The Primary Structure of a Tetradecapeptide Containing the Cysteine Residue Linking \(\gamma M\)-Globulin Subunits*

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SUMMARY

A procedure was developed to alkylate the cysteine involved in the \(\mu\)-chain bond joining the 8 S subunits of 19 S \(\gamma M\)-globulins. The method used permitted the interchain and intrachain disulfide bonds in reduced IgM to re-form and the intersubunit sulfhydryl groups to be specifically alkylated with 1\(^{14}\)C-iodoacetamide. A minimally labeled subunit containing 3.4 moles of S-carboxamidomethyl-cysteine per 200,000 g did not dissociate significantly into heavy and light chains in 8 M urea. Heavy chains isolated from this subunit contained about 95% of the radioactive label. After tryptic digestion of the \(\mu\)-chain, about two-thirds of the label was found in a large glycopeptide. Most of the label in this material was found in a chymotryptic tetradecapeptide product of it. This latter peptide isolated from four macroglobulins had identical amino acid composition and peptide mapping characteristics. The sequence of it is:

\[
\text{Ser-Ala-Val-Gly-Glu-Ala-Ser-Ile-Cys-Glu-Asp-Asn-Asn-Trp}
\]

A second \(\mu\)-chain peptide with relatively low specific activity has a composition similar to that reported for the region of \(\mu\)-chains which contains the half-cystine which forms the disulfide bond which joins the light chain. No radioactivity was found in the \(S\)-carboxamidomethylcysteine liberated by carboxypeptidase A treatment from radiolabeled \(\mu\)-chain. This indicates that the intersubunit bond is not formed by a cysteine residue penultimate to the carboxyl terminus of \(\mu\)-chains as previously suggested.

Immunoglobulins of the \(\gamma M\) class exist largely as proteins sedimenting at 18 to 19 S. They appear to be composed of 5 identical subunits of 180,000 to 200,000 mol wt joined together by disulfide bonds between the heavy chains of adjacent subunits (2, 3). The conversion of 10 S \(\gamma M\) globulins to 8 S subunits by reduction with mercaptans is accompanied by changes in biological properties (4-9). Extensive reaggregation of subunits takes place upon removal of the mercaptan and the re-formation of protein sedimenting about 19 S is accompanied by restoration of various antibody activities (10, 11). When 8 S subunits of two different \(\gamma M\)-globulins are reaggregated, the 19 S molecules formed possess some of the properties of both of the original molecules (12-14). The formation of such hybrid \(\gamma M\)-globulins suggests that the structures of the 8 S subunits in the region involved in their linkage to adjacent subunits would be somewhat similar and thus indicates that the peptides containing the intersubunit cysteine residues are also similar. The present report presents the results of a study directed to this consideration.

MATERIALS AND METHODS

\(\text{IgM}^1\) proteins were separated from macroglobulinemic sera by precipitation at low ionic strength. The precipitated protein was dissolved in 0.15 M sodium chloride solution, any insoluble material was removed by centrifugation, and the solution was again digested sufficiently to effect precipitation. Four such successive procedures were carried out on each IgM preparation. The macroglobulin in one serum source (H.E-IgM) was soluble in biological properties (4-9). Extensive reaggregation of subunits takes place upon removal of the mercaptan and the re-formation of protein sedimenting about 19 S is accompanied by restoration of various antibody activities (10, 11). When 8 S subunits of two different \(\gamma M\)-globulins are reaggregated, the 19 S molecules formed possess some of the properties of both of the original molecules (12-14). The formation of such hybrid \(\gamma M\)-globulins suggests that the structures of the 8 S subunits in the region involved in their linkage to adjacent subunits would be somewhat similar and thus indicates that the peptides containing the intersubunit cysteine residues are also similar. The present report presents the results of a study directed to this consideration.

1 The abbreviations used are: IgM, symbol for immunoglobulin recommended by a conference on human immunoglobulins sponsored by the World Health Organization (19); IgM\(_{\text{un}},\) reduced, unlabeled 8 S form of the IgM; IgM\(_{\text{rad}},\) reduced protein (IgM\(_{\text{rad}}\)) from which the excess disassociating mercaptan had been removed and an oxidation period permitted before the alkylation procedure was initiated; IgM\(_{\text{shr}},\) IgM\(_{\text{shr}}\) protein that has undergone polymerization to molecules sedimenting faster than 8 S.
All radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Company) using the dioxane scintillation solution described by Bray (18). Results are reported for column effluents either as disintegrations per min per ml or as specific radioactivities (dpm/ml X 10^6)/(E_b_280 X Protein concentrations were based on optical densities at 280 nm, assuming an E_b_280 value of 12 for all components. Molecular weights of 200,000 and 67,000 were used for the 8 S IgM subunits and p-chains, respectively (3).

