# Molecular Dissection of Immunoglobulin G. Conformational Interrelationships of the Subunits of Human Immunoglobulin G†

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ABSTRACT: Circular dichroic (CD) and infrared (ir) spectroscopic examinations of human immunoglobulin G (IgG) and its chemically and enzymatically defined subunits suggest the presence of β-sheet structure within the immunoglobulin molecule. The CD spectrum from 210 to 250 nm of human IgG could be resolved into two component spectra consisting of a major contribution at 217 nm and a minor contribution centered between 225 and 235 nm. The minor contribution apparently arises independently in the Fc region of the molecule. No hydrogen bonding of the type associated with β-sheet structure exists between the Fab and Fc subunits. Ir analysis suggests the presence of the β-sheet type of structure between the heavy and light chains of the IgG molecule. The C-terminal constant region of the heavy chain represented by component II and F'c exhibited a broad CD spectrum which exhibited a minimum at 224 nm. Although component II failed to exhibit a CD band attributable to the presence of β-sheet structure, ir analysis indicated the presence of this class of secondary structure and the lack of α helix. The structural features which give rise to the 224-nm band were found to be susceptible to disorganization by guanidine-HCl, as was the organized structure of both IgG and isolated light chains. Reduction and alkylation of the intrachain disulfide linkages of normal light chains did not influence the denaturation spectra as determined in guanidine-HCl. Enzymatic fragments, produced by tryptic hydrolysis between the variable and constant portions of the light chain, exhibited a CD spectrum not unlike the spectrum of intact light chains examined under identical solvent conditions, suggesting independent folding of these subunits. The lack of interactions between both major and minor enzymatic subunits of IgG is consistent with previous observations on the susceptibility of IgG to enzymatic proteolysis, and with the concept of regional differentiation of function within the antibody molecule.

Previous spectroscopic studies of immunoglobulins employing optical rotatory dispersion (ORD), circular dichroism (CD), and infrared analysis (ir) have demonstrated that this class of macromolecule was virtually devoid of α helix and contained considerable amounts of β-sheet structure (Sarkar and Doty, 1966; Ross and Jirgensons, 1968; Cathou *et al.*, 1968; Abaturov *et al.*, 1969). Secondary structure similar to β sheet has been detected in the immunoglobulins of nine species of lower vertebrates representing phylogenetic deviations from the evolutionary line leading to the mammals (Litman *et al.*, 1971). Furthermore, the CD spectra of both human IgA and IgM, as well as all four subclasses of human IgG, exhibit prominent negative dichroism bands in the spectral region assigned to the β-sheet structure (Litman *et al.*, 1971; Frommel *et al.*, 1970). All of these immunoglobulins exhibit CD spectra which lack detectable contributions from the α-helix conformation.

The CD bands assignable to the β-sheet structure are apparently not dependent on the structural integrity of the complete immunoglobulin molecule. Bence-Jones proteins, isolated from the urine of patients afflicted with multiple myeloma and consisting only of the light-chain portion of the immunoglobulin molecule, exhibit a prominent, negative CD band, interpretable as representing β-sheet structure (Ikeda *et al.*, 1968; Ghose and Jirgensons, 1971b; Kincaid and Jirgensons, 1972). Fab and Fc subunits derived from both human and rabbit IgG also exhibit bands assignable to β structure (Cathou *et al.*, 1968; Doi and Jirgensons, 1970; Litman *et al.*, 1970; Ghose and Jirgensons, 1971a).

In a preliminary article (Litman *et al.*, 1970), we confirmed the presence of β-sheet structure in the isolated Fab and Fc regions of the IgG molecule, and noted it to be qualitatively detectable in both heavy and light chains. The apparent lack of β-sheet structure in a fragment consisting of roughly half of an Fc region suggested interdomain (Edelman and Gall, 1969) interaction in formation of the β-sheet structure. In this article we report circular dichroism and infrared spectroscopic studies aimed at further analysis of β structure in the immunoglobulin molecule and elucidation of the conformational interactions within and between the major subunits of the immunoglobulin molecules.

## Materials and Methods

*Analytical Methods.* Analytical ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge equipped with Schlieren phase plate optics, electronic speed control, and automatic temperature regulator. Calculations of sedimentation velocities were performed as described by Schachman (1957).

Cellulose acetate electrophoresis at pH 8.2 was carried out in a Beckman Microzone electrophoresis apparatus using the manufacturer's membranes, buffers, and suggested running times. The dried membranes were stained with Ponceau Red, and scanned in a densitometer.

Immunoelectrophoresis was performed with only minor modification of the method originally described by Scheid-

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egger (1955). Noble special agar in 0.05 M barbital buffer, pH 8.6, was used.

Polyacrylamide gel electrophoresis, employing pH 9.5 separating gels, was performed as described (Davis, 1964). Acid–urea polyacrylamide gel electrophoresis was performed by the method of Neville (1967). N-Terminal amino acids were determined by the method of Gros and Labouesse (1969). Following reaction of the protein with dansyl[1] chloride (Pierce Chemical Co., Rockford, Ill.), the protein was precipitated with $Cl_3CCOOH$, washed, and hydrolyzed in 6 N HCl at 110° before application to polyamide sheets (Chun Chek-ken, Trading Co., Taiwan, Formosa) and subsequent chromatography. The dansyl amino acids were positively identified by parallel reference runs with dansyl amino acid standards.

*Purification of Immunoglobulin G.* IgG was purified from normal human serum by ion exchange chromatography on DEAE-Sephadex. Serum (20 $cm^3$) was dialyzed for 18 hr against a 1000 volume excess of 0.0175 M sodium hydrogen phosphate, pH 6.6, at 4°. The fine precipitate which formed under these conditions was removed by slow speed centrifugation and the supernate material was applied to a 2.4 × 40 cm column of DEAE-Sephadex in the same buffer. The fall-through material was found to represent IgG applying the criteria of immunoelectrophoresis, polyacrylamide gel electrophoresis, and analytical ultracentrifugation.

