Linear Alignment of the Cyanogen Bromide Fragments of Heavy Chain from Strain 13 Guinea Pig Immunoglobulin G2†

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ABSTRACT: Eight fragments from a CNBr digest of γ_2 chain derived from strain 13 guinea pig immunoglobulin G2 have been aligned. These eight fragments appear to account for the entirety of the γ_2 chain. The positions of the fragments were determined from analysis of five methionine-containing peptides which overlapped the six fragments extending from ≃N-140 to the carboxyl terminus and from the direct sequence analysis of intact γ_2 chain beginning from the amino terminus. The alignment of these fragments permits the rigorous positioning of those specifically affinity labeled CNBr fragments derived from antibody.

Primary structural analyses of human myeloma proteins have suggested that, among the members of a given subclass, the C-terminal three-quarters of their heavy chains have a "constant" sequence and that the sequence of N-terminal ≃120 residues of each, called the "variable" region, is different (Press and Hogg, 1969; Steiner and Porter, 1967; Edelman *et al.*, 1969). It has been inferred that these "variable" regions form the antibody combining site. To more directly localize sections of heavy chain involved in site formation and to correlate their sequences with antigen binding specificity we have begun by determining the primary structure of the entire γ_2 chain of normal strain 13 guinea pig IgG2,[1] a vast mixture of antibodies. To this end, we have isolated eight CNBr fragments of γ_2 chain which appear to account for its entirety (Birshtein *et al.*, 1971a,b; Turner and Cebra, 1971; Birshtein and Cebra, 1971; D. Benjamin, unpublished data). Five of these fragments (C-1-b, C-1-c, C-3, C-4, and C-5) have been shown to each have a single sequence (Birshtein *et al.*, 1971b; Turner and Cebra, 1971; D. Tracey and T. Trischmann, unpublished data). The remaining three fragments (C-1-n, C-1-a_1, and C-1-a_2) each have sections of variable primary structure (Birshtein and Cebra, 1971; D. Benjamin, unpublished data; A. Ray, unpublished data). Further, C-1-n, C-1-a_1, and C-1-a_2 are the only fragments of the eight which carry significant affinity label when they are derived from specifically affinity-labeled anti-DNP antibodies (Ray and Cebra, 1972). Thus, positioning these variable fragments within the γ_2 chain seems fundamental to the linear localization of those residues involved in antigen binding site formation. We report here the alignment of the eight CNBr fragments of γ_2 chain by the identification of overlapping methionine-containing peptides isolated from various enzymic digests of the intact heavy chain.

Materials and Methods

Tryptic Digest of γ_2 Chain. Totally reduced and carboxymethylated heavy chain (γ_2) was prepared from IgG2 and digested with trypsin as previously reported (Birshtein *et al.*, 1971b). The digest was applied to a column of Sephadex G-50 (3.5 × 180 cm) equilibrated in 0.05 M NH_4HCO_3 (Figure 1). Each of the five pools was recycled through the same column before further purification steps.

Complete Reduction of Disulfide Bonds and Aminoethylation of the Resulting Cysteine Residues. IgG2 (400 mg) was dissolved in 25 ml of 7 M guanidine·HCl, 0.1 M in Tris-acetate (pH 8.0). Dithiothreitol (193 mg) was added to 0.05 M, and the solution was kept at room temperature for 2 hr. Ethylenimine (0.7 ml) was then added in a tenfold molar excess over the dithiothreitol, and the pH was maintained at 8.0 for 30 min. The mixture, containing completely reduced, aminoethylated heavy and light chains, was then applied to a column (2.5 × 100 cm) of G-200 Sephadex equilibrated in 5 M guanidine·HCl.

^{14}C-Carboxymethylation of Methionine Residues. Completely reduced and aminoethylated γ_2 chain (220 mg) was dissolved in 10 ml of 5 M guanidine·HCl, and the pH was adjusted to 3.6 with concentrated HCl. Iodoacetic-*1*-^{14}C acid (300 μCi) was added, and the mixture was held at 37° for 36 hr. Cold iodoacetic acid was then added in a tenfold molar excess with respect to methionyl residues, and the mixture was incubated at 37° for an additional 24 hr. The γ_2 chain was then freed of reagents by dialysis against 3 changes (6.0 l. each) of 0.1 M ammonium formate buffer (pH 3.6) over 24 hr followed by exhaustive dialysis against distilled water.

Tryptic and Chymotryptic Digestion of γ_2 Chain Containing Aminoethylcysteine and S-Carboxymethylmethionine. The completely reduced, aminoethylated, and carboxymethylated γ_2 chain (prepared as above) in distilled water was concentrated to 17.0 ml by vacuum dialysis. This γ_2 chain was then digested with trypsin as described above. Following tryptic digestion, when no further uptake of NaOH was being recorded by the autotitrator (Radiometer), 4.0 mg of α-chymotrypsin (5.0 mg/ml in H_2O) was added in 1.0-mg portions. Digestion was continued until there was no further uptake of NaOH, and the digest was freeze-dried.