The mercaptans used as reducing agents in these investigations were dithiothreitol (Calbiochem), ethyl mercaptan, and 2-mercaptoethanol (Eastman). The latter reagent was redistilled before use. Reductions were carried out under nitrogen in Tris-KCl buffer, pH 8.0 for 1 hour at 0°C unless otherwise specified. Iodoacetamide (Aldrich) was recrystallized from a 3:1 mixture of acetone and n-hexane. Iodoacetamide-13C (Nuclear-Chicago) with a specific activity of 26.8 mCi per mmole was crystallized with 100 times its weight of the once recrystallized 13C-iodoacetamide. The specific activity of the resultant dried crystals was 0.23 mCi per mmole. Alkyations were carried out in the dark at pH 8.0 for 30 min at 0°C. The low molecular weight reactants and products were separated from protein by filtration through a column of Sephadex G-25.

Complete reduction of the minimally labeled protein was effected by incubating it for 4 hours at 37°C with 0.5 M mercaptoethanol in Tris-HCl buffer, pH 8.0, that was 8 M in urea. Sufficient 14C-iodoacetamide was then added to provide a 10% excess over the mercaptoethanol with the pH being maintained at 8 by the addition of Tris. The solutions were then exhaustively dialyzed against deionized water or 0.2 m ammonium bicarbonate buffer, pH 7.8.

Digestions were carried out with calcium-stabilized trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone and with three times crystallized p-chymotrypsin (Worthington) according to previously published procedures (19, 20). Twice crystallized pepsin (Nutritional Biochemicals) was employed at an enzyme to substrate ratio of 1:100 and digestions were carried out in 0.5 N acetic acid for 24 hours at 37°C. The reaction was stopped by adjustment of the pH to 8 with 1 m ammonium hydroxide.

Peptide mapping was performed as described by Bennett (21). The labeled peptides of the tryptic digests of p-chains were located radiographically by laying sheets (14 x 17 inch) of Kodak Royal Blue medical X-ray film (Eastman Kodak Company) on the peptide map and by exposing it for 3 weeks.

The ion exchange resins employed for peptide separations were AG 50W-X2 and AG 1-X8 (Bio-Rad, Richmond, California) and IR 120 (type 15A, Beckman Instruments).

Amino acid analyses were performed by the automated method of Spackman, Stein, and Moore (22) using a Spinco model 120 apparatus equipped for accelerated analysis and increased sensitivity (23). The samples were dried in Pyrex ignition tubes and

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**Fig. 1.** Results of ultracentrifugal and urea starch gel electrophoretic studies of A.U.-IgM reduced with 0.05 M mercaptoethanol and alkylated with levels of iodoacetamide (left hand vertical axis figures) equivalent to 1 to 10% of the mercaptoconcentration. The control sample (0%) was allowed to reaggregate without alkylation. A, photos were taken after 40 min of centrifugation at 8,040 rpm; B, urea starch gel electrophoretogram. The positions of the light chain (L), heavy chains (p), IgM monomer (8 S), and aggregated protein (AGG.) are indicated.

**Fig. 2.** Results of ultracentrifugal and urea starch gel electrophoretic studies of H.E.-IgM which was alkylated after various times of reoxidation. The control sample (50 hours) was allowed to reaggregate without alkylation. A, photos were taken after 45 min of sedimentation at 8,040 rpm; B, acid urea starch gel electrophoretogram.
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FIG. 3. Results of gel filtration of A.U.-IgMγM-Akt (250 mg in 35 ml) on a column (2.5 x 51 cm) of Sephadex G-25 equilibrated with Tris HCl buffer, T/2 = 0.2, pH 8.0. A flow rate of 15 ml per hour was employed and 5.0-ml fractions were collected. The bar indicates the fractions which were pooled.

A.

B. (-)

8S AGG.

CONT.

ALK.

FIG. 4. Results of ultracentrifugal and urea starch gel electrophoretic studies of A.U.-IgMγM-Akt (Akt) and its unalkylated control (Cont.). A photo was taken after 54 min of centrifugation at 22,000 rpm; B, acid urea starch gel electrophoretogram (both samples in duplicate).