*Preparation of Heavy and Light Chains.* Heavy and light chains were prepared from IgG which had been dialyzed against 0.15 M Tris-HCl–0.15 M NaCl, pH 8.0, and reduced with dithiothreitol at 15-fold excess over the interchain half-cystine residue. Reduction was allowed to proceed for 40 min at 23° in a glass-stoppered test tube. Alkylation of the reduced interchain half-cystine residues was performed with iodoacetamide at a twofold molar excess over dithiothreitol for 20 min at 0°. The reduced and alkylated IgG was dialyzed against 1 N propionic acid for 6 hr prior to application to a 2.3 × 140 cm column of Sephadex G-200 equilibrated with the same solvent. The distinct heavy- and light-chain peaks were concentrated and dialyzed successively against 0.1% acetic acid (1000 volume excess for 12 hr) and 0.02 M sodium acetate–acetic acid, pH 5.5 (1000 volume excess for 12 hr), at 0°. The isolated light chains were shown to be free of heavy-chain contaminants based on behavior in acid–urea polyacrylamide gel electrophoresis, double diffusion in agar gel, and analytic gel filtration on Sephadex G-150 in 6 M guanidine-HCl (Heico, Inc., Delaware Water Gap, Pa.).

To reduce intrachain disulfides, light chains were first dialyzed against 6 M guanidine-HCl–0.30 M Tris-HCl, pH 8.0, for 24 hr at 23°. Dithiothreitol dissolved in the guanidine-HCl buffer was added to a 100-fold molar excess over intrachain half-cystine and reduction was allowed to proceed for 2 hr in stoppered test tubes. Alkylation, performed with iodoacetamide added dry in a twofold molar excess over dithiothreitol, was allowed to proceed for 18 hr at 4°. This procedure was capable of reducing and alkylating 3.8 of the theoretical four half-cystine residues per molecule of previously isolated light chains as determined by alkylation with [$^{14}$C]-iodoacetamide. The reduced and alkylated preparations were dialyzed against 4.8 M guanidine-HCl prior to spectral analysis.

*Preparation of Enzymatic Fragments and Subfragments.* Light chains were cleaved with trypsin to produce "half" light-chain fragments by a modification of the procedure described by Karlsson *et al.* (1969). Light chains, isolated by the procedure described above, were dialyzed against 1.0 M Tris-HCl, pH 8.0, for 18 hr at 4°. L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin (Worthington Biochemicals, Freehold, N. J.) dissolved in the pH 8.0 buffer was added to the light-chain preparations at an enzyme to substrate ratio of 1:500 (w/w) and proteolysis was allowed to proceed in a stoppered test tube for 60 min at 37°. Soybean trypsin inhibitor (Worthington Biochemicals, Freehold, N. J.) was added at a 1.5-fold molar excess over trypsin and the entire mixture was applied to a 1.5 × 140 cm column of Sephadex G-100 in 1.0 M Tris-HCl–1.0 M NaCl, pH 8.0. The distinct chromatographic peak eluting after the bulk of the intact light chains was identified as representing half light chains based on both electrophoretic mobility and N-terminal amino acid analysis.

Fab and Fc were obtained by digestion of human IgG with papain in the presence of cysteine by the method of Porter (1959). A 3-hr digestion was performed and the proteolysis was terminated by addition of iodoacetamide in 50% excess over cysteine. The digest was immediately applied to a column of Sephadex G-100 in phosphate-buffered saline at pH 7.3. The second peak which emerged from the column, representing subunits with molecular weights of 50,000, was subjected to zone electrophoresis in starch and separated into Fab and Fc components. The possibility of free carbohydrate contributing to the CD spectra was averted by extensive dialysis of the Fab and Fc prior to spectral analysis. Fab and Fc were characterized by analytical gel filtration in 6 M guanidine-HCl, polyacrylamide gel electrophoresis and double diffusion in agar gel employing antisera monospecific for Fab and Fc. $Fab'_2$ was obtained from IgG by digestion with pepsin as described by Nisonoff *et al.* (1960).

Pepsin component II was obtained from IgG at pH 4.2 in 0.2 M sodium acetate–acetic acid by digestion with pepsin as described by Nisonoff *et al.* (1960). Enzymatic digests were applied to columns of Sephadex G-75. The peak emerging at an elution volume/void volume ratio of 1.95 was found to represent component II based on analytical gel filtration in 6 M guanidine-HCl, polyacrylamide gel electrophoresis at both neutral and acid pH, and by double diffusion reactions in agar as described by Frommel and Hong (1970). At neutral pH, component II behaved as a noncovalent dimer.

F'c was recovered from papain digests of IgG by a combination of ion exchange chromatography on carboxymethylcellulose and gel filtration (Poulik, 1966). F'c also was characterized by gel filtration in 6 M guanidine-HCl, polyacrylamide gel electrophoresis and double diffusion reactions in agar gel. Like component II, the F'c behaved as a noncovalent dimer in neutral aqueous solvents.

*Spectroscopic Techniques.* Protein concentrations were determined by both a modified Folin–Ciocalteu method (Lowry *et al.*, 1951) and from the uv absorption spectra of the individual proteins determined in a Cary 1605 recording spectrophotometer using appropriate extinction coefficients.