Ion-Exchange Chromatography on Columns of Dowex 1-X2 or Dowex 50-X8. Ion-exchange chromatography of peptides obtained from tryptic digests of γ_2 chain was carried out as previously described (Birshtein *et al.*, 1971b). Ion-exchange chromatography of the tryptic–chymotryptic digest of aminoethylated–carboxymethylated γ_2 chain was carried out on a column (2.5 × 12.0 cm) of Dowex 50-X8 (AA-15, Beckman Instruments) as follows. The freeze-dried digest

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[1] The nomenclature is in accord with that proposed by the Conference on Nomenclature for Animal Immunoglobulins, Prague, June 1969.

TABLE I: Methionine-Containing Peptides and Peptides Derived from Them by CNBr Cleavage and Tryptic or Chymotryptic Digestion.[a]

	T3-4	T3[c]	T3[d]	T4[c]	T4[d]	T11-12-13	T11	Ch19	Ch20	Ch21	T26-27	Ch22
Lys	0.90	0.90	1			1.2	1.0			1.0		0.98
His	2.8	1.7	3									
Arg						1.0			1.0		1.0	
CMCys	1.0			0.60	1							
Asp	2.2	1.1	1	1.1	1	1.8	1.2	1.3	1.0		0.96	
Thr	2.9	0.89	1	1.7	2	0.94			0.89		1.8	1.9
Ser	2.0	0.80	1	2.0	2	1.7	0.89		1.3	1.4	1.0	3.1
Glu	3.2	2.1	2	1.2	1	1.1	1.0		1.1			1.1
Pro						2.7		1.1	1.8		0.93	0.94
Gly	1.0			2.0	1							
Ala	1.9	1.2	1	1.1	1							
Val	2.9	1.1	1	1.6	2	1.1		1.0				1.9
Met	1.0					0.79		0.71			0.84	0.71[e]
Ile											0.89	
Leu	1.1	1.0	1			1.9	1.0		1.8		1.96	1.0
Tyr	1.0			0.70	1	0.94		0.94				
Hsr				0.90	1							
Trp	+			+	1							
Mobilities[b]												
pH 3.6	+0.39	+0.77		+0.13		+0.21	+0.37	+0.07	+0.45	+0.89	+0.13	+0.31

[a] Compositions are reported as moles of amino acid per mole of peptide. [b] Mobilities given relative to lysine = +1.0, neutral amino acid = 0. [c] Composition of T3 and T4 derived from CNBr cleavage of tryptic peptide T3-4. [d] Compositions from known sequences of the N terminus of C-5 and the C terminus of C-4. [e] *S*-Carboxymethylmethionine on hydrolysis gives rise to homoserine, homocysteine, and methionine—value given is the approximate sum of these three.

was dissolved in 0.01 N HCl and applied to the column equilibrated in 0.01 N HCl. The column was developed at 1.6 ml/min initially with 0.01 N HCl. After those peptides not retarded by the column were released, the column was developed with pyridinium acetate buffer (0.05 M in pyridine, pH 2.4). Development was continued until no further peptides were released, after which the column was developed sequentially with two different linear gradients as follows: gradient 1, 750 ml of 0.05 M pyridinium acetate buffer (pH 2.4)–750 ml of 1.0 M pyridinium acetate buffer (pH 4.0); gradient 2, 750 ml of 1.0 M pyridinium acetate buffer (pH 4.0)–750 ml of 2.0 M pyridinium acetate buffer (pH 5.0). Fractions were collected every 7.5 min from the portions of the effluent not used for analysis. A portion of the effluent (0.16 ml/min) was automatically analyzed by reaction with ninhydrin after alkaline

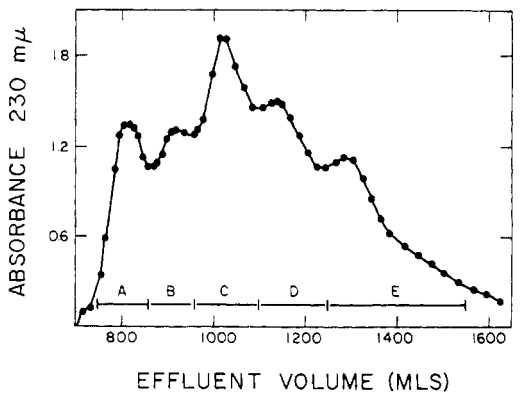

FIGURE 1: Elution profile of the fractionation of tryptic peptides from γ_2 chain on a column of Sephadex G-50 equilibrated in 0.05 M NH_4HCO_3. Details of the procedure are found in the text. The absorbance of the fractions (10 ml) was read at 230 mμ.

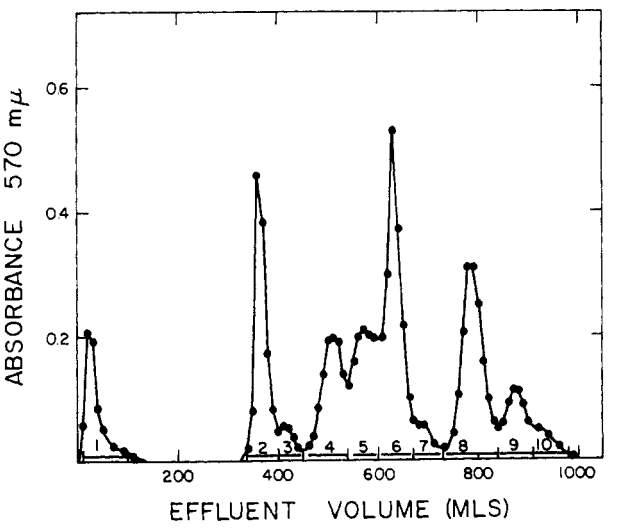

FIGURE 2: Separation of the tryptic peptides of γ_2 chain contained in pool B (Figure 1) on a column of Dowex 1-X2. Details of the procedure are found in the text. The column was developed at 1.6 ml/min with a continuous gradient of pyridinium–acetate buffers. A portion of the effluent (0.16 ml/min) was automatically analyzed by the ninhydrin reaction after alkaline hydrolysis. The absorbance of the reaction mixture was monitored at 570 mμ. Fractions were collected for 7.5 min each.