1.0 ml of 6 N hydrochloric acid was added. The tubes were evacuated at a pressure of 10 to 30 μ of mercury for 30 min at room temperature, sealed, and then heated at 110 °C for 20 hours unless otherwise indicated. The precautions used in evacuating the tubes were designed to minimize destruction of ς-carboxymethylcysteine. Effluents from the colorimeter outlet were assayed for radioactivity.

Amino-terminal sequences were established by sequential Edman degradations (24) and analysis of the residual peptide (25) or by identification of the phenylthiohydantoin derivatives by chromatography on silica gel sheets (Eastman). The Solvent Systems IV and V of Jeppsson and Sjöquist (26) were employed. Carboxyl-terminal sequences were determined by treatment of the peptides with carboxypeptidase A (27) and analysis of the amino acids in the digests.

RESULTS

Reduction and Limited Alkylation of IgM—When IgMγM protein is alkylated with iodoacetamide at molar levels equal to only a few per cent of the mercaptan employed, little reaggregation of the subunits to 19S and other polymeric forms occurs upon subsequent removal of the excess mercaptan (3). Such a result as illustrated by the ultracentrifuge data in Fig. 1 indicates that the cysteines involved in the intersubunit bonds are more readily alkylated than 2-mercaptoethanol. If the intersubunit cysteines are also more reactive than the inter- and intrachain cysteines, it might be possible to find conditions under which only the intersubunit sulfhydryl groups are alkylated. The results of urea starch gel experiments presented in Fig. 1 reveal that a large portion of the subunits remaining after limited alkylation of IgMγM dissociated into γ and L-chains in 8 M urea, thus indicating that some of the cysteines involved in interchain bonds had also been alkylated. Similar results were obtained when ethyl mercaptan...
or dithiothreitol were employed as the reducing agent. Therefore, this method was not suitable for specifically alkylating the cysteines involved in the intersubunit bonds.

Reduction, Removal of Mercaptoan, and Alkylation—The reduction products of 19 S IgM in which all disulfide bonds have been cleaved can be dissociated into \( \mu \)- and \( \lambda \)-chains in 8 \( \times \) M urea. The stability of the 8 S subunits in aqueous solution indicates that there are strong noncovalent interactions between their polypeptide chains in contrast to the minimal interactions between the subunits. Therefore, the cysteines which are normally paired in interchain and intrachain disulfides are probably held in close proximity in the reduced subunit IgM\(_{\text{nr}}\). These disulfides should be readily re-formed when the mercaptan dissociating agent is removed, whereas the intersubunit disulfides would be expected to re-form at a much slower rate.

To test this, macrooglobulins were reduced with 0.05 \( \times \) M mercaptoethanol for 1 hour at 0\( ^{\circ} \) and the mercaptan was then removed by gel filtration. The solution of IgM\(_{\text{nr}}\) was stored at 0\( ^{\circ} \) and aliquots were alkylated after 6, 9, 12, and 23 hours by the addition of iodoacetamide at molar levels from 5 to 10 times that of the protein intersubunit sulfhydryl group concentration. These solutions were then dialyzed to remove the low molecular weight reactants and products and the samples then subjected to ultracentrifugal and urea starch gel electrophoretic analyses. The data for a typical experiment with H.E.-IgM are presented in Fig. 2. The extent of reaggregation was proportional to the length of time between reduction and alkylation. The sample that was alkylated at 9 hours contained a relatively large amount of 8 S subunit but underwent little dissociation into \( \mu \)- and \( \lambda \)-chains in 8 \( \times \) M urea. This suggests that the major portion of interchain disulfides, but only a small fraction of intersubunit disulfides, had re-formed. Similar results were obtained with the four other macrooglobulins, but the optimum time of incubation of the IgM\(_{\text{nr}}\) protein between removal of the mercaptan reducing agent and alkylation to give IgM\(_{\text{nr}}\) was different for each.