Circular dichroism spectra were determined in either a Cary 60 recording spectropolarimeter equipped with CD attachment or a Durrum-Jasco SP-245 (used for the guanidine-HCl denaturation experiments). Cylindrical cells of path length 0.5, 1.0, and 2.0 cm were used for the determination of the CD spectra in systems with low solvent absorption, and 0.1 mm cells were used for analyses involving the 4.8 M guanidine-HCl solutions. For calculation of results presented as molar ellipticity, $[\theta']$, we used the relationship $[\theta'] = \theta M/100lc$ where $\theta$ is the observed ellipticity in degrees, $M$ is the mean residue molecular weight, taken as 108 for all im-

[1] Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

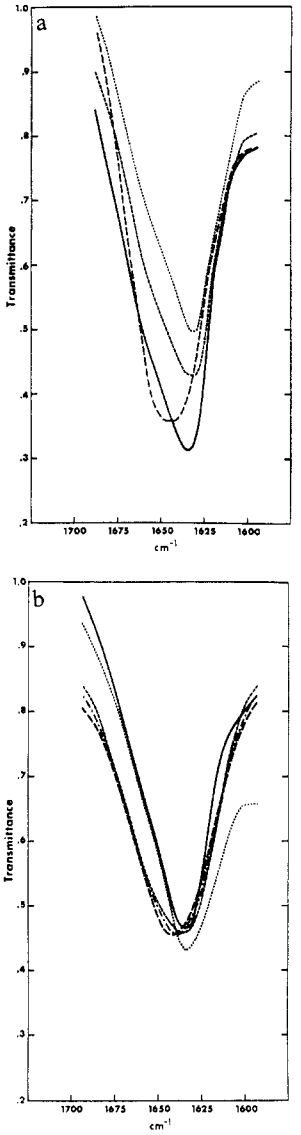


FIGURE 1: (a) Infrared spectra in $D_2O$ solution of various reference proteins and a purified IgG myeloma protein: (—·—) IgG myeloma protein; (– – –) bovine serum albumin; (- - -) carbonic anhydrase; (· · ·) α-chymotrypsin. (b) Infrared spectra in $D_2O$ solution of IgG and various IgG fragments: (—) IgG; (– – –) γ-type heavy chain; (- - - -) light chain; (· · ·) component II; (– – – – –) composite spectra consisting of an equimolar mixture of heavy (γ) and light chains. The experimentally determined spectra were identical with the calculated spectra for an equimolar mixture of heavy and light chains.

munoglobulins and immunoglobulin subunits (Crumpton and Wilkinson, 1963), *l* is the pathlength of the sample, and *c* is the concentration of the sample in grams/milliliter.

Infrared spectra were determined in a Perkin–Elmer Model 52i ir spectrophotometer utilizing $CaF_2$ cells. The proteins or their subunits were dialyzed exhaustively against 0.001 M KCl buffer, quick frozen, and immediately lyophilized. After drying over a bed of $P_2O_5$ in the presence of concentrated $H_2SO_4$, the proteins were dissolved in a minimal quantity of $D_2O$ and H → D exchanged overnight. The proteins were again freeze-dried and redissolved in $D_2O$ at a pD 5.4. The concentrations were adjusted to be 3 mg/ml by the addition of the $D_2O$ at pD 5.4, and spectra were determined at 23°. All of the proteins and subunits, with the exception of the heavy chains, were readily soluble following this treatment. The addition of DCl was found necessary to assure complete solubility of the heavy chains.

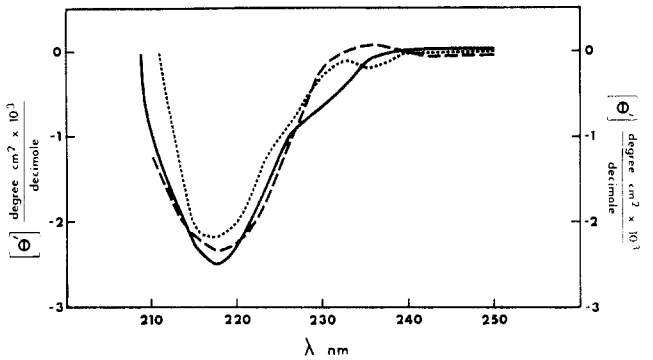


FIGURE 2: Circular dichroism spectra of IgG (—), Fab′$_2$ (– – –), and Fab (- - - -) in 0.02 M sodium hydrogen phosphate–0.15 M NaCl, pH 7.3.

*Myeloma and Heavy-Chain Disease Proteins.* An IgG1 myeloma protein with λ-type light chains was generously provided by Dr. H. H. Fudenberg. The protein was cleaved with trypsin to produce Fab and Fc subunits as described (Edelman *et al.*, 1968). Heavy-chain disease protein was generously provided by Dr. M. D. Poulik.

## Results

Figure 1a illustrates the amide I frequencies in $D_2O$ of an IgG1 myeloma protein and several other proteins included for reference. A distinct similarity is readily observable between the IgG1 myeloma, α-chymotrypsin, and carbonic anhydrase, the secondary structure of the latter two proteins being largely dominated by β-sheet structure. Bovine serum albumin exhibits a markedly different spectra consistent with the presence of considerable α helix in the secondary structure of this protein. The spectra of these latter three proteins are in close agreement with the published values of Timasheff *et al.* (1967).

Figure 1b provides the amide frequencies in $D_2O$ of several immunoglobulin fragments, as well as intact IgG. Particularly notable in these spectra is the absence of significant absorption in the 1650-$cm^{-1}$ spectral region, consistent with an absence of α helix. When analyzed in parallel with the immunoglobulin and immunoglobulin subfragments, pancreatic ribonuclease, a protein known to be largely devoid of α helix, actually exhibited more absorption in the 1650-$cm^{-1}$ region than did the immunoglobulins (not illustrated). Heavy chains and light chains exhibited spectra which did not reconstitute the spectra of the IgG myeloma protein from which they had been chemically derived. This latter point is of extreme importance in that, unlike CD spectra, the interpretation of ir spectra, based on relative intensity of parts, is not as sensitive to concentration or subpopulation interactions, a matter of concern in the interpretation of results obtained with the former technique.