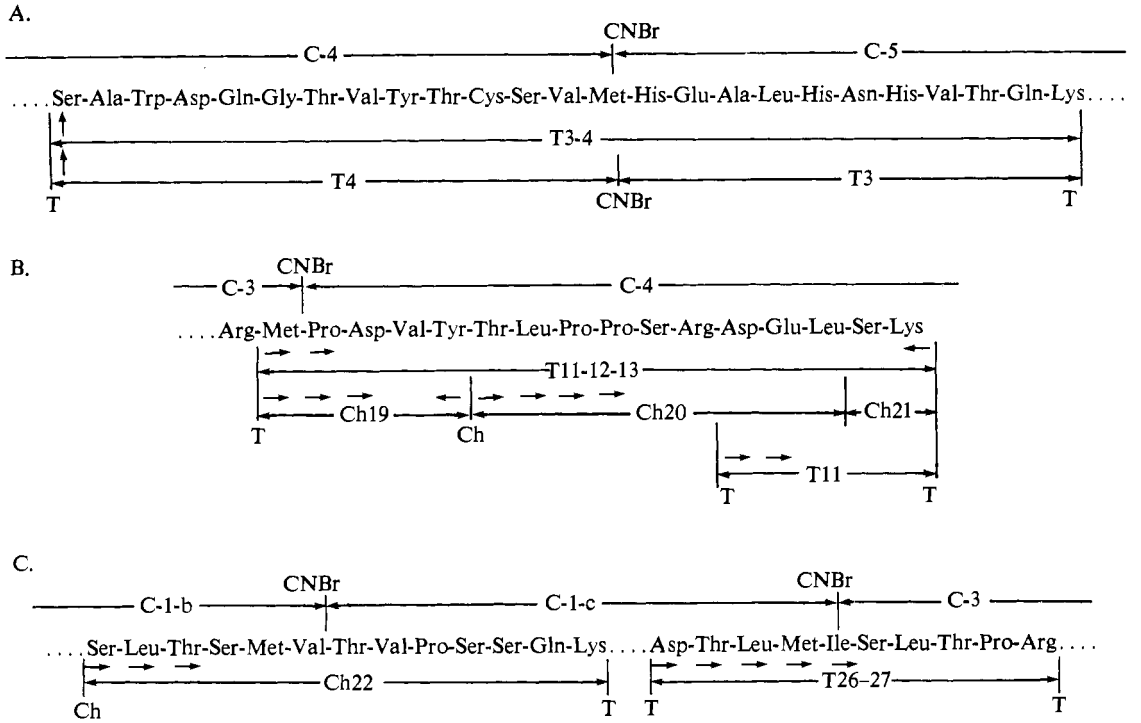

FIGURE 3: A schematic representation of the procedures and resulting data used to align the CNBr fragments, C-1-b, C-1-c, C-3, C4, and C5, which account for the ≃303 C-terminal residues of guinea pig γ_2 chain. Exact composition of peptides is given in Table I. The symbol → indicates removal of a residue by the Edman degradation procedure; the symbol ← indicates identification of a residue by treatment with carboxypeptidase A or B; the symbol ↑ indicates identification of a residue by dansylation.

hydrolysis in a Technicon AutoAnalyzer. Further purification of carboxymethylated methionine peptides was by gel filtration and high-voltage electrophoresis as described in the text or by ion-exchange chromatography on Dowex 1-X2 as previously described (Birshtein *et al.*, 1971b).

Other Methods. High-voltage electrophoresis at pH 3.6 and 6.5, as well as amino acid sequence analysis of peptides, was carried out as previously described (Turner and Cebra, 1971). Fractions were desalted either by freeze-drying from volatile buffers, by rotary evaporation, or by gel filtration through Sephadex G-25 in 0.05 M formic acid followed by freeze-drying. Enzymes and other materials were as previously described (Birshtein *et al.*, 1971a; Turner and Cebra, 1971). The nomenclature used for peptides from γ_2 chain has also been described previously (Turner and Cebra, 1971). The sulfur-containing peptides were identified after paper electrophoresis by dipping the paper into a solution of platinic iodide (Easley, 1965).

Results

Position of CNBr Fragment C-5. The sequence of this octadecapeptide has been reported (Turner and Cebra, 1971) and must occupy the extreme carboxyl-terminal end of γ_2 chain since it is the only fragment from the CNBr digest which lacks a homoserine residue.

