was concluded that it included TPS2. In addition to the hinge, two other sets of cysteic acid peptides were isolated from the same diagonal map and their sequence was established by the dansyl-Edman procedure as follows

TPS 1 Cys-Pro-Asp-Gly-Lys

TPS 1a Glx(Cys-Pro-Asp-Gly)Lys

TPS 3 Asp-Leu-Cys-Gly-Cys-Tyr-Ser

TPS 3a Asp(Leu-Cys-Gly)

The presence of aspartic acid rather than asparagine in these peptides is based on the mobilities of peptides TPS1, TPS3, and TPS3a. The finding of peptide TPS1a in two positions on the map with different mobilities is probably the result of partial deamidation of glutamine. Peptide TPS3a is derived from TPS3 which includes TPα3 (Table I).

The amino acid compositions of the remaining carboxymethylcysteine peptides (TPα3, TPα5, and TPα6) (Table I) were identical or very similar (TPα4) to peptides sequenced previously in an IgA1 myeloma protein. These peptides, which were identified by homology with the previously characterized peptides, were not sequenced and are listed in Table IV. The amount of TPα1 recovered was not sufficient to permit further characterization.

Discussion

We previously characterized the labile disulfide bonds of an IgA1 myeloma protein (Wolfenstein *et al.*, 1971). In the present study, we have isolated the carboxymethylcysteine peptides obtained by similar treatment of an IgA2, Am2(+) myeloma protein and are thus able to compare the two subclasses (Table IV). As expected, a peptide corresponding to the one involved in the heavy–light disulfide bridge in IgA1 (TPα1) could not be found in IgA2, Am2(+). While its absence had already been noted in the diagonal map of this protein (Mihaesco *et al.*, 1971), it was not possible using this method to exclude either its presence with another function or the possible existence of a related peptide with a small difference in composition which could have affected its electrophoretic mobility and thus precluded its detection. These possibilities were ruled out in the present study since neither this peptide nor a related one were isolated from IgA2, Am2 (+) under the conditions described above. The absence of the heavy–light peptide has been of help in identifying this subclass by chemical typing (Frangione and Franklin, 1972).

Several of the peptides obtained were identical for both subclasses; these included: the pentapeptide TPα3, the tetra-

peptide TPα5 and the C-terminal peptide (TPα6). The last was obtained without the C-terminal Tyr due to peptic digestion, a finding which is in agreement with the results of Prahl *et al.* (1971). Peptide TPα4 shows a high degree of homology with that of IgA1; the two nonidentical residues can be explained by single nucleotide base changes in the codons.

Peptides TPα4 and TPα5 are homologous to those forming intrachain disulfide bridges present in the Fc region of μ chain (Frangione *et al.*, 1971a) and γ chains of different subclasses (Frangione *et al.*, 1969). Therefore it is possible that these peptides are involved in an intrachain bridge, labile under the conditions of the study.

While the similarities between the cysteic acid peptides of α1 and α2 chains were marked, two differences were noted. Firstly, the IgA2, Am2(+) α chain contained a peptide (TPα1) which seems to be different from any peptide in IgA1. Secondly, the hinge peptide (TPα2) was significantly smaller than that from the α1 chain and lacked carbohydrate. However, it resembled it in being rich in proline and containing three cysteine residues. In addition, as shown in Table IV, their sequences were identical for the first 8 amino acid residues. While the complete sequence of the hinge peptide from the α1 chain is not yet available, it seems likely that significant homology may again appear at the C terminus after a gap of 12–13 residues.

While it would be desirable to provide a model for the function of the cysteine residues in the hinge region, it is not possible to do so with the results currently available since (a) after digestion with three different enzymes two other peptides (TPS1 and TPS3) were always found in the same region of the diagonal map as the hinge (TPS2) (Figure 3); (b) these peptides were not separated from each other even by performing the diagonal maps at pH 6.5. These two facts strongly suggest that they are bridged. The high content of cysteines of these peptides makes it difficult to establish which of the cysteines are bound to each other. To be able to give a model it would be necessary to break down some of these peptides but so far several attempts with different enzymes have been unsuccessful. This resistance to proteolysis is not surprising in view of the unique sequence of five prolines and two cysteines in a row in the hinge peptide.

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