Figure 2 represents the CD spectra of IgG, Fab′$_2$, and Fab. All three spectra are characterized by a prominent negative ellipticity band centered near 217 nm. In addition, the spectra of intact IgG are further characterized by a broad CD band occurring between 225 and 235 nm. The absence of this latter band in the spectra of Fab′$_2$ and Fab suggests that it may either have arisen independently in the Fc region of the molecule or been generated by an Fab–Fc interaction in the intact

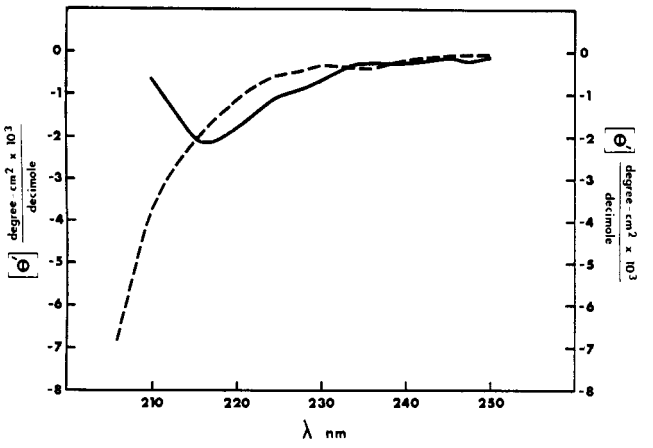


FIGURE 3: Effect of guanidine-HCl on the circular dichroism spectrum of IgG in 0.02 M sodium hydrogen phosphate–0.15 M NaCl, pH 7.3: (—) guanidine-HCl not present and (- - - -) 4.8 M guanidine-HCl present.

molecule. The possibility that an Fab–Fc interaction accounts for the band was excluded when these two enzymatically (trypsin) generated components, derived from an IgG1 myeloma, were analyzed in a mixture at a Fab:Fc ratio of 2:1. The spectra were found to be coincident with the spectra of intact myeloma protein (not illustrated).

Figure 3 demonstrates the loss of organized structure of IgG in 4.8 M guanidine-HCl, pH 7.3. Loss of structure occurred rapidly and, within the limits of experimental precision, was apparently complete within 1 hr.

Figure 4 provides the CD spectra of Fc, heavy-chain disease protein, and isolated heavy chains of the $\mu$ and $\gamma$ class. By examination of the calculated Fc spectra, it is apparent that the broad negative ellipticity band noted in the CD spectra of IgG between 225 and 235 nm arises within this region of the molecule. Of further note is the decreased molar ellipticity in the spectra of both the Fc and the heavy-chain disease protein when compared to the Fab portion of

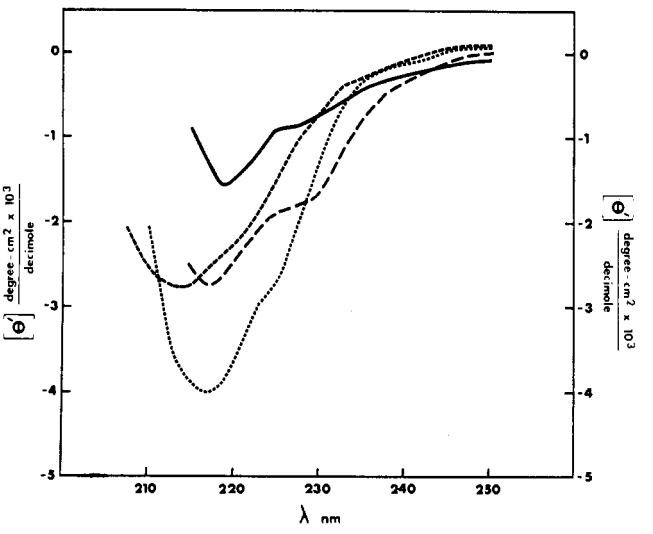


FIGURE 4: Circular dichroism spectra of Fc (—), heavy-chain disease protein (– · –), $\gamma$-type heavy chain (- - - - -), and $\mu$-type heavy chain (· · ·). Fc and heavy-chain disease protein were analyzed in 0.02 M sodium hydrogen phosphate–0.15 M NaCl, pH 7.3. $\gamma$- and $\mu$-type heavy chains were analyzed in 0.02 M sodium acetate–0.15 M NaCl, pH 5.5.

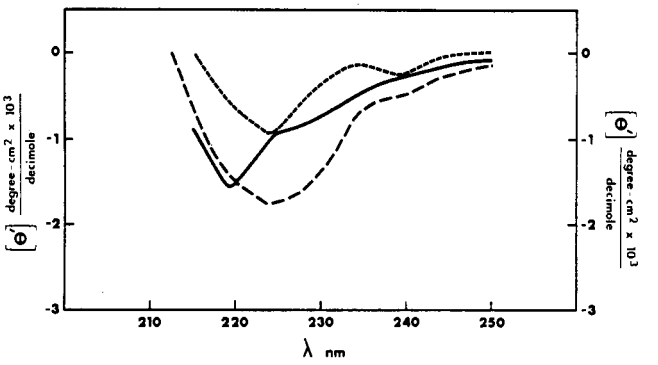


FIGURE 5: Circular dichroism spectra of Fc (——), component II (– – –), and F′c (- - - - -) in 0.02 M sodium hydrogen phosphate–0.15 M NaCl, pH 7.3.