Position of CNBr Fragment C-4 and Isolation of the Tryptic Peptide T3-4. Heavy chain was digested with trypsin, and the digest was applied to a column of Sephadex G-50 in 0.05 M NH_4HCO_3 (Figure 1). Each pool of effluent was recycled through the same column and a portion was taken for amino acid analysis. Pool E contained negligible amounts of methionine and will not be discussed further. Pool B was freeze-dried and dissolved in 3% pyridine, and its peptides were resolved on a column of Dowex 1-X2. The methionine-containing peptide T3-4 was purified from pool 4 (Figure 2) by paper electrophoresis at pH 3.6 and gave the analysis shown in Table I. The N-terminal amino acid of this peptide was found to be serine by dansylation. Intact peptide T3-4 (0.5 μmole) was then dissolved in 3.0 ml of 70% formic acid and cleaved after addition of 60 mg of CNBr and incubation for 5 hr at room temperature. Following CNBr treatment, the reaction was stopped by dilution with 10 volumes of distilled water, and the solution was freeze-dried. Component peptides T3 and T4 were subsequently purified by paper electrophoresis at pH 3.6. The compositions of these peptides are given in Table I along with those of T3 and T4 calculated from the known sequences of C-5 (Turner and Cebra, 1971) and C-4 (T. Trischmann, unpublished data), respectively (see Figure 3A). The N-terminal residue of peptide T4 was confirmed as serine by dansylation. Thus, the fragment C-4 is positioned penultimate to C-5 and the C-terminal sequence of fragments in γ_2 chain is C-4→C-5.

Position of CNBr Fragment C-3 and Isolation of the Tryptic Peptide T11-12-13. Pool C (Figure 1) was freeze-dried, dissolved in 3% pyridine, and applied to a column of Dowex 1-X2 (Figure 4). Subsequent preparative paper electrophoresis of pool 7 (Figure 4) yielded the methionine-containing peptide T11-12-13 (Table I). Sequential Edman degradation revealed the N-terminal sequence to be Met-Pro-. Digestion of intact T11-12-13 with carboxypeptidase B revealed lysine to be the C-terminal residue (Figure 3B). Peptide T11-12-13 was then digested with α-chymotrypsin and the component peptides (Figure 3B) Ch19, Ch20, and Ch21 were purified by paper electrophoresis at pH 3.6. The compositions (Table I) of these peptides accounted for the entirety of T11-12-13. Peptide Ch19 contained methionine and is therefore the N-terminal portion whereas Ch21 contained lysine and ac-

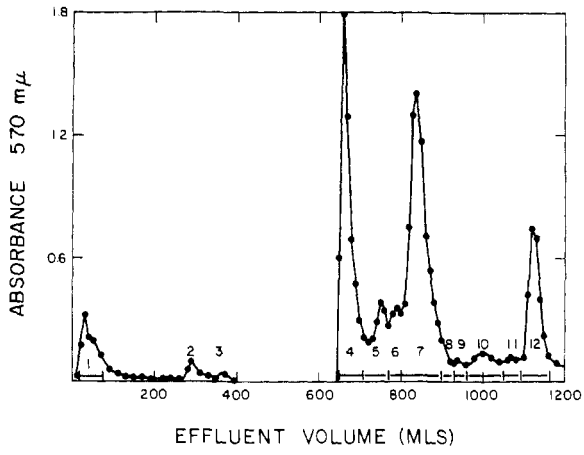

FIGURE 4: Separation of the tryptic peptides of γ_2 chain contained in pool C (Figure 1) on a column of Dowex 1-X2. Details are as in Figure 2.

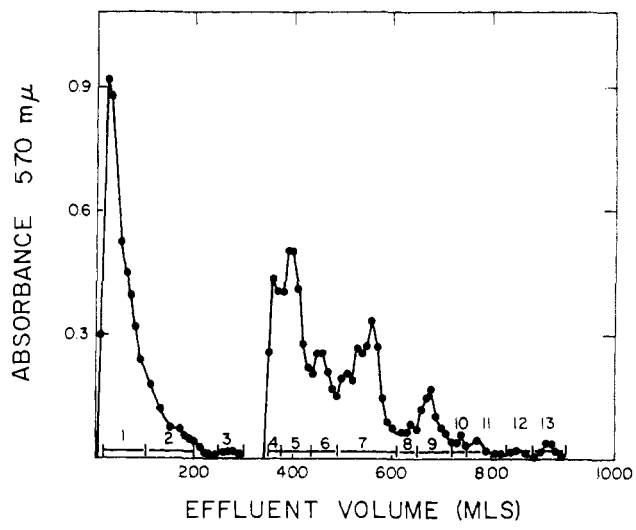

FIGURE 5: Separation of the tryptic peptides of γ_2 contained in pool D (Figure 1) on a column of Dowex 50-X8. Details of the procedure are found in the text. The effluent was monitored as indicated in the legend to Figure 2.

counts for the C terminus. Each of these chymotryptic peptides was subjected to sequence analysis as shown in Figure 3B. Peptide T11 was obtained by paper electrophoresis at pH 6.5 following tryptic digestion of intact T11-12-13. Its composition is given in Table I and sequence in Figure 3B. The N-terminal sequence of C-4 is known (T. Trischmann, unpublished data) and corresponds to the homologous portion of T11-12-13. The C-terminal sequence of C-3 has not yet been rigorously established. However, this fragment carries the heavy-chain carbohydrate moiety and has been tentatively positioned by comparison with known sequences of γ_2 chain from other species (Hill *et al.*, 1967; Cunningham *et al.*, 1969). Furthermore, the methionine N-terminal residue of T11-12-13 indicates the presence of a basic residue penultimate to homoserine of the adjacent fragment. Tryptic digestion of C-3 yields large amounts of homoserine (60%) and a tetrapeptide, Gly-Ala-Pro-Arg, which by comparison with γ_2 chain of other species, fits penultimate to the homoserine in this fragment (D. Tracey, unpublished data). Thus, fragment C-3 can be positioned immediately N terminal to C-4 and the C-terminal sequence of fragments in γ_2 chain is -C-3→C-4→C-5.