When experiments were performed with larger quantities of IgM to provide sufficient subunit for extensive study, it was found necessary to incubate the reduced protein for longer periods of time prior to alkylation. In a typical experiment, 300 mg of A.U.-IgM in 15 ml was reduced and the mercaptan removed as previously described. After 30 hours \( 1^{\text{14C}} \)-iodoacetamide was added at a molar level 5 times that calculated for the intersubunit sulfhydryl group. The excess alkylating agent was removed by gel filtration and about 30% of the total radioactivity was eluted with the protein as shown in Fig. 3. A sample of IgM\(_{\text{nr}}\) that was allowed to reaggregate without alkylation served as the control. Ultracentrifuge and acid urea starch gel electrophoretic assays of these samples gave the results shown in Fig. 4. About 55% of the protein remained in the 8 S form in the IgM\(_{\text{nr}}\) sample, whereas in the control about 90% had re-aggregated to IgM\(_{\text{nr}}\) forms. Neither sample showed significant dissociation into \( \mu \)- and \( \lambda \)-chains in the urea starch gel electrophoretic evaluation. This indicates that the interchain disulfides had essentially re-formed and only the intersubunit cysteine had been appreciably alkylated by the \( 1^{\text{14C}} \)-iodoacetamide.
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CHROM. (-1 PH 1.8 ELEC. b) +

FIG. 8. Peptide maps of a tryptic digest of μ-chains prepared from A.U.-IgM Min.-Air. A, with electrophoresis at pH 1.8 and B, at pH 6.5; C, of tryptic peptide T1 isolated from labeled A.U. μ-chains. The encircled spot in the upper right-hand portion of the peptide maps in B and C denotes the location of a phenol red marker. The radiolabeled peptide is encircled with a dashed line.

Isolation of Minimally Alkylated Subunits—The A.U.-IgM Min.-Air protein was passed through a column of 6% Gelrose (Litex, Glostrup, Denmark) in order to separate the 8 S subunits from IgM Air, in the mixture with the results presented in Fig. 5. The first component eluted consisted of aggregated protein and the second was the labeled 8 S IgM Min.-Air subunit. The results of ultracentrifugal and urea starch gel electrophoretic studies of this preparation of 8 S subunit and another similar sample from a different IgM are presented in Fig. 6. The amount of radioactivity associated with the 8 S subunits in this experiment was equivalent to 3.4 moles of 14C-labeled S-carboxyamidomethylcysteine per 200,000 g of IgM Min.-Air. If only the intersubunit cysteines had been alkylated, 2 residues should have been found. The greater labeling probably reflects some alkylation of intrachain and interchain cysteines. The IgM Air components contained only 1.7 moles of labeled cysteine derivative per 200,000 g. The specific radioactivities of various preparations of IgM Min.-Air, ranged from 2.0 to 4.5 moles of 14C-labeled S-carboxyamidomethylcysteine per 200,000 g. Preparations with the lower levels of labeling did not dissociate in 8 M urea, but they often provided too little material for further study. More highly labeled preparations dissociated extensively into μ- and L-chains in 8 M urea.

Preparation of μ- and L-chains from Minimally Labeled Subunits—The A.U.-IgM Min.-Air protein was completely reduced with mercaptoethanol and alkylated with an excess of 14C-iodoacetamide. The light and heavy chains were separated by gel filtration with the result shown in Fig. 7. The radioactive label in the heavy chain component was equivalent to 1.45 residues of cysteine per 67,000 g and the amount in the light chains was less than 0.07 mole/22,000 g. This is in agreement with previously published data (28, 29) which suggests that the intersubunit link is between μ-chains of adjacent subunits.

Isolation of Labeled Tryptic Peptide—Tryptic peptide maps of the labeled μ-chains derived from A.U.-IgM Min.-Air, are presented in Fig. 8. Autoradiographic studies revealed a single major radioactive spot which is encircled on the peptide maps. This material gave a faint yellow stain with ninhydrin and its mapping properties were typical of a glycopeptide. The soluble portion of a tryptic digest which contained 91% of the 14C-label of the μ-chain was fractionated on a column of Sephadex G-50. It can be seen from the result presented in Fig. 9 that most of the radioactivity was eluted in the void volume. The fraction designated T1 contained 61% of the label, or 0.9 of the 1.45 residues of 14C-carboxyamidomethylcysteine per molecule of μ-chain. A minor radioactive component (T2) contained 20% of the label or 0.3 residue per molecule of μ-chain.