the molecule. The heavy-chain disease protein exhibited a somewhat higher negative ellipticity at 217 nm than did the Fc fragment. The inclusion of at least a portion of Fd in the heavy-chain protein could provide an explanation for this observed difference (Terry and Ohms, 1970). The two heavy-chain preparations yielded somewhat different spectra. This difference could arise either through possession of distinct, unique conformations or could be accounted for by differences in solution behavior of the two preparations. The $\mu$-chain preparation analyzed was largely monomeric (24 hr after being transferred from 0.1% acetic acid to 0.02 M sodium acetate–acetic acid–0.15 M NaCl, pH 5.5, over 90% of the protein sedimented at 7 mg/ml as a monomeric species). The $\gamma$-chain preparations employed were somewhat erratic in polymeric behavior. A maximum of 70% sedimented as monomer at 7 mg/ml, and the remainder distributed as polymers of increasing size 24 hr after being transferred from 0.1% acetic acid to 0.02 M sodium acetate–acetic acid–0.15 M NaCl, pH 5.5. Mixing type experiments, although helpful in discerning intersubunit conformational interactions, were not attempted with equimolar portions of heavy and light chains due to these uncontrollable self-association factors.

Figure 5 illustrates the CD spectra of Fc in comparison with two of its component subunits, component II and F′c.

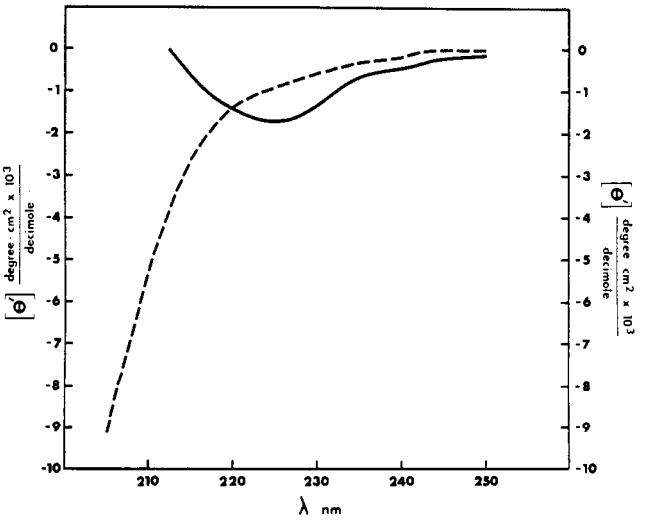


FIGURE 6: Effect of guanidine-HCl on the circular dichroism spectrum of component II in 0.02 M sodium hydrogen phosphate–0.15 M NaCl, pH 7.3: (——) guanidine-HCl not present and (– – –) 4.8 M guanidine-HCl present.

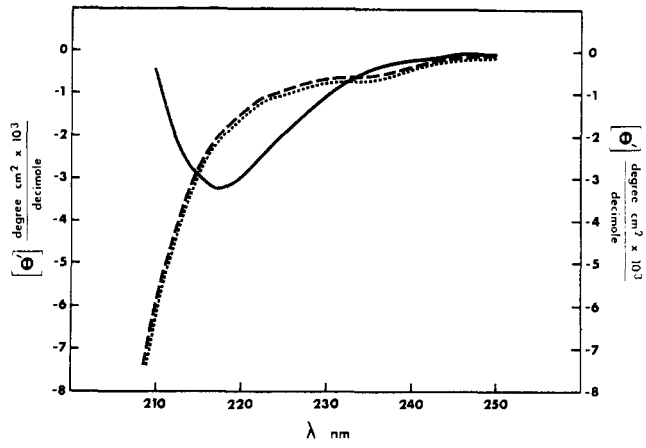


FIGURE 7: Circular dichroism spectrum of light chains derived from normal IgG and effect of guanidine-HCl on the circular dichroism spectrum of light chains with intact and reduced and alkylated intrachain disulfide linkages: (——) light chains in 0.02 M sodium acetate–0.15 M NaCl, pH 5.5; (– – –) light chain in the presence of 4.8 M guanidine-HCl; and (· · ·) light chain with reduced and alkylated intrachain disulfide linkages in the presence of 4.8 M guanidine-HCl. Guanidine solutions were buffered at pH 5.5 with sodium acetate.

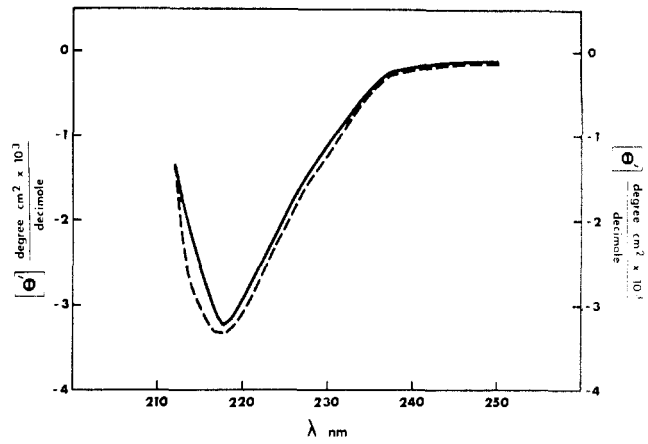


FIGURE 9: Circular dichroism spectra of (—) intact and (· · ·) half-light chains analyzed in 0.05 M Tris-HCl–1.0 M NaCl, pH 8.0.

The CD spectra of Fc can readily be resolved into two contributions centered at 217 nm and between 225 and 230 nm. Neither component II nor F'c possesses a distinct CD band at 217 nm but rather is comprised of a minimal contribution at 240 nm and a major contribution with a negative ellipticity maximum at 224 nm. The component II spectra were more intense than were the spectra of the structurally shorter F'c subunit.

Figure 6 illustrates the disorganization of component II in 4.8 M guanidine-HCl. The prominent CD band centered near 224 nm collapses and a typical denaturation spectra is revealed. This disorganization supports the contention that the 224-nm band is representative of an organized molecular structure.