Isolation of the Tryptic Peptide T26-27 and the Position of CNBr Fragment C-1-c. The sequence of fragment C-1-c has been reported (Turner and Cebra, 1971). This fragment is known to possess the proline rich "hinge" region as well as those half-cystine residues involved in the heavy–heavy interchain disulfide bonds (Oliveira and Lamm, 1971). Pool D (Figure 1) was recycled through a column (2.2 × 180 cm) of G-25 Sephadex in 0.05 M NH_4OH. The elution diagram showed an ascending shoulder imposed on the peak of peptide-rich effluent. The main fraction contained all the methionine and was obtained by pooling effluent to exclude the peptides represented in the diagram by the shoulder. The fraction was freeze-dried, dissolved in 0.05 M pyridinium acetate (pH 2.4), and applied to a column of Dowex 50-X8 (Figure 5). Peptide T26-27 was purified from pool 4 (Figure 5) by paper electrophoresis at pH 3.6, and its composition is given in Table I. The partial sequence of this peptide (as revealed by Edman degradation) is compared to the sequence of the C-terminal tryptic peptide of C-1-c (Turner and Cebra, 1971) and the N-terminal tryptic peptide from C-3 (D. Tracey, unpublished data) in Figure 3C. It is clear that T26-27 overlaps these two cyanogen bromide fragments. Thus, the C-terminal sequence of fragments in γ_2 chain is thus extended as -C-1-c→C-3→C-4→C-5.

Isolation of Peptide Ch22 and Position of CNBr Fragment C-1-b. The sequence of the CNBr fragment C-1-b has been reported as has its isolation joined together with C-1-c by an interfragment disulfide bond (Birshtein *et al.*, 1971b). Since the C-terminal tryptic peptide of C-1-b was 27 residues long it was expected that the methionine-containing peptide overlapping C-1-b with another fragment would be among the largest peptides in the tryptic digest of γ_2 chain. However, this overlap peptide could not be isolated directly. Instead, heavy chain was isolated from completely reduced, aminoethylated IgG2, and its methionines were labeled by carboxymethylating them with iodoacetic-^{14}C acid. This labeled derivative of γ_2 chain was digested consecutively with trypsin and then chymotrypsin and the peptides were first fractionated on a column (2.5 × 12 cm) of Dowex 50-X8. The fractions obtained were assayed for radioactivity to identify those with peptides containing carboxymethylated methionine, and the profile seen in Figure 6 was obtained. Pool 3 contained the majority of the radioactive peptides (70%). This pool was dried by rotary evaporation, dissolved in 0.05 M NH_4OH, and applied to a column (2.2 × 180 cm) of G-25 Sephadex equilibrated in 0.05 M NH_4OH (Figure 7). Pool 2 (Figure 7) was further fractionated on a column of Dowex 1-X2 (0.9 × 90 cm) (Figure 8) with pyridinium acetate buffer. After preparative paper electrophoresis at pH 3.6, pool C (Figure 8) yielded the peptide Ch22 corresponding to the overlap of C-1-b and C-1-c. The composition of this peptide with carboxymethylated methionine is given in Table I, and its partial sequence is shown in Figure 3C, along with the corresponding regions of C-1-b and C-1-c. The alignment of the CNBr fragments accounting for the ≃303 C-terminal residues of γ_2 chain is thus -C-1-b→C-1-c→C3→C4→C5.

Isolation of the Tryptic Peptide T36-37 and Position of CNBr Fragment C-1-a_2. Pool A (Figure 1) yielded peptide T36-37 after fractionation on Dowex 1-X2. The isolation and sequence of this methionine-containing peptide were reported by Birshtein *et al.* (1971b). Intact T36-37 was digested with chymotrypsin and with thermolysin, and the resulting peptides

