Interchain Disulfide Bridges of Guinea Pig γ_2-Immunoglobulin*

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ABSTRACT: The peptides of guinea pig γ_2-globulin (the principal immunoglobulin class in guinea pigs) containing the interchain disulfide bridges were isolated by diagonal electrophoresis of enzyme digests of both whole γ_2-globulin and γ_2-globulin that had been mildly reduced and alkylated with iodoacetic-^{14}C acid, and were then sequenced. γ_2-Globulin contains three inter-H-chain disulfide bridges in the "hinge" region.

The L chain is linked to another part of the H chain, in the neighborhood of residue position 135.

The principal immunoglobulin class of guinea pigs is γ_2-immunoglobulin (Benacerraf *et al.*, 1963). It can fix complement (Bloch *et al.*, 1963) and bind to macrophages (Berken and Benacerraf, 1966), but is unable to mediate anaphylaxis in guinea pigs, a property of γ_1-immunoglobulin (Ovary *et al.*, 1963; Stechschulte *et al.*, 1967). Structural studies at the level of antigenic determinants (Nussenzweig and Benacerraf, 1966), peptide maps (Lamm *et al.*, 1967), and amino acid compositions (Lamm, 1969) have demonstrated that guinea pig γ_1- and γ_2-globulins are very similar or identical in their Fab portions but dissimilar in their Fc portions, which are responsible for the so-called biological properties of immunoglobulins. Recent work suggests that the NH_2-terminal region of the Fc fragment may be involved in biological activities (Utsumi, 1969; Kehoe and Fougereau, 1969). In addition, the amino acid sequences around the interchain disulfide bridges are of interest for comparative studies. We now report the isolation and characterization of the interchain disulfide bridge peptides of guinea pig γ_2-globulin. For their isolation we have used the diagonal electrophoresis method of Brown and Hartley (1966). This approach has proved to be useful for immunoglobulins in a number of instances (for example, Frangione *et al.*, 1969).

Materials and Methods

γ_2-Immunoglobulin. Guinea pig γ-globulins were obtained from Pentex (these are almost wholly γ_2 (Nussenzweig *et al.*, 1966)) or from antibodies against the DNP[1]-hapten (Lamm and Lisowska-Bernstein, 1968). The γ_2 antibodies were separated from the γ_1 on DEAE-cellulose (Oettgen *et al.*, 1965).

Reduction and Radioactive Alkylation. For some diagonal peptide maps (see below) guinea pig γ_2-globulin (10 mg/ml) was reduced with 0.0013 M DTE in 0.5 M Tris-HCl (pH 8.2) for 90 min at 37° under N_2. The reduced protein was alkylated with 0.0038 M iodoacetic-^{14}C acid (New England Nuclear), 0.5 mCi/mmole, at room temperature for 90 min, and then dialyzed against water.

In order to learn whether native, unreduced guinea pig γ_2-globulin has appreciable available sulfhydryl groups, a sample of intact γ_2-globulin was exposed to 0.04 M iodoacetic acid at pH 8.2 for 90 min at room temperature. It was then well dialyzed in the cold against water, lyophilized, and analyzed for its CMCys content after hydrolysis *in vacuo*.