The T1 component was further purified by paper electrophore-
sis and then peptide mapped to give the result shown in Fig. 8C.
This material had the same chromatographic, electrophoretic, and staining properties as the 14C-containing peptide which was located by autoradiography. The amino acid composition of T1 is presented in Table I. Arginine was found in the smallest amount and was assumed to represent 1 residue. The acid hydrolysate contained 1.2 moles of residues of 14C-label per mole of peptide but only 0.8 residue of S-carboxymethylcysteine was found. The relatively large amount of cystine (1.7 residues of half-cystine) may indicate that the peptide contains one of the disulfide loops of the p-chain. The presence of a relatively large amount of hexosamine in the hydrolysate confirms the glycoprotein nature of this material.

Abel and Grey (30) have suggested that the cysteine residue penultimate to the carboxyl-terminal tyrosine of p-chains forms the intersubunit linkage. Because Peptide T1 contains no tyrosine or methionine, it does not appear to be located at the carboxyl-terminal position. When µ-chain isolated from A.U.-IgM[Akk] subunit was treated with carboxypeptidase A, tyrosine was released rapidly, followed by unlabeled S-carboxamidomethylcysteine and several other amino acids, in agreement with previous studies (30). Upon similar treatment of the µ-chain of V.I.-IgM[Akk], serine was released, but little tyrosine and no S-carboxamidomethyl cysteine. These results suggest variability in the carboxyl-terminal region of µ-chains and confirm that the specifically alkylated cysteine is not the penultimate residue of these chains.

The radioactive component of Fraction T2 (see Fig. 9) was separated by chromatography on a column of AG 50W-X2. The labeled peptide found was further purified by paper chromatography using the upper phase of a butanol-acetic acid-water (4:1:5) mixture and then by chromatography on AG 1-X8 resin. The amino acid composition of this peptide (T2) is included in Table I. The data are most consistent with a tryptic peptide containing a single S-carboxamidomethylcysteine residue with a lower specific radioactivity. Its composition is similar to that reported by Pink and Milstein (31) and by Beale and Buttress (32) for the portion of the µ-chain containing the cysteine residue linked to the light chain.

**Isolation of Specifically Labeled Chymotryptic Peptide**—The size of the 14C-labeled tryptic peptide T1 necessitated further cleavage to provide a smaller fragment more suitable for initial sequence analysis. This peptide was digested with chymotrypsin and the products were fractionated on a column of Sephadex G-25. As shown by the data of Fig. 10A, most of the radioactivity (88%) was eluted in a single peak which was well separated from the other peptide material. The radioactive component was further fractionated on anion and cation exchange resins. These results are presented in Fig. 10, B, C, and D. In the final chromatographic step a component of constant specific radioactivity, designated as T2C, was eluted as a single symmetrical peak. A peptide map of an aliquot of this material, shown in Fig. 11A, reveals a single acidic peptide. Its amino acid composition is presented in Table II. The data are most consistent with a peptide containing 14 amino acids, but fractional residues of S-carboxamidomethylcysteine and half-cystine are present.

An attempt was made to determine whether the cysteine noted had been formed from the S-carboxamidomethylcysteine. Aliquots of Peptide T1C were subjected to a digestion for 20, 60, and 190 hours. The decrease in serine was nearly linear with

### Table I

| Amino acid                  | Peptide T1 | Peptide T2 |
|----------------------------|------------|------------|
| Lysine                     | 19.5       | 4.3        |
| Histidine                  | 4.5        | 1.0        |
| Arginine                   | 3.4        | 0.8        |
| S-Carboxymethylcysteine    | 56.2       | 12.5       |
| Aspartic acid              | 33.8       | 7.5        |
| Threonine                  | 45.5       | 10.1       |
| Serine                     | 38.8       | 8.6        |
| Glutamine acid             | 13.0       | 2.9        |
| Proline                    | 18.2       | 4.0        |
| Alanine                    | 34.1       | 7.6        |
| Half-cysteine              | 7.7        | 1.7        |
| Valine                     | 15.1       | 3.4        |
| Methionine                 | 19.6       | 4.4        |
| Isoleucine                 | 11.4       | 2.5        |
| Leucine                    | 12.0       | 2.7        |
| Tyrosine                   | 34.0       | 7.5        |
| Phenylalanine              | 5.3        | 1.2        |
| 
| Hexosamines²               | 34.0       | 7.5        |
| Tryptophane³               | 6.7        | 0.3        |
|
| nmoles residues            | nmoles residues |
|----------------------------|-----------------|
| 19.5                       | 4.3             |
| 4.5                        | 1.0             |
| 3.4                        | 0.8             |
| 56.2                       | 12.5            |
| 33.8                       | 7.5             |
| 45.5                       | 10.1            |
| 38.8                       | 8.6             |
| 13.0                       | 2.9             |
| 18.2                       | 4.0             |
| 34.1                       | 7.6             |
| 7.7                        | 1.7             |
| 15.1                       | 3.4             |
| 19.6                       | 4.4             |
| 11.4                       | 2.5             |
| 12.0                       | 2.7             |
| 34.0                       | 7.5             |
| 5.3                        | 1.2             |

* Residues are expressed as molar ratios to arginine.
* Tryptophane was determined spectrophotometrically.
* Not determined.