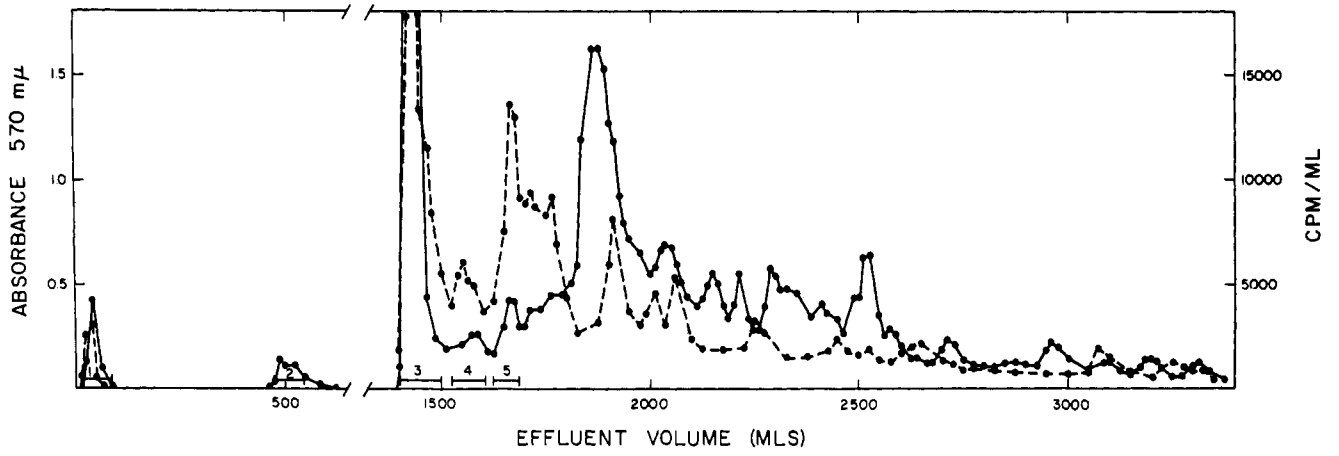

FIGURE 6: Separation of the peptides from a tryptic and chymotryptic digest of γ_2 chain on a column of Dowex 50-X8. Details of the procedures are found in the text. The column was developed sequentially with 0.01 N HCl–0.05 M pyridinium acetate (pH 2.4), and then with two linear pyridinium acetate gradients. A portion of the effluent was automatically anlayzed by the ninhydrin reaction after alkaline hydrolysis. The absorbance of the reaction was monitored at 570 mμ as shown by the solid line. The dashed line indicates the radioactivity as measured on 0.1-ml aliquots of every other tube. Fractions were collected for 7.5 min each.

were isolated by paper electrophoresis following gel filtration on Sephadex G-25. The compositions of these peptides have been reported (Birshtein *et al.*, 1971b), and their sequence is given in Figure 9A. Tryptic digestion of fragments C-1-b and C-1-a_2 (Birshtein *et al.*, 1971b; Ray and Cebra, 1972) yielded peptides corresponding in composition and sequence (Figure 9A) to the C- and N-terminal parts of T36-37, respectively. Thus this peptide overlaps the CNBr fragments C-1-a_2 and C-1-b giving a C-terminal alignment so far of C-1-a_2→C-1-b→C-1-c→C-3→C4→C5.

Position of CNBr Fragments C-1-n and C-1-a_1. No further methionine-containing peptides were isolated that overlapped fragments not already aligned, and therefore, fragments C-1-n and C-1-a_1 were positioned by other means. Intact, completely reduced and carboxymethylated γ_2 chain was subjected to automatic and sequential Edman degradation in a Beckman Model 890 sequencer. The thiazolinone derivatives were converted to the phenylthiohydantoin derivatives and the latter identified as previously described (Birshtein and Cebra, 1971). The sequence thus obtained is shown in Figure 9B. The isolation and sequence of fragment C-1-n have been elucidated (D. Benjamin, unpublished data) and, based on the identity of its sequence with that determined by automatic analysis for intact γ_2 chain, C-1-n can be positioned at the extreme N terminus of γ_2 chain (Figure 9B). Furthermore, an 11 residue peptide (Ch25, Figure 9B) having an N-terminal pyrrolidonecarboxylic acid residue has been isolated from a digest of whole heavy chain (D. Benjamin, unpublished data) and partially sequenced to support the assignment of alternative residues of pyrrolidonecarboxylic acid and Glu at position N-1 of γ_2 chain.

The seven CNBr fragments so far considered account for about 394 of the ≃443 residues of guinea pig γ_2 chain. The sequence of a 49-residue CNBr fragment (C-1-a_1) has been reported (Birshtein and Cebra, 1971). No overlapping methionine-containing peptides have been isolated that overlap this fragment with one of the others. It has therefore been placed in order directly N terminal to C-1-a_2 and C terminal to C-1-n, since there is no other relationship to the other fragments possible for this unique fragment.

Discussion

Eight fragments from a CNBr digest of γ_2 chain of guinea pig IgG2 have been placed in the order: NH_2-C-1-n→C-1-a_1→

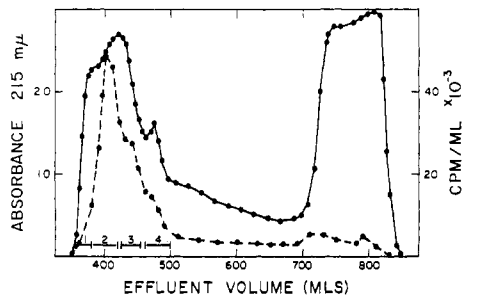

FIGURE 7: Elution profile of the fractionation of peptides of γ_2 chain contained in pool 3 (Figure 6) on a column of Sephadex G-25 equilibrated with 0.05 M NH_4OH. Details of the procedure are given in the text. The absorbance of the fractions (5 ml) was read at 215 mμ. The dashed line indicates the radioactivity as measured on 0.1-ml aliquots of every other tube.