The CD spectra of light chains in an aqueous solvent are illustrated in Figure 7. Under the neutral aqueous conditions, 90% of the light chains sedimented as a monomeric population. A typical denaturation spectra was observed for the light chains upon interaction with 4.8 M guanidine-HCl. The oxidation state of the two cystine residues per light chain was shown not to affect the denaturation spectra within experimental limits as the CD spectra of fully reduced and alkylated light chains were virtually identical with the CD spectra of the nonreduced and alkylated light chains.

Figure 8 shows the chromatographic resolution of light chains cleaved by trypsin to yield half-light chains and low molecular weight peptides. Cellulose acetate electrophoresis of the second peak, representing proteins in the molecular weight range 9000–12,000, revealed two electrophoretically distinct components whose migrations corresponded to those reported by Karlsson *et al.* (1969) for the mobility of variable and constant regions of the light chain. Polyacrylamide gel electrophoresis in acid and urea showed the material in peak II to migrate as two distinct populations with molecular weights of between 10,000 and 12,000.

Figure 9 illustrates the CD spectra of intact and half-light chains analyzed in 1.0 M NaCl–0.05 M Tris, pH 8.0. In this buffer system, the half-light chains were shown to be monomeric by analytical gel filtration. The spectra of the half-light chain mixture were slightly different than the spectra of the light chains from which they were derived. The heterogeneous nature of the intact light chains and the restriction of effective proteolysis to only a portion of this heterogeneous population may have accounted for this phenomenon.

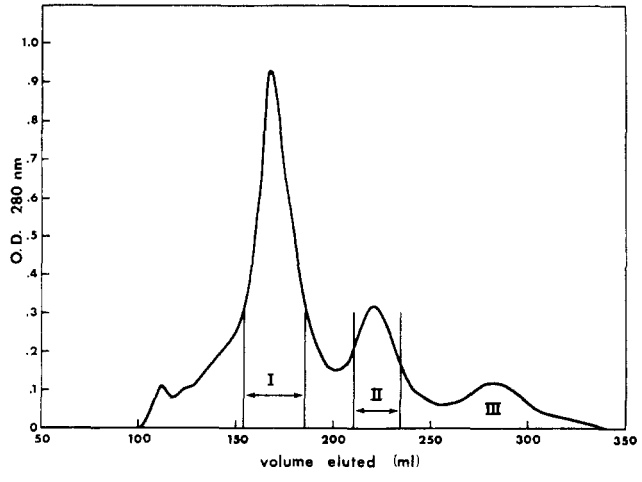


FIGURE 8: Gel filtration of trypsin digest of light chains derived from normal IgG. 1.5 × 140 cm column of Sephadex G-100 in 1.0 M Tris-HCl–1.0 M NaCl, pH 8.0. The flow rate was 12 ml/hr.

## Discussion

While analysis of the primary structure of immunoglobulins has progressed rapidly in the past decade, progress in understanding the relationships between secondary, tertiary, and quaternary structure has hardly kept pace. The profuse molecular heterogeneity of immunoglobulin populations, the difficulties involved in obtaining and analyzing crystal forms of myeloma proteins, and the lack of adequate technology for effectively analyzing macromolecular conformation in the solution state have made analysis of immunoglobulin conformation technically difficult. Thus, incisive data about the conformational relationships within the immunoglobulin molecule have not been forthcoming. We chose to approach the problem of immunoglobulin conformation through circular dichroic examination of the intact IgG molecule as well as its chemically and enzymatically derived major and minor subunits. Our aim in these investigations was to analyze the

overall secondary structure of the immunoglobulin molecule, assess the conformational interrelationships of the molecule's subunits, and identify the structural origins of certain minor effects displayed in the circular dichroic spectra.

Little doubt exists that the β-sheet conformation is the predominating form of organized structure in the immunoglobulin molecules. From ORD, ir, and CD spectroscopic analyses of immunoglobulin structure, it is apparent that the solution conformation of IgG is devoid of significant α helix and that both IgG and its subunits display spectral bands whose assignment to the β-sheet conformation can be made utilizing the isolated spectra of amino acid homopolymers as a reference (Greenfield and Fasman, 1969). Extensive studies, based on CD of IgG myeloma proteins representative of the four major heavy-chain subclasses, as well as studies directed at assessing the solution conformation of immunoglobulins isolated from the sera of numerous species representative of critical periods of vertebrate evolution, attest to the preservation of β-sheet structure in virtually all immunoglobulin forms.

The strongest support for our conclusion regarding the prevalence of β-sheet structure comes from the ir studies reported here as well as those of Abaturov *et al.* (1969). The dominance of the β-sheet associated frequencies is substantiated by empirical correlation to the observed frequencies in proteins whose secondary structure has been determined by X-ray diffraction. The narrower range of extinction coefficients for single transitions eliminates, to a large extent, interference by side-chain functions.

The interaction of the IgG molecule with guanidine-HCl results in a loss of organized structure, providing further evidence that the 217-nm band represents some feature of the secondary or tertiary structure of the protein, and agrees with previous findings of Doi and Jirgensons (1970). Our data indicate that the guanidine denaturation of isolated light chains is affected little by the integrity of the two intrachain disulfide linkages present in this mol wt 22,000 subunit. Whether the kinetics of guanidine denaturation are at all affected by the integrity of these disulfides is uncertain. The similar findings by Doi and Jirgensons (1970) and our own of guanidine-induced denaturation of IgG are not in agreement with the conclusions reached by Cathou *et al.* (1968) who contended that β-sheet structure persists in rabbit IgG after prolonged exposure of the molecule to concentrated solutions of guanidine-HCl. Our finding (unpublished), that the denaturation of rabbit IgG proceeds in a manner identical with that reported for human IgG, excludes the possibility that the rabbit IgG molecule is unique with regard to interactions with this protein denaturant. The discrepancies between the findings of solvent-induced denaturation and β-sheet preservation are difficult to explain at present.