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**Fig. 10.** Isolation procedures for the separation of the labeled peptide T1C from a chymotryptic digest of tryptic peptide T1 from the \( \mu \)-chain of A.U.-IgM. A, results of filtration of the digest (0.4 \( \mu \)mole) on a column (1.4 \( \times \) 25 cm) of Sephadex G-25 that was equilibrated with ammonium acetate buffer, \( \Gamma/2 = 0.1 \), pH 4.0. A flow rate of 5 ml per hour was employed and 1.0-ml fractions were collected. B, the radioactive fraction isolated in A was chromatographed on a column (0.6 \( \times \) 15 cm) of AG 1-X8 resin. A linear pH and salt gradient was established by the addition of 60 ml of 2 M pyridine acetate buffer, pH 5.0, to 60 ml of 0.2 M pyridine acetate, pH 6.5. The flow rate was 12 ml per hour and 1.33-ml fractions were collected. The material indicated by the bar was pooled. C, material pooled in B was applied to a column (0.6 \( \times \) 15 cm) of I.R. 120 resin and eluted with a pH-salt gradient established as described for B. A 2 M pyridine acetate buffer, pH 5, was added to 0.2 M pyridine acetate, pH 3.1. A flow rate of 10 ml per hour was employed, 0.74 ml fractions were collected, and the radioactive fractions were pooled as indicated. D, the material pooled in C was chromatographed on a column (0.6 \( \times \) 15 cm) of AG 50W-X2 resin. A continuous pH and salt gradient was established by adding 0.4 M pyridine acetate buffer, pH 3.7, to 0.2 M pyridine acetate, pH 3.1, as described under B. The flow rate was 12 ml per hour. Aliquots of all fractions of each chromatogram were subjected to alkaline hydrolysis and assayed by the ninhydrin reaction.
length of hydrolysis and extrapolation to zero time gave 2.0 residues. The recovery of S-carboxymethylcysteine was variable and did not decrease with increasing length of hydrolysis. Treatment of the peptide with 0.05% thiodiglycol for 4 hours at 37° before hydrolysis of the samples as previously described appeared to improve the yield of S-carboxymethylcysteine and no cystine was found. This result suggests that the variable recoveries of S-carboxymethylcysteine experienced may be due to acid degradation of sulfoxide forms (33). A sample of Peptide TIC1 was hydrolyzed after 6 months of storage at −20°. No S-carboxymethylcysteine was present in the 6 M HCl hydrolysate, although the amount of radioactivity was equivalent to 1.0 residue of the labeled derivative.

The chymotryptic peptide TIC1 was isolated from three other IgM proteins using similar minimal labeling and isolation procedures. The peptide maps obtained from these other three IgM proteins were indistinguishable from the one obtained from the A.U.-IgM derived chymotryptic peptide. The peptide maps obtained for the pure TIC1 peptide derived from A.U.-IgM and from H.E.-IgM are shown in Fig. 11, A and B, respectively. Small amounts of peptide were used in the experiments shown in Fig. 11. The weakly stained ninhydrin positive spots which also show intense radioactivity are encircled in this figure. The amino acid compositions of the peptides from the H.E., V.I., and S.L.-IgM sources are included with the data for TIC1 of A.U.-IgM in Table II. The compositions of all four peptides are seen to be identical if the sum of the S-carboxymethylcysteine and half-cystine residues are taken as one in each case. The differences in the recovery of S-carboxymethylcysteine from the four peptides are not significant in view of the variations experienced upon repeated analysis of one peptide. These data indicate that the region surrounding the intersubunit bond is the same in the four IgM proteins studied and that this is a constant feature of their structure.

