Mobile Fc Region in the Zie IgG2 Cryoglobulin: Comparison of Crystals of the F(ab')$_2$ Fragment and the Intact Immunoglobulin†

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ABSTRACT: A human IgG2(κ) immunoglobulin (Zie) and the bivalent antigen-binding fragment [F(ab')$_2$] derived from it by peptic hydrolysis have been crystallized in forms suitable for x-ray diffraction studies. Both the parent protein and the fragment have cryoglobulin properties. The IgG2 molecule crystallized at 15 °C in 0.1 M sodium borate–0.05 M sodium chloride, pH 7.0, and the F(ab')$_2$ fragment crystallized in 0.02 M sodium phosphate, pH 7.4. The crystals were nearly isomorphous. The space group was $I222$ or $I2_12_12_1$, with a = 100.3, b = 153.1, and c = 188.5 Å for the IgG2 protein and a = 99.6, b = 150.7, and c = 192.7 Å for the F(ab')$_2$ fragment. Precession photographs of the $hk0$, $h0l$, and $0kl$ projections were markedly similar for the two molecules. A comparison of 409 pairs of reflections from the 6.5-Å shells of the two crystals indicated a mean difference (R_F) in amplitudes of 0.155. The striking similarities indicate that the structural features contributing to the diffraction patterns are nearly the same in the two crystals. We conclude that the Fab' regions have similar conformations in the fragment and parent molecule and that the Fc region does not contribute significantly to the diffraction pattern of the IgG2 protein. The ability of the Fc region to adopt more than one conformation with respect to the Fab' arms in the crystal lattice adds to previous chemical and crystallographic evidence for the biological importance of the flexibility of immunoglobulin molecules.

An IgG immunoglobulin consists of four polypeptide chains, two light (mol wt ~22 500) and two heavy chains (mol wt ~50 000). Each light chain is composed of two domains (V_L and C_L) of about 110 amino acid residues, and each heavy chain contains four domains (V_H, C_H1,C_H2, and C_H3). The domains in each chain are connected by segments of extended polypeptide backbone. The segment between the C_H1 and C_H2 domains is called the hinge region and is believed to be responsible for much of the segmental flexibility of immunoglobulins (Noelken et al., 1965; Cathou & Dorrington, 1975). Within the hinge region are half-cystine residues contributing to interheavy chain disulfide bonds. IgG proteins from subclasses 1 and 4 have two such bonds, while members of subclass 2 have four (see Milstein & Pink, 1970). IgG3 molecules appear to have as many as 11 interheavy chain bonds (Michaelsen et al., 1977). In both the human Mcg (Fett et al., 1973) and Dob (Lopes & Steiner, 1973) IgG1 proteins, the hinge region is deleted, and interchain disulfide bonds between heavy chains are absent. The normal disulfide bonds between heavy and light chains are also missing in the Mcg and Dob proteins, but the two light chains are covalently linked in each immunoglobulin.

The presence of a hinge region has become very important in the crystallographic analyses of immunoglobulins. In the human Kol IgG1 protein, with an intact hinge region and a normal pattern of interchain disulfide bonds, the Fc part of the molecule occupied more than one position in the crystal lattice and was not clearly defined in the 5-Å electron density map (Colman et al., 1976). In contrast, the Fc region of the Dob IgG1 immunoglobulin appeared as a distinct feature in both the 6-Å electron density map and in electron micrographs (Sarma et al., 1971; Labaw & Davies, 1971; Silverton et al., 1978). Moreover, the preliminary electron density maps of the hingeless Mcg IgG1 protein currently under investigation in our laboratories also showed an ordered Fc region.

In the present article we describe a dual protein system suitable for direct comparison of the conformations of the Fab' arms of an IgG2 cryoglobulin (Zie) and its divalent F(ab')$_2$ fragment. The results of the comparison will also be used to assess the contribution of the Fc region to the diffraction pattern of the IgG2 protein.

Materials and Methods

Preparation of the IgG2 Protein. The plasma from a patient (Zie) with multiple myeloma was kindly provided by Dr. W. Pruzanski of the University of Toronto. The IgG2 protein was initially obtained by cryoprecipitation from plasma placed in an ice bath for 30 min. The precipitate was recovered by centrifugation at 0 °C for 10 min at 10 000 rpm and resuspended in ice-cold 0.15 M sodium chloride buffered with 0.05 M sodium phosphate, pH 7.4. After 10 min the precipitate was collected by centrifugation, rewashed with the cold phosphate-buffered saline, and again recovered by centrifugation. The precipitate was then dissolved in the buffered saline at 37 °C for 30 min, and the solution was centrifuged at 25 °C for 10 min at 10 000 rpm. The supernate was concentrated at 4 °C by ultrafiltration in a Diaflo cell (Amicon Corp.) to 10–12 mg per mL. Crystals which formed during concentration were dissolved at 37 °C and the solution was passed through a Nalgene filter at the same temperature. The filtered solution was stored at 4 °C in sterile tubes and sent to Utah by air for recrystallization and x-ray diffraction experiments.

Crystals which formed during transportation and storage were removed by centrifugation. The supernate was dialyzed against 0.1 M borate–0.15 M sodium chloride, pH 8.4, at 22 °C for 24 h. The solution was concentrated to 20–22 mg per mL and crystallized by microdiffusion (Zeppezauer et al., 1968) at 15 °C against 0.1 M borate–0.05 M sodium chloride,

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pH 7.0. Parallelopiped crystals elongated along the *c* axis and bounded by {110} faces were obtained in sizes appropriate for x-ray analysis (i.e., 0.2 mm in the shortest dimension) in about 2 months.

Preparation of the F(ab′)$_2$ Fragment. The fragment was prepared by hydrolysis of the Zie IgG2 protein with porcine pepsin (2× crystallized, Worthington Biochemical Corp.). The hydrolysis was carried out in 0.1 M sodium acetate, pH 4.3, for 7 h at 37 °C, with an enzyme:substrate ratio of 1:100 by weight. The reaction was stopped by increasing the pH to 9.0 with a few drops of 2 M Tris-HCl, pH 9.0, and 1 M NaOH. The F(ab′)$_2$ fragment was separated from the other constituents of the digest by gel filtration over a 5.0 × 90 cm column of Sephadex G-150, equilibrated at pH 7.4 and 4 °C with 0.05 M Tris-HCl–0.15 M NaCl containing 0.002% Hibitane.

Prior to crystallization, samples of the fragment were concentrated to 10–12 mg per mL, warmed to 37 °C, and filtered through Nalgene membranes and stored in sterile tubes. These tubes were transported to Utah by air. The solutions were placed in microdiffusion tubes and dialyzed at 15 °C against 0.02 M sodium phosphate, pH 7.4. Crystals of suitable size for diffraction experiments appeared in about 1 month. The crystals were more elongated but were otherwise similar in appearance to those of the parent IgG2 protein.

Purities of the Constituents of Crystals. After crystals were used in diffraction studies, they were removed from their capillaries, dissolved, and subjected to sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis (Laemmli, 1970). The electrophoretic patterns were compared with those of the mother liquor from which the crystals were grown. Electrophoresis was conducted in the presence of 2-mercaptoethanol on 1.5-mm slabs of 10% polyacrylamide gels in 0.1% (w/v) $NaDodSO_4$ and 0.025 M Tris–0.192 M glycine, pH 8.3.

Density Measurements. The densities of the crystals of the IgG2 and F(ab′)$_2$ proteins were determined in gradients of bromobenzene–xylene mixtures (Low & Richards, 1952). Droplets of sucrose or sodium phosphate of known densities were used as standards.

Diffraction Data Collection. Preliminary experiments indicated that the diffraction patterns for the two types of crystals extended only to a resolution of about 6 Å. For the initial comparison 5° precession photographs were taken of the *hk*0, *h*0*l*, and 0*kl* zones of the two crystals. Then intensity measurements of reflections within the 6.5-Å shells were made with a Syntex $P2_1$ diffractometer. Of approximately 3500 reflections in this shell for the IgG2 protein, only about 900 reflections were observed at the 3σ level. A file containing the *h*, *k*, *l* indices of these observed reflections was created, and their intensities were measured again. The crystals were extremely stable in the x-ray beam, with the intensities of three standard reflections decreasing by only 8% in 70 h of exposure (Cu Kα radiation, 40 kV, 15 mA).

The entire 6.5-Å data set and a truncated data set corresponding to the file of observed reflections of the IgG2 crystal were also collected for a crystal of the F(ab′)$_2$ fragment. Since the standard reflections for the F(ab′)$_2$ crystal showed no significant decreases in intensity in 170 h for exposure to the x-ray beam, it was possible to repeat the data collection for the truncated set several times.

All data were corrected for geometric factors, absorption effects, and background. After data reduction 409 pairs of scaled and matched reflections were used for comparisons of the IgG2 and F(ab′)$_2$ proteins.

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

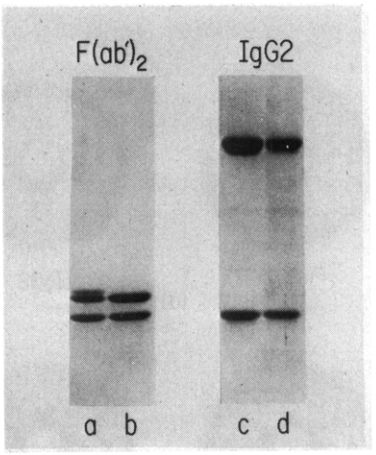

FIGURE 1: Electrophoretic patterns of mother liquor (a and c) and dissolved crystals (b and d) of the Zie F(ab′)$_2$ and IgG2 proteins. Electrophoresis was carried out with $NaDodSO_4$–polyacrylamide gels in the presence of 2-mercaptoethanol. The samples of mother liquor were removed from tubes containing the crystals used for diffraction (see Figure 2). After diffraction the crystals were dissolved in the electrophoresis buffer. The mother liquor (a) over the F(ab′)$_2$ crystal contained two heavy chain fragments (two top bands) and one light chain (bottom band). The minor heavy chain component was larger than the major constituent by about 1000 daltons. Only the major constituent appeared in the crystal (b). The IgG2 protein in both the mother liquor (c) and crystal (d) consisted of single species of intact heavy chains (upper band) and light chains (lower band).

Results and Discussion

Purities of the IgG2 and F(ab′)$_2$ Proteins. The use of cryoprecipitation to isolate the IgG2 myeloma protein was advantageous because the mixture of normal immunoglobulins remained in the serum. Cryoprecipitates of the Zie IgG2 protein consistently migrated as a single band in starch gel electrophoresis and showed only one precipitin line after immunoelectrophoresis when either anti-normal human sera or anti-IgG sera were employed (Laschinger, 1976). Of major importance to the work presented here was the fact that the IgG2 samples were free of contaminating enzymes, including plasmin. For example, sterile solutions of the IgG2 protein incubated at 37 °C for 1 week remained free of any degradation products (Laschinger, 1976).

The electrophoretic patterns for the samples of F(ab′)$_2$ and IgG2 proteins used in the diffraction experiments are shown in Figure 1. The mother liquor of the F(ab′)$_2$ sample consisted of two molecular species, with one of the heavy chain fragments larger than the other by about 1000 daltons. However, the crystal was composed exclusively of molecules with the shorter heavy chain fragment. This is an example of the power of crystallization to discriminate between closely related molecules.

Both the mother liquor and the crystal of the IgG2 protein contained a single component with κ-type light chains and full-length γ2 heavy chains.

Diffraction Patterns for Crystals of the IgG2 and F(ab′)$_2$ Proteins. Representative precession photographs for the crystals are presented in Figure 2. Note the striking similarities in the diffraction patterns. The *mmm* symmetry and the systematic extinctions ($h + k + l$ = odd) indicated an orthorhombic space group, $I222$ or $I2_12_12_1$, in each case.

The unit cell dimensions measured from the precession photographs were $a = 100.3 \pm 0.4$, $b = 153.1 \pm 1.3$, and $c = 188.5 \pm 0.6$ Å for the IgG2 protein, and $a = 99.6 \pm 0.1$, $b = 150.7 \pm 0.2$, and $c = 192.7 \pm 1.1$ Å for the F(ab′)$_2$ fragment. The unit cell volumes calculated from these cell dimensions

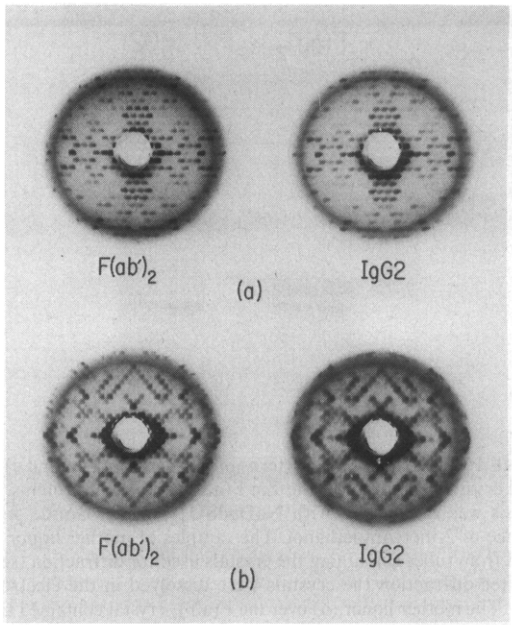

FIGURE 2: The 5° precession photographs of the $h0l$ (a) and $0kl$ (b) projections of crystals of the Zie $F(ab')_2$ and IgG2 proteins. Note the striking similarities in the patterns for the two proteins.

were 2.89(4) and 2.89 (2) $\times 10^6$ Å^3, respectively.

The values for the densities were 1.096 ± 0.005 and 1.067 ± 0.005 g/mL. These observed densities were equal to calculated values if half-molecules of the IgG2 and $F(ab')_2$ proteins were taken as the asymmetric units (of which there are eight per unit cell). With the symmetry operations in the space group $I222$ or $I2_12_12_1$ the half-molecules are related by crystallographic two-fold axes of rotation.

The fractional volumes of solvent in the crystals were high (V_{solv} = 0.75 and 0.83 for IgG2 and $F(ab')_2$ crystals) in comparison with most proteins. For example, the Mcg Bence-Jones orthorhombic form, the Bence-Jones trigonal form, and the IgG1 orthorhombic form of crystals had V_{solv} values of 0.46, 0.60, and 0.61 (Edmundson et al., 1971). V_{solv} values for other proteins have ranged from 0.27 to 0.65, with the average being about 0.43 (Matthews, 1968). With the small concentrations of the Zie proteins in such large