As contributions assignable to the β-sheet conformation dominated both the ir and CD spectra of IgG, our attention was directed at analyzing the origin of this particular form of polypeptide folding within the immunoglobulin molecule. Three critical questions to which we addressed ourselves were: does the β-sheet structure arise independently within the major subunits of the antibody molecule; does the formation of β-sheet structure require cooperative interactions between the major subunits of the antibody molecule; or does the formation of β-sheet structure rely on both independent and cooperative interactions within and between the definable subunits of the antibody molecule? To answer these questions the molecule had to be degraded into a series of definable subunits by chemical and enzymatic means, CD and/or ir analyses had to be made of each subunit, and the relationship of these subunits to the overall conformation of the intact immunoglobulin molecule had to be ascertained. While this approach could represent a relatively simple one with many macromolecules, a number of complications exist in applying this technique to the analysis of immunoglobulins. (1) The heterogeneous nature of normal IgG makes the CD or ir spectrum of this molecule a composite of the spectra of member immunoglobulins belonging to each of the four major heavy-chain subclasses; thus, the CD and ir spectra do not reflect molecules with a single amino acid sequence, but rather the contributions of a mixture of primary structural forms. (2) Certain subclasses of IgG are not as susceptible as others to partial digestion with proteolytic enzymes (Jefferis *et al.*, 1968); thus, the CD spectra of Fab and Fc do not represent the total population of Fab and Fc conformations, but only represent the conformation of those subclasses of IgG susceptible to proteolysis. (3) Certain isolated subunits of IgG tend to polymerize or aggregate after purification; *e.g.*, component II behaves as a noncovalent dimer, heavy chains aggregate in neutral aqueous buffers, and half-light chains aggregate unless they are kept in buffers with high ionic strengths.

Through the appropriate uses of both CD and ir, the employment of certain partial denaturing solvents, and the comparative analyses of homogeneous myeloma proteins rather than normal IgG, we were able to circumvent many of these problems.

The component spectra of Fab and Fc appeared to be markedly different. Fab and a covalent dimer of Fab, $Fab'_2$, exhibit similar CD spectra with respect to the major negative contribution centered near 217 nm. This suggests that hydrogen bonding of the β-structure type does not exist between the Fab subunits of IgG. The CD spectrum of Fc is unique from the spectra of the Fab subunits. Less β-sheet structure per unit mass is indicated by comparing the spectra of Fc to Fab directly. Since a compensating transition of opposite (+) elliptical sign could account for the diminuation in the 217-nm band, we cannot be entirely assured that less β structure does exist in the Fc. Detailed examination of the 250–300-nm range has failed to detect any unusual bands or band systems that could also be reflected in the 210–250-nm range (Doi and Jirgensons, 1970; Ghose and Jirgensons, 1971a). Again, this finding does not conclusively prove that such bands do not exist. The most logical explanation, however, for the diminuation of the 217-nm band in the Fc is that this portion of the molecule possesses less β structure.

The minor band between 225 and 230 nm seen in the CD spectrum of Fc is also seen in the CD spectra of the intact IgG molecule, heavy chains, and heavy-chain disease protein, but not in the spectra of light chains, Fab, or $Fab'_2$. We can confidently say that the structural feature which gives rise to this contribution originates in the Fc portion of the molecule. A further search for the origin of this band was possible, as the C-terminal half of the Fc, component II, can be cleaved from the IgG molecule by controlled digestion with pepsin. This fragment, along with F'c, a somewhat abbreviated form of component II, exhibited CD spectra which were largely devoid of 217-nm contributions and consisted of a single broad negative band centered at 224 nm. Two points were evident from this experiment: (1) the 224-nm band could readily account for the presence of the minor band in the CD spectrum of Fc; (2) since the C-terminal half of the Fc failed to exhibit a 217-nm band, either the N-terminal half of the Fc was the complete source of the 217-nm band or interactions be-

tween the N-terminal half and C-terminal half of the Fc resulted in formation of the β-sheet structure.

Component II in $D_2O$ revealed an ir spectrum consistent with the presence of β-sheet structure. Furthermore, the possibility that the 224-nm band resulted from an isolated chromophoric influence which did not directly depend on the maintenance of secondary structure was negated when the 224-nm band collapsed after component II interacted with guanidine-HCl. Whether or not cooperative interactions between component II and the N-terminal half of the molecule result in formation of β-sheet structure must await isolation and conformational analysis of that portion of the Fc.

The existence of noncovalent bonding between heavy and light chains has been inferred from a study of solvent-mediated chain dissociation patterns (Zimmerman and Grey, 1972). The type of noncovalent bonding has not yet been established and seems to vary in degree between the immunoglobulins of various classes within a given species or between the same class of immunoglobulin found in different species.

Although the CD spectra of isolated heavy and light chains both reveal intense negative contributions at 217 nm, which, within the framework of this paper, constitutes evidence for the presence of the β-sheet structure, the relationship of these two spectra to the CD spectrum of IgG is difficult to ascertain. Due to the uncontrollable self-association tendencies within heavy-chain populations, light-chain populations, and between heavy- and light-chain populations, the CD spectral summation experiments, capable of detecting intersubunit β-sheet structure formation, were discarded in favor of the ir spectral summation approach. The interpretation of ir, which is largely independent of the effects of intersubunit interactions, indicated different spectra for heavy and light chains. Summation of the heavy- and light-chain spectra in a 1:1 ratio did not reconstruct the CD spectra of IgG analyzed under identical conditions. The lack of heavy-chain subclass preference in the reduction–alkylation procedure used to obtain the heavy and light chains negates any subclass enhancement phenomenon as the explanation for the failure of the isolated heavy- and light-chain spectra to reconstitute the spectrum of IgG. It is possible that chemical modification of the heavy and light chains by reduction and alkylation of the interchain disulfides accounts for the calculated spectral differences, although inter-heavy-chain and -light-chain bonding of the class associated with β-sheet structure formation is a plausible interpretation of this difference.

Several proteolytic enzymes, including enzymes of both broad and restricted specificity, are able to cleave normal light chains between their variable (V) and constant (C) regions (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969). As we have shown, separation of the half-light chains from undigested light chains can be readily effected under conditions of partial denaturation. When analyzed in the presence of 1.0 M NaCl, an approximately equimolar mixture of V and C regions exhibited a CD spectrum only slightly different from the spectrum of intact light chains analyzed under the same solvent conditions. These results are similar to those reported by us earlier for normal light chains (Litman *et al.*, 1971) and by Ghose and Jirgensons (1971b) and Björk *et al.* (1971) for the V and C regions of both normal and myeloma light chains. Independent folding seems to characterize the conformation of isolated V and C regions, a fact which fits observations of the inter V–C restricted proteolysis, as well as of the compactness of structure exhibited by half-light chains on hydrodynamic analysis (Karlsson *et al.*, 1969).

Freedom from ordered forms of sedondary structure, *e.g.*, α helix, and from intersubunit β-sheet structure formation between the enzymatically defined subunits analyzed, characterizes the solution state conformation of the immunoglobulin molecule. Where interaction between subunits is necessary for biological function, *e.g.*, formation of the antigen combining site by heavy and light chains, certain evidence suggests stabilization of such processes by intersubunit β structure. The patterns of conformational interactions within the antibody molecule are entirely consistent with our current concepts of both intermolecular cooperative effects in generating biological activity and regional differentiation of function within the antibody molecule.

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# Derivative Spectroscopy Applied to Tyrosyl Chromophores. Studies on Ribonuclease, Lima Bean Inhibitors, Insulin, and Pancreatic Trypsin Inhibitor†

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ABSTRACT: The spectroscopic properties of the tyrosyl chromophores in several tryptophan-free proteins (ribonuclease, lima bean inhibitors, insulin, and pancreatic trypsin inhibitor) have been examined using derivative spectroscopy. These are compared to a model chromophore, *N*-acetyl-L-tyrosine ethyl ester (AcTyrOEt). In the simplest case of a lima bean inhibitor having only a single tyrosyl chromophore, presumably largely exposed to solvent, the derivative bands are considerably sharper and of larger amplitude than would be expected if it resided in a *normal* aqueous environment. In the case of other proteins containing more than one chromophore, the situation is more complicated due to the possibility of spectral heterogeneity resulting from different local environments of the individual chromophores. When this spectral heterogeneity is partially removed for ribonuclease by selective acetylation, it can also be seen that the spectrum for both the three "accessible" and the three "inaccessible" chromophores is again much sharper than anticipated from the corresponding spectra of AcTyrOEt in water or in other liquid hydrogen-bonding solvents. Although covered up to a large extent by broadening from heterogeneity, it is probable that the same situation exists for the other proteins which were examined. Possible explanations for the relatively low degree of environmental broadening of the spectra of protein chromophores are discussed, including the possibility that any "solid-like" character of the protein or the surrounding solvent will minimize configurational fluctuations about native protein chromophores and the possibility that the chromophores are not completely hydrogen bonded. Either of these factors could lead to reduced spectral broadening. Also, the solvent perturbation spectra (25% ethylene glycol) of ribonuclease and its acetylated derivatives have been measured and compared to the equivalent spectrum of AcTyrOEt. It is found that the three accessible chromophores give rise to a perturbation spectrum which is somewhat at variance with the model spectrum. More significantly, there is a moderately large perturbation spectrum for the three inaccessible chromophores which bears little resemblance to the model spectrum and which could arise from factors not explicitly taken into account in the normal analysis of solvent perturbation data.

Ultraviolet spectroscopy is the most commonly used technique for studying protein conformational transitions and for investigating the local environment of the aromatic chromophores of tyrosine and tryptophan residues. In spite of the large dependence on this technique, very little is actually known about the spectroscopic properties of *individual protein chromophores* since most proteins contain a number of chromophores of different type, as well as multiple chromophores of the same type. Even for multiple chromophores of the same type (*e.g.*, the six tyrosyl chromophores in ribonuclease), it is expected that the spectra of the individual chromophores will not be identical due to nonidentity of their local environments within the native protein. If the spectrum of each chromophore within a protein is slightly different (*i.e.*, shifted along the wavelength scale) from the other chromophores of the same type, then the observable or composite spectrum of the protein will be broadened due to this nonsuperposition of the individual spectra which contribute to the composite spectrum. *Spectral heterogeneity* of this type can give rise to protein spectra which do not accurately reflect the spectroscopic properties of the individual chromophores, even when dealing with chromophores of only a single type.

Environmental heterogeneity in native proteins is of interest in itself, but spectroscopic complications arising from this must be overcome before any definitive information on the spectra of the individual chromophores can be obtained. As mentioned above, there is at present no information in the literature on the spectra of single chromophores in native proteins and it has commonly been assumed that their spectroscopic properties can be adequately reproduced from the spectra of suitable model chromophores in hydrogen-bonding solvents such as water, alcohols, and other organic solvents. The purpose of this work is to examine more carefully the $\pi \rightarrow \pi^*$ transition of the phenolic chromophore in both model compounds and proteins in order to reach more definite conclusions concerning spectral heterogeneity in proteins and the effects of local environment on individual chromophore spectra. The large breadth of the absorption curve associated with tyrosyl chromophores in proteins has generally made it

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