Products of Peptic Digestion—In order to provide smaller fragments for structural studies, 0.35 μmole of the chymotryptic tetradecapeptide from one source (H.E.-IgM) was digested with pepsin. The digest was fractionated on a column of AG 50W-X2 and the two major components (P1 and P2) were resolved. Chromatography of Fraction P1 on IR 120 resin and then on AG 1-X8 resin further separated it into an unlabeled peptide, termed TIC1P1, and a small amount of the parent peptide. The amino acid composition of TIC1P1, presented in Table III, is con-

### Table II

**Amino acid compositions of specifically labeled chymotryptic peptides (TIC1) from four γM-globulins**

| Amino acid                  | A.U.-IgM | V.I.-IgM | H.E.-IgM | S.L.-IgM |
|-----------------------------|----------|----------|----------|----------|
|                             | m moles  | residues | m moles  | residues | m moles  | residues | m moles  | residues |
| S-Carboxymethylcysteine     | 7.0      | 0.8      | 3.6      | 0.8      | 50.7     | 1.0      | 42.2     | 0.9      |
| Half-cystine                | 4.1      | 0.4      | N.D.     |          | 6.9      | 0.1      |          |          |
| Aspartic acid               | 32.5     | 2.8      | 12.9     | 2.8      | 172.0    | 2.9      | 139.0    | 2.9      |
| Serine                      | 23.4     | 2.1      | 10.1     | 2.2      | 124.0    | 2.1      | 96.6     | 2.0      |
| Glutamic acid               | 23.4     | 2.1      | 9.5      | 2.1      | 116.0    | 1.9      | 104.0    | 2.2      |
| Glycine                     | 12.6     | 1.1      | 5.7      | 1.2      | 64.0     | 1.1      | 54.4     | 1.1      |
| Alanine                     | 23.4     | 2.1      | 9.1      | 2.1      | 121.0    | 2.1      | 102.0    | 2.0      |
| Valine                      | 11.3     | 1.0      | 4.5      | 1.0      | 62.0     | 1.0      | 49.0     | 1.0      |
| Isoleucine                  | 11.7     | 1.0      | 4.6      | 1.0      | 59.0     | 1.0      | 49.3     | 1.0      |
| Tryptophan                  | N.D.     |          |          |          |          |          |          |          |

*Residues are expressed as molar ratios to isoleucine.

**Not determined.**

Serine values are corrected for 10% destruction as determined for Peptide S.L. TIC1. The levels of valine and of isoleucine found after hydrolysis of this peptide for 20, 44, 50, and 130 hours were essentially the same.

Tryptophan was determined spectrophotometrically.
The major radioactive Fraction P2 was rechromatographed on IR 120 resin and one labeled component was eluted. The peptide map of this material, designated T1C1P2, is presented as Fig. 11D. The amino acid composition of this peptide, included in Table III, is most consistent with a heptapeptide that is at least 90% pure. The amino acids of this peptide and of Peptide T1C1P1 account for those comprising the parent tetradecapeptide. About 0.14 amole of T1C1P2 was available for further study.

Comparison of the peptide map of the T1C1 peptide of H.E.-IgM in Fig. 11B with the peptide maps of the two heptapeptides derived from it (C and D of Fig. 11) reveals that the amionic mobility of T1C1P2 at pH 6.5 is about twice that of T1C1P1 and slightly greater than that of the parent peptide T1C1. These mobilities are consistent with net charges of -1 for T1C1P1, -2 for T1C1P2, and -3 for T1C1. This indicates that the glutamyl residue in T1C1P2 must be present as the free acid and that 2 of the 4 dicarboxylic acid residues of T1C1P2 are present in the amide form.

The complete sequence of the chymotryptic tetradecapeptide of H.E.-IgM in Fig. 11B with the peptide maps of the two heptapeptides derived from it (C and D of Fig. 11) reveals that the anionic mobility of T1C1P2 at pH 6.5 is about twice that of T1C1P1 and slightly greater than that of the parent peptide T1C1. These mobilities are consistent with net charges of -1 for T1C1P1, -2 for T1C1P2, and -3 for T1C1. This indicates that the glutamyl residue in T1C1P2 must be present as the free acid and that 2 of the 4 dicarboxylic acid residues of T1C1P2 are present in the amide form.

Sequence of Chymotryptic Peptide—Edman degradations of T1C1 were employed to determine its NH2-terminal sequence. Subtractive analysis of the residual peptide after 1 degradative cycle revealed that serine was the NH2-terminal amino acid. Direct identification of the phenylthiohydantoin derivatives after each of the next 4 cycles established a partial sequence of Ser-Ala-Val.