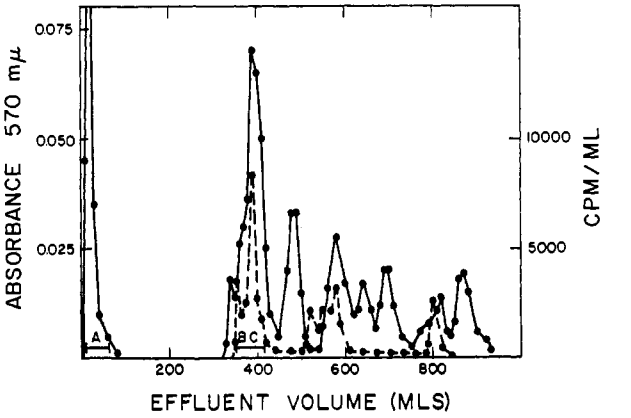

FIGURE 8: Separation of the peptides of γ_2 chain contained in pool 2 (Figure 7) on a column of Dowex 1-X2. Details are as in Figure 2. Solid line indicates the absorbance of the effluent at 570 mμ following the ninhydrin reaction. The dashed line represents the radioactivity as measured on 0.1-ml aliquots of every other tube. Fractions were collected for 7.5 min each.

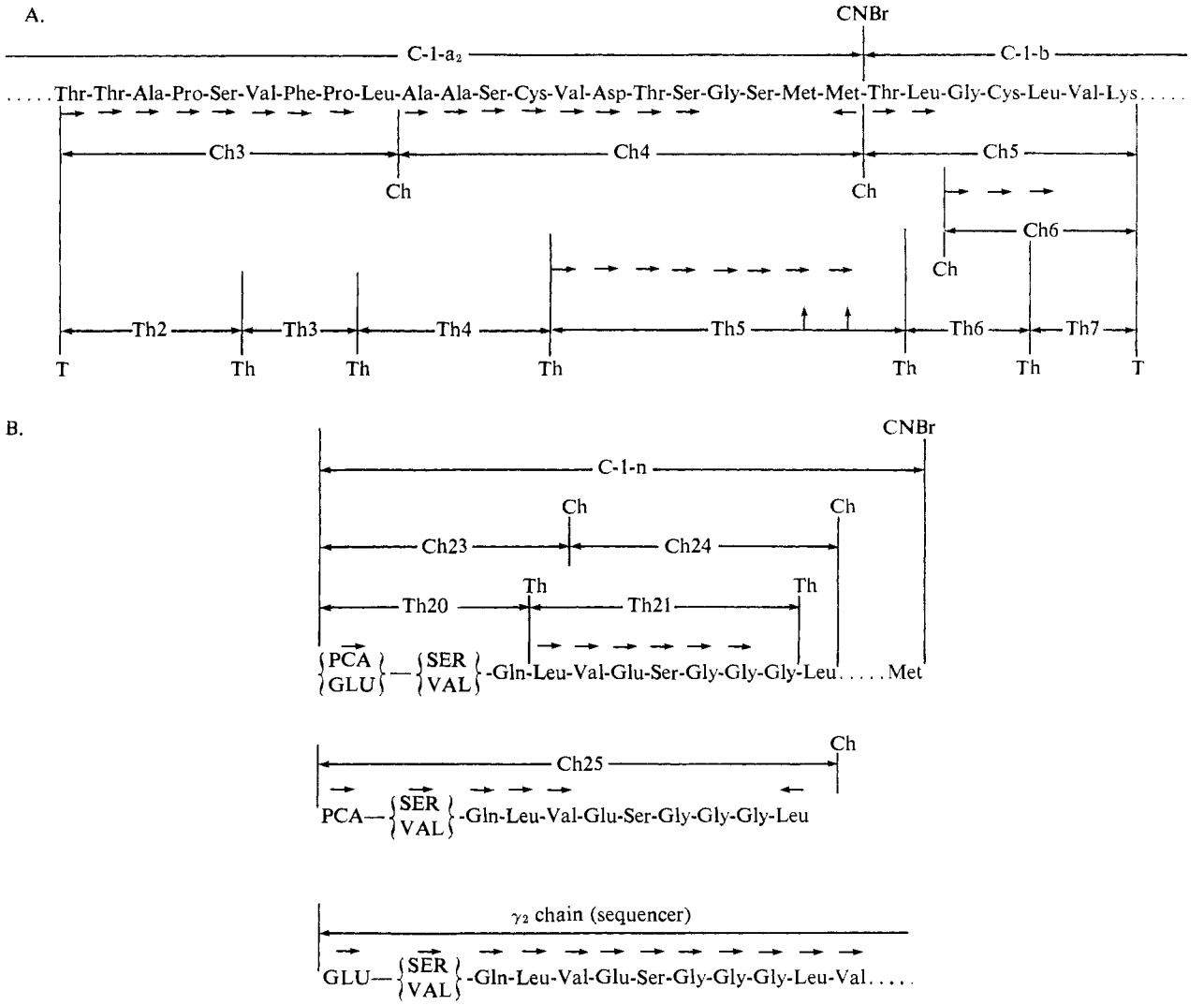

FIGURE 9: A schematic representation of the procedures and resulting data used to position the CNBr fragments C-1-a_2 and C-1-n. Details are as in Figure 3 except that the symbol, →, also indicates removal of the pyrrolidonecarboxylic acid residue with the enzyme pyrrolidonecarboxylic acid hydrolase (see Birshtein *et al.*, 1971b).

C-1-a_2→C-1-b→C-1-c→C-3→C-4→C-5-COOH. All of these fragments have been rigorously aligned except C-1-a_1, which was positioned between C-1-n and C-1-a_2 but not shown to be contiguous with either. Although it is possible that additional, smaller CNBr fragments may yet be found that also occur in γ_2 chain between C-1-n and C-1-a_2, comparison of the sequences of C-1-n, C-1-a_1, and C-1-a_2 with sequences of human γ chain (Press and Hogg, 1969; Cunningham *et al.*, 1969) suggests that it is unlikely that such fragments have been missed. It is probable that C-1-a_1 itself completely accounts for all residue positions present in guinea pig γ_2 chain between C-1-n and C-1-a_2.

The task of isolating all methionine overlap peptides from a tryptic digest of γ_2 chain containing a minimum of 52 peptides was formidable and was not completely accomplished. However, those methionine peptides that were isolated directly, usually by using as a final guide the platinic iodide stain for sulfur-containing peptides on paper, were supplemented by other peptides with radiolabeled methionines from a tryptic–chymotryptic digest. This method of carboxymethylating the methionines with iodoacetic-^{14}C acid (Wilkinson, 1969) proved invaluable in permitting the easy monitoring of methionine overlap peptides through many steps in their isolation from a complex mixture. Not only was the critical peptide overlapping C-1-b→C-1-c isolated in this way but also peptides which confirmed the alignment of C-1-c→C-3 and C-1-a_2→C-1-b.

The five CNBr fragments with constant sequences were positioned in the C-terminal three-quarters of guinea pig γ_2 chain as expected from homology with the sequences of rabbit γ_2 chain (Hill *et al.*, 1967; Cebra *et al.*, 1968) and of human myeloma γ chains (Press and Hogg, 1969; Steiner and Porter, 1969; Edelman *et al.*, 1969). Perhaps of most interest was the positioning of the two largest CNBr fragments, C-3 and C-4, such that they each account for most of a "domain," C_H2[1] and C_H3, respectively. Both of these fragments retain an intrachain disulfide bond. They may lend themselves to attempts to correlate structure of guinea pig γ_2 chain with such biologic activities as cytophilia for macrophages (Berken and Benacerraf, 1966) and activation of the complement system (Bloch *et al.*, 1963).

Knowledge of the positions of CNBr fragments within γ_2 chain and of the disulfide bonds of IgG2 enabled the development of a simpler procedure which permits the isolation of all CNBr fragments from a digest of the intact IgG2 molecule after only one or two fractionation steps (Birshtein and Cebra,

1971). This procedure is proving practical for isolation of those fragments associated with antigen-binding specificity from small amounts of specific antibodies.

Finally, perhaps the most immediately valuable feature of the alignment of the CNBr fragments is that it permits the rigorous placement of those fragments having a variable primary structure. These three fragments, C-1-n, C-1-a_1, and C-1-a_2, account for the N-terminal $\simeq$140 residues. In the accompanying paper (Ray and Cebra, 1972) we have shown that these three fragments are those which are specifically affinity labeled when anti-DNP antibody is reacted with *m*-nitrobenzenediazonium tetrafluoroborate. The data presented here permit the affinity label to be rigorously localized in the N-terminal quarter of heavy chain.

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Localization of Affinity-Labeled Residues in the Primary Structure of Anti-Dinitrophenyl Antibody Raised in Strain 13 Guinea Pigs†

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ABSTRACT: Several preparations of anti-dinitrophenyl antibody raised in strain 13 guinea pigs were modified with the affinity label, *m*-nitrobenzenediazonium-^{14}C fluoroborate. The molar ratio of modified residues in heavy and light chains was about 8:1, respectively. Fragments accounting for the entire length of the heavy chain (γ_2 chain) were isolated from CNBr digests of both whole antibody and separated γ_2 chain. The specifically modified residues were clearly localized to the three fragments C-1-n, C-1-a_1, and C-1-a_2, comprising the amino-terminal 140 residues of γ_2 chain. Each of these three fragments from "normal" γ_2 chain contains a segment with a variable primary structure. The predominant modified residue in affinity-labeled antibody was tyrosine. The positions of the azotyrosine residues appear to be within or just without those segments found to be variable in "normal" γ_2 chain. The fragments C-1-n and C-1-a_2 are linked in the parent molecule by a disulfide bond between half-cystine residues N-22 and N-96 and thus their variable segments would be close in the intact antibody molecule.

Two approaches have been used to locate the antigen binding sites in immunoglobulin molecules and, concomitantly, to deduce the primary structural basis of antibody specificity.

One method is based on the known dependence of protein conformation on amino acid sequence (Epstein *et al.*, 1963) and the corollary that the heterogeneity of antigen binding sites within an immunoglobulin population must be reflected by positions having alternative amino acid residues. Hence, comparison of the sequences of a set of homogeneous myeloma proteins or the determination of the primary structure of one polymorphic form of "normal" immunoglobulin from inbred animals would be expected to define regions of variable primary structure which presumably lie within or determine the conformation of the combining site. By comparison of sequences of human myeloma proteins, variable regions (V_H and V_L) have been localized to the amino-terminal half of light chain (Titani *et al.*, 1965; Hilschmann and Craig, 1965) and the amino-terminal quarter of heavy chain (Press and Hogg, 1969; Cunningham *et al.*, 1969; Wikler *et al.*, 1969). Positions in heavy chain having alternative residues have likewise been found only in its N-terminal quarter when

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