Diagonal Electrophoresis. This was done by the method of Brown and Hartley (1966). The mixture of peptides derived from γ_2-globulin by enzyme digestion was applied to a 46 × 57 cm sheet of Whatman No. 3MM chromatography paper and electrophoresed at 3000 V. Next, a 3-cm wide strip was cut along one margin and oxidized with performic acid vapor (50 ml of formic acid + 2.5 ml of 30% H_2O_2) for 4 hr in a desiccator at room temperature. The oxidized strip was sewn to another sheet of 46 × 57 cm paper; the portion of the recipient sheet under the peptide-containing strip (*i.e.*, the second thickness) was cut away; and electrophoresis was performed under the same conditions as before oxidation, but in a direction perpendicular to the first electrophoresis. The resulting diagonal map was stained with ninhydrin (1 g of ninhydrin, 600 ml of ethanol, 80 ml of collidine, and 200 ml of acetic acid), and for radioactive peptides an autoradiograph was made by placing Kodak RB54 X-Ray film next to the map. Peptides shown to be "off the diagonal" (*i.e.*, those whose electrophoretic mobility was altered by oxidation) were then isolated from the remainder of the first electrophoresis sheet (*i.e.*, minus the 3-cm wide strip used to complete the diagonal map); the strip known from the diagonal map to contain the desired cystine or CMCys (Harris, 1967) peptide was cut out, oxidized, and reelectrophoresed. The locations of pertinent peptides were identified by autoradiographs or by staining a guide strip with ninhydrin. Peptides were eluted with 0.02 N NH_4OH or water. Recoveries averaged about 15%.

Radioactivity Counting. Aqueous aliquots were dissolved in a toluene-based counting solution containing 5% (v/v) Bio-Solv-3 (Beckman) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Amino Acid Analysis. This was done by the method of Spackman *et al.* (1958), after 18–24-hr hydrolysis in 6 N HCl *in vacuo*, with a Spinco Model 120C amino acid analyzer equipped with high-sensitivity cuvets and recorder. Values

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[1] Abbreviations used are: DNP, 2,4-dinitrophenyl; DTE, dithioerythritol; CMCys, *S*-carboxymethylcysteine; $CMCysSO_2$, *S*-carboxymethylcysteine sulfone; Cya, cysteic acid; $MetSO_2$, methionine sulfone; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

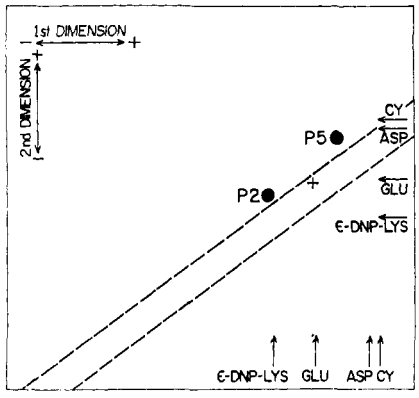

FIGURE 1: Diagonal peptide map at pH 3.5 of a peptic digest of guinea pig γ_2-globulin that had been partially reduced and alkylated with iodoacetic-^{14}C acid. P2 and P5 are the two major radioactive peptides off the diagonal band, which lies within the confines of the dashed lines. The change in mobility of P2 and P5 results from the oxidation of CMCys to $CMCysSO_2$ (Harris, 1967). The + in the diagonal band indicates the lines of origin for the two electrophoreses. The arrows indicate the positions of certain markers (see Methods).

are expressed relative to one of the residues taken as 1.0. $CMCysSO_2$ values were always low and not reliable indicators of the half-cystine content; other workers using similar methods (*e.g.*, Frangione and Milstein, 1968) have also observed this.

Dansyl-Edman Method. For sequencing peptides the procedure of Gray (1967) was used except that dansylamino acids were identified by thin-layer chromatography on either silica gel G (Press *et al.*, 1966) or polyamide sheets (Woods and Wang, 1967; Pink *et al.*, 1970). When the amount of material permitted, the identification of the dansylamino acids was checked by amino acid analysis after the next Edman degradation (subtractive Edman procedure). The net charge of peptides at pH 6.5 was determined according to Offord (1966) with mobilities expressed relative to aspartic acid taken as −1.0 (mobilities of acidic and basic peptides are listed − and +, respectively). The decrease in negative charge of a peptide after Edman degradation (and the decrease in radioactivity in the case of radioactively alkylated peptides) was helpful in locating half-cystine residues which could not be identified readily as the dansyl derivative.

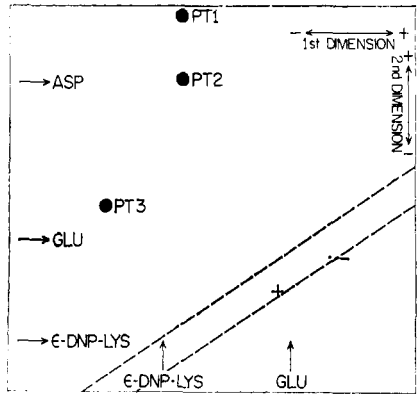

FIGURE 2: Diagonal peptide map at pH 3.5 of a peptic–tryptic digest of whole γ_2-globulin. Nonpertinent peptides off the diagonal are omitted.

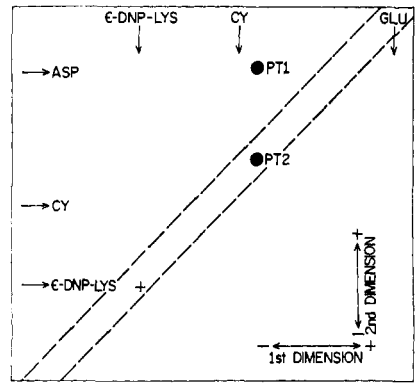

FIGURE 3: Diagonal peptide map at pH 6.5 of a peptic–tryptic digest of whole γ_2-globulin. Peptide PT2, the partner of PT1, was not apparent on this map (since it was in the diagonal band), but was isolated from the designated area by subsequent electrophoresis at pH 3.5. Nonpertinent peptides are omitted.

Enzyme Digestions. Digestion with pepsin (Worthington twice crystallized), 2.5–5% by weight of γ-globulin, was done in 5% formic acid for about 15 hr at 37°. Digests were lyophilized. Tryptic digests were made with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin (Worthington) in 1–2% NH_4HCO_3 for 6 hr at 37°. For whole γ-globulin 2% by weight of trypsin was used, and for peptides 10%. Carboxypeptidase A (DFP-treated, Worthington) digestion was carried out in 0.15 M NH_4HCO_3 for 5 hr at room temperature using 20 μg of enzyme/0.01 μmole of peptide. Digestion was stopped by adding glacial acetic acid, and the digest was taken to dryness in an evacuated desiccator. The whole digest was then applied to the amino acid analyzer.

High-Voltage Paper Electrophoresis. This was done with Whatman No. 1 or 3MM chromatography grade paper in Savant LT-48A tanks. The following buffers were used: pH 6.5 (pyridine–acetic acid–water, 33:1:300, v/v), pH 3.5 (pyridine–acetic acid–water, 1:11:190, v/v), and pH 1.9 (formic acid–acetic acid–water, 1:4:28, v/v). Certain markers were used to help in the identification of peptides: ink from a red Pentel pen (mobility ~ aspartic acid at pH 6.5), xylene cyanole FF (mobility relative to aspartic acid 0.4 at pH 6.5, and 1.4 at pH 3.5), and ε-DNP-L-lysine (neutral).

TABLE I: Amino Acid Composition of Peptide P2 from a pH 3.5 Diagonal Peptide Map of Mildly Reduced and Radioactively Alkylated Guinea Pig γ_2-Globulin.[a]

	P2	P2-T1	P2-T2	P2-T3
Lys	1.0		0.6	
Arg	1.0	1.0		
$CMCysSO_2$	1.1		0.6	0.2
Asx	2.1		1.0	1.0
Thr	2.1		1.7	
Glx	2.1		1.0	1.0
Pro	8.0	1.0	4.0	3.0
Ile	0.9	1.0		

[a] Also, the compositions of the tryptic peptides derived from peptide P2.

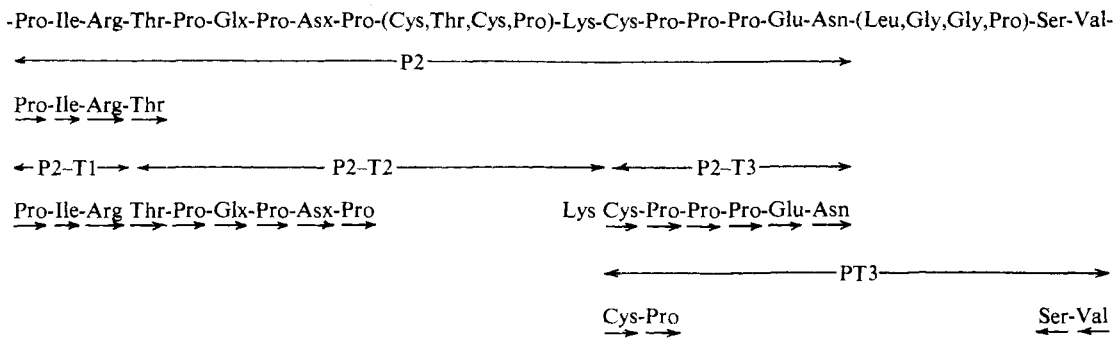

FIGURE 4: Summary of the peptides derived from the hinge region of the H chain of γ_2-globulin. (→) Residue identified by the dansyl-Edman procedure. (←) Residue identified after release by carboxypeptidase A. The order of the residues within parentheses is taken from Turner and Cebra (1971).

Results

Number of Interchain Disulfide Bridges in Guinea Pig γ_2-Globulin. γ_2-Anti-DNP antibodies were reduced with 0.01 M DTE for 1 hr at room temperature, and then alkylated at 0° with a 20% excess of iodoacetic-^{14}C acid (Lamm and Lisowska-Bernstein, 1968). The protein was next dialyzed in the cold against 0.01 M Tris-buffered saline (pH 8.2) and then against 4 M guanidine hydrochloride at room temperature, following which it was passed at room temperature through a column of Sephadex G-200 in 4 M guanidine hydrochloride. The center of the H-chain peak and the rear three-quarters of the L-chain peak were dialyzed against 0.01 M NH_4HCO_3. Aliquots were taken for liquid scintillation counting and protein determination (Lowry *et al.*, 1951). The H chains had a specific activity of 77,000 cpm/mg (4.08×10^6 cpm/μmole), and the L chains 45,000 cpm/mg (1.01×10^6 cpm/μmole). Thus, the ratio of radioactivity in H and L chain was 4:1 on a molar basis. On the assumption that only interchain disulfide bridges are easily reduced (*i.e.*, reduced in the absence of denaturants) and since there is only one H to L disulfide bridge (Lamm and Lisowska-Bernstein, 1968), it was tentatively concluded that there are three inter-H-chain bridges.

In γ_2-globulins that had been exposed to iodoacetic acid without prior reduction (see Methods), only 0.28 mole of CMCys was found per mole of γ_2-globulin, based on the composition reported by Lamm (1969), and in diagonal maps of radioactively alkylated γ_2-globulin (not previously reduced), no radioactive peptides were observed. Therefore, the radioactively alkylated half-cystines (above) and the half-cystine residues to be described below are thought to occur in disulfide bridges in the native molecule and not to be present as cysteine.

TABLE II: Amino Acid Compositions of Peptic–Tryptic Peptides from a pH 3.5 Diagonal Peptide Map of γ_2-Globulin.

	PT1	PT2	PT3
Cya	1.0	0.9	0.8
Asx		1.0	1.1
$MetSO_2$		0.7	
Thr		0.8	
Ser	0.9	2.4	1.2
Glx	1.0		1.0
Pro			3.4
Gly		0.9	2.0
Val		0.8	1.0
Leu			1.0

Inter-H-Chain Disulfide Bridges. Guinea pig γ_2-globulin (Pentex) was mildly reduced and alkylated with iodoacetic-^{14}C acid, and then digested with pepsin (see Methods). Diagonal electrophoresis was performed at pH 3.5 (Figure 1). The major radioactive peptide off the diagonal, P2, was subsequently purified at pH 6.5 (mobility ~ -0.2), and in some cases at pH 1.9 also.

The composition of peptide P2 is shown in Table I. Because of the ratios of the component amino acids found, it was considered possible that peptide P2 was only one-half the size indicated in Table I and that Lys and Arg were alternatives in the same position. If this were true the Ile content would also have been nonintegral but could have perhaps been accommodated either as an alternative to a Pro or as the result of a partial peptic split at one end of the peptide. In order to resolve these questions the amino groups of P2 were maleylated, a procedure which converts their potential charge from positive to negative (Butler *et al.*, 1969). Maleic anhydride (25-fold excess over amino groups) was added to 0.06 μmole of peptide in 0.2 M borate buffer (pH 9.0) at 0°. After 10 min the maleylated peptide was desalted on Sephadex G-25 in 0.02 N NH_4OH. Aliquots of maleylated P2 were electrophoresed at pH 3.5 (mobility cyanole FF) and pH 6.5 (mobility -0.7). No other peptides were observed by autoradiography. The remainder of the maleylated P2 was purified by electrophoresis at pH 6.5 and analyzed with the same results as before (*cf.* Table I). It was concluded that the Lys and Arg occurred in separate positions in the same peptide.

Peptide P2. The first four residues determined by the dansyl-Edman technique were Pro-Ile-Arg-Thr-. Peptide P2 was digested with trypsin and then electrophoresed at pH 6.5 yielding three ninhydrin-positive peptides, two of which were radioactive (Table I).

Peptide P2-T1. This was a nonradioactive, basic peptide with a mobility of $+0.55$. The sequence was found to be Pro-Ile-Arg, the N-terminal three residues of peptide P2.

Peptide P2-T2. This was an acidic, radioactive peptide with a mobility of -0.24. It was further purified by electrophoresis at pH 1.9. Its N-terminal residue was Thr, which established P2-T2 as being next to P2-T1 in peptide P2. The sequence was Thr-Pro-Glx-Pro-Asx-Pro-($CMCysSO_2$,Thr, $CMCysSO_2$,Pro)-Lys. Lys was positioned at the C terminus because P2-T2 was a tryptic peptide, and the order of the residues in parentheses was taken from Turner and Cebra (1971). The exact number of $CMCysSO_2$ residues was not

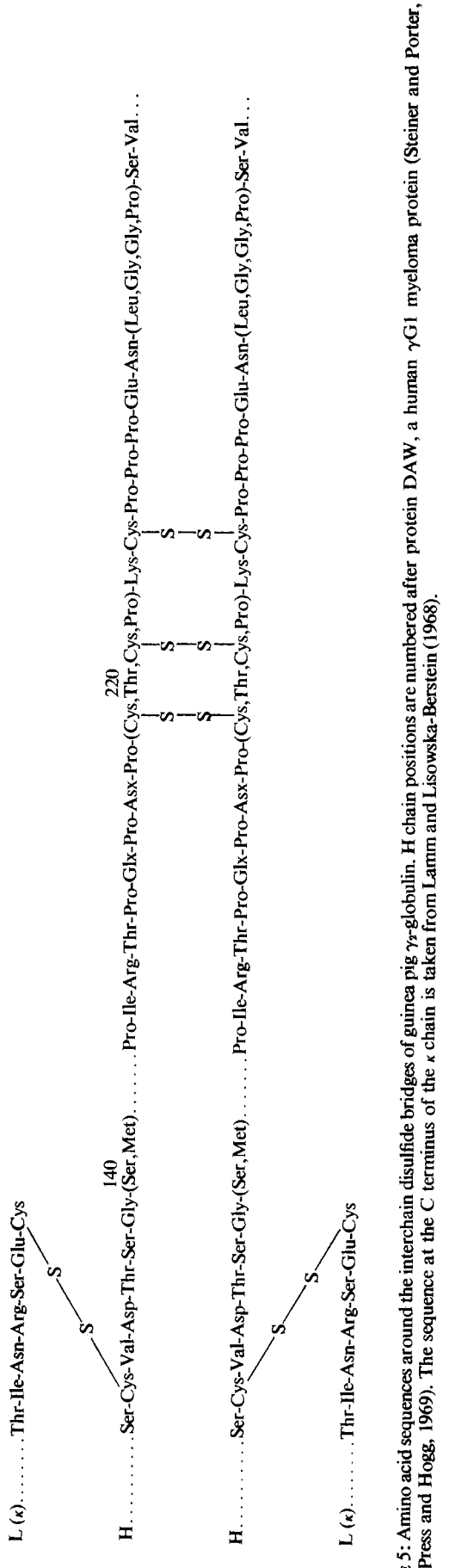

FIGURE 5: Amino acid sequences around the interchain disulfide bridges of guinea pig γ_2-globulin. H chain positions are numbered after protein DAW, a human γG1 myeloma protein (Steiner and Porter, 1967; Press and Hogg, 1969). The sequence at the C terminus of the κ chain is taken from Lamm and Lisowska-Berstein (1968).

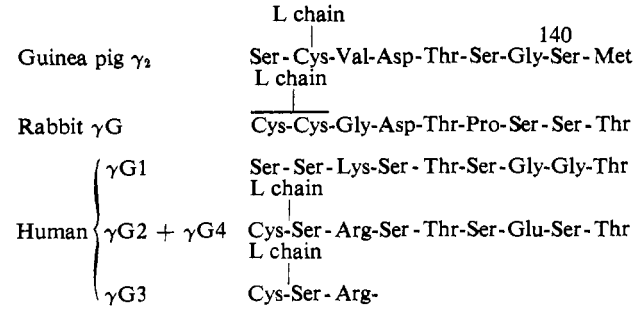

FIGURE 6: Comparative amino acid sequences around the Cys in guinea pig, rabbit, and human γG-globulin H chains which is linked to the L chain. Residue numbering is according to protein DAW (Press and Hogg, 1969). In human γG1, Cys-221 is the link to the L chain. Data are from: guinea pig, the present study and Birshtein *et al.* (1971); rabbit, O'Donnell *et al.* (1970); human γG1, Press and Hogg (1969) and Edelman *et al.* (1969); human γG2, γG3, and γG4, Frangione *et al.* (1969) and Pink *et al.* (1970).

certain. Turner and Cebra (1971) found two half-cystines in this region. The amino acid analysis indicated the presence of hexosamine although analyses of the parent peptide, P2, never showed hexosamine (we have no explanation for this discrepancy). Because of limited material and uncertainty with regard to the carbohydrate moiety, the nature of the side chains of the Asx and Glx residues was not established with certainty. In view of the two Cys residues (Turner and Cebra, 1971) and the mobility of -0.24 at pH 6.5, it is likely that they occur as the amides.

Peptide P2-T3. This was a radioactive, acidic peptide (mobility -0.92), which comprised the C terminus of the parent peptide P2. The sequence was found to be $CMCysSO_2$-Pro-Pro-Pro-Glu-Asn. The evidence for $CMCysSO_2$ at the N terminus is: (a) no dansylamino acid was identified at this position and (b) after the first Edman degradation the electrophoretic mobility decreased to -0.31 and 70% of the radioactivity was lost. The nature of the Glu and Asn residues was determined as follows. After the fourth Edman degradation the dipeptide Glx-Asx had a mobility of -0.65 (indicative of 1 net negative charge), and after the fifth Edman cycle the last amino acid was neutral.

All of the half-cystines of peptide P2 were found to be involved in inter-H-chain disulfide bridges (see below).

Disulfide Bridge between the H and L Chains. Diagonal peptide maps were made of peptic–tryptic digests of guinea pig γ_2-globulin. Following digestion with pepsin in 5% formic acid, the peptides were lyophilized and then digested with trypsin in 2% NH_4HCO_3. Diagonal electrophoresis was carried out at pH 3.5 and the results were confirmed by a pH 6.5 diagonal map.

Diagonal Map at pH 3.5. The map is illustrated in Figure 2. Peptides PT1 and PT2 off the diagonal had had the same electrophoretic mobility after the first dimension (before oxidation), and were therefore most probably partners in the same disulfide bridge. They were purified at pH 6.5. Their compositions are listed in Table II.

Peptide PT1. This acidic peptide had a mobility of -1.0 and the sequence Ser-Glu-Cya, which corresponds to the C-terminal tryptic peptide of guinea pig κ chains (Hood *et al.*, 1967; Lamm and Lisowska-Bernstein, 1968). This finding indicated that PT2, its partner in the diagonal map, is the H-chain peptide linked to the L chain.

Peptide PT2. This was an acidic peptide with a mobility of -0.62. The amino acid sequence was found to be Ser-Cya-

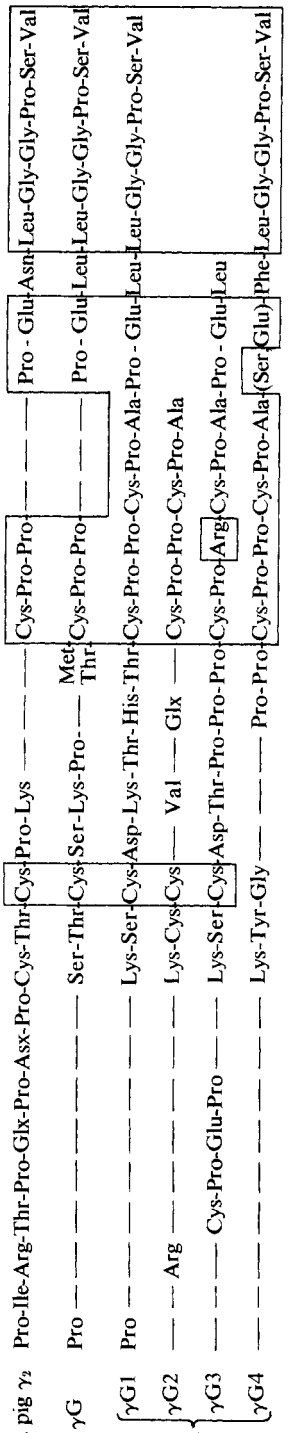

FIGURE 7: Comparative sequences in the hinge regions of various H chains: guinea pig γ_2-globulin from the present study and Turner and Cebra (1970); rabbit γG from Fruchter *et al.* (1970); human γG1 from Steiner and Porter (1967), Frangione and Milstein (1967), and Edelman *et al.* (1969); and human γG2, γG3, and γG4 from Frangione *et al.* (1969) and Pink *et al.* (1970). The placement of the gaps (large dashes), which were introduced to maximize homologies, is arbitrary in some instances. Residues that appear to be invariant, or nearly so, are blocked together. All the Cys residues form inter-H-chain bridges except 221 in rabbit, which is part of an intrachain bridge (O'Donnell *et al.*, 1970), and 221 in human γG1, which is linked to the L chain. The numbering is according to protein DAW (Steiner and Porter, 1967; Press and Hogg, 1969).

Val-Asp-Thr-Ser-Gly-(Ser,$MetSO_2$). Methionine sulfone was released after digestion with carboxypeptidase A (11% yield). The electrophoretic mobility showed the Asx to be aspartic acid (see Offord, 1966). The sequence of peptide PT2 is in agreement with Birshtein *et al.* (1971).

Peptide PT3. This peptide had no partner in the diagonal map. It was purified at pH 6.5 (mobility −0.49), and had the composition given in Table II. The high proline content and lack of a partner suggested that PT3 forms an inter-H-chain symmetrical disulfide bridge in the hinge region. The first two residues by the dansyl-Edman technique were Cya-Pro. Valine and serine were obtained in 100 and 67% yields, respectively, after digestion with carboxypeptidase A. Peptide PT3 apparently includes peptide P2-T3 plus an additional six residues. Its sequence is therefore Cya-Pro-Pro-Pro-Glu-Asn-(Leu,Gly,Gly,Pro)-Ser-Val (the order of the residues in parentheses is taken from Turner and Cebra, 1971).

Diagonal Map at pH 6.5. This is illustrated in Figure 3. Peptide PT1, the C terminus of the κ chain, was readily identified but it did not have a partner off the diagonal. However, the expected mobility of the H-chain portion of the H–L disulfide bridge peptide (*i.e.*, peptide PT2 isolated from the pH 3.5 diagonal map as described above) would have placed it in the main diagonal band of the pH 6.5 diagonal map. Consequently, an attempt was made to isolate this same peptide, PT2, from the portion of the pH 6.5 diagonal band postulated to contain the partner of PT1, the κ-chain peptide (see Figure 3). Indeed, peptide PT2 was isolated from this region by electrophoresis at pH 3.5.

Isolation of the H-Chain Portion of the H–L Disulfide Bridge after Mild Reduction and Radioactive Alkylation. Peptide P5 shown on the pH 3.5 diagonal map after mild reduction and alkylation with iodoacetic-^{14}C acid (Figure 1) was purified by electrophoresis at pH 3.5 after oxidation. Its composition was the same as PT2 (Table II) except for $CMCysSO_2$ instead of Cya. The fact that this peptide, P5 or PT2, was labeled by radioactive alkylation after mild reduction is consistent with its identification *via* diagonal electrophoresis without prior reduction as the H-chain peptide linked to the L chain.

In Figure 4 are summarized the peptides from the hinge region of the H chain of γ_2-globulin, and Figure 5 shows the sequences around the interchain disulfide bridges of γ_2-globulin. The findings agree with those of Turner and Cebra (1971) and Birshtein *et al.* (1971).

Discussion

In the present work the peptides containing the interchain disulfide bridges of guinea pig γ_2-globulin were identified and isolated by the technique of diagonal electrophoresis. When the γ_2-globulin was first mildly reduced and then alkylated with iodoacetic-^{14}C acid, four easily labeled half-cystine residues were found per H chain. This suggested, but did not prove, that there are three inter-H-chain cystine bridges and two H–L interchain bridges (one H–L bridge for each H–L chain pair) in the intact molecule. Identification of the H chain half-cystine which is joined to the L chain was obtained by identifying and isolating the H-chain peptide which was the partner of the peptide containing the C-terminal half-cystine of the κ chain. The same H-chain peptide was isolated from separate diagonal maps run at two different pH's, 3.5 and 6.5. The remaining three easily reduced half-cystines of the H chain are located within a six-residue segment in the hinge region. The half-cystine at residue position 227, protein DAW numbering scheme (Steiner and Porter, 1967; Press

and Hogg, 1969) (position 224 in Figure 5), probably forms a symmetrical interchain bridge because it was found in a peptide, PT3, which lacked a partner on the diagonal map. From steric considerations the half-cystines at positions 219 and 221 cannot be linked to one another in an intrachain bridge. If these two half-cystines had been joined in disulfide bridges to other half-cystine residues elsewhere, there should have been two additional radioactively alkylated (after mild reduction) half-cystine peptides in addition to those found. Furthermore, evidence was obtained that there are four easily reduced H-chain half-cystines in γ_2-globulin (see above), and these were all accounted for. Thus, it is likely that Cys-219 and Cys-221 partake in inter-H-chain bridges with each other. Whether they are joined in parallel or anti-parallel fashion is not known.

In Figure 6 the guinea pig, rabbit, and human γG sequences adjacent to the H-chain half-cystine which is linked to the L chain are compared. The amino acid sequence in the hinge region of the H chain of guinea pig γ_2-globulin is compared to that of rabbit γG and the four subclasses of human γG-immunoglobulin in Figure 7. Although certain homologies and differences are apparent (see also Pink *et al.*, 1970, and Turner and Cebra, 1971), more sequence data will be needed to see if there are any obvious correlations between primary structure and biological activity.

Acknowledgment

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