Edman degradations of Peptide T1C1P2 were then carried out to determine the order of amino acids in the carboxyl-terminal half of the tetradecapeptide. The phenylthiohydantoin derivatives were identified directly after each of the first 4 cycles and the results were confirmed by subtractive analysis of the residual peptide. On the basis of these data the following partial sequence could be assigned for T1C1P2: Ile-Cys-Glu-Asp-(Asx3,Trx)Glu. Digestion of T1C1 with carboxypeptidase A indicated a carboxy-terminal sequence of: Asx3,Trx. The results of Edman and carboxypeptidase degradation experiments, along with the amino acid composition and net charge of -2 for Peptide T1C1P2 were consistent with a sequence of: Ile-Cys-Glu-Asp-Asn-Asn-Trx.

The complete sequence of the chymotryptic tetradecapeptide must then be:

Ser-Ala-Val-Glu-Ala-Asp-Ile-Cys-Glu-Asp-Asn-Asn-Trx

It is apparent that this peptide was isolated in the form of the carboxamidomethylcysteine derivative.

DISCUSSION

Two general approaches to the specific labeling of the cysteine residues form the S subunit disulfide bonds of the 19 S molecule have been utilized. One of them is the selective reduction of these bonds and the subsequent labeling of the liberated sulfhydryl groups. This approach has been used with varying success by employing reducing agents such as cysteine (29), dithiothreitol (32), and mercaptoethanolamine (34). The use of such an approach by Chapin, Cohen, and Press (35) first indicated that the subunits of IgM were linked through their heavy chains. However, as reported by Miller and Metzger (29), in their extensive study, specific disulfide bonds of γM-globulins cannot be selectively cleaved. This approach thus has limitations.

In our work, the differences in the rate of re-formation of inter- and intrasubunit disulfide bonds rather than in the extent of reduction of these bonds, were used as the basis for specifically alkylating the intersubunit cysteine. This approach entails a different shortcoming, namely that disulfide interchange could have taken place and that the re-oxidation of residual sulfhydryl groups are not representative of those that make up the inter-subunit disulfide bonds.

The approach we have employed, however, seemed to be a logical one for several reasons. The re-formation of disulfides within the IgM subunits appears to be favored by noncovalent interactions between the polypeptide chains which serve to hold cysteine residues in close proximity and thus permit reoxidation to their original form. The regeneration of most of the biological activities of 19 S subunits upon reaggregation of their S subunits (10, 11) and the ability to form mixed IgM hybrids (12-14, 36) also suggests that significant disulfide interchange does not take place upon reoxidation of these immunoglobulins. It would be anticipated that disulfide interchange would greatly change the tertiary structure of these molecules and induce marked changes in their biological properties.

The formation of stable mixed disulfides of the protein and reducing thiol (23, 37) results in variable levels of protein sulfhydryl groups being available for alkylation. In the present experiments, the protein was reduced with an excess of mercaptoethanol to favor the generation of the thiol anion forms of the reduced protein. Extensive formation of mixed disulfides of the protein and 2-mercaptoethanol should not have occurred because this requires a relatively high level of diethanol disulfide in the reaction mixture (37). The thiol was rapidly removed by gel filtration and
the reduced protein was allowed to reoxidize for a sufficient time to permit re-formation of the inter-subunit disulfides. The inter-subunit sulphydryl groups could then be alkylated to prevent further reaggregation of the subunits to polymeric forms. Nearly complete reaggregation occurred in control samples which were not alkylated. Because only one S-carboxymethylcysteine in the S S minimally alkylated form of the IgM had a specific radioactivity about 1.0, this residue most likely forms the inter-subunit bond. Further evidence for the specificity of the alkyla-
tion is the lower specific radioactivity of the aggregated components compared to that of the S S subunits. In addition, the chymotryptic peptide T1C1 isolated from minimally alkylated S S subunits was completely labeled, whereas the same peptide isolated from mixtures of subunits and aggregated components contained only 50 to 60% as much label.

The amino acid composition of the labeled tryptic glycopeptide T1 is not identical with any of the cysteine-containing peptides of the μ-chain reported by Beale and Buttress (32) but is similar to their Peptides u4 and u5 which are believed to contain the inter-subunit bond near the carboxyl terminus of the μ-chain. It is also in agreement with a recent results of preliminary carboxypeptidase degradations of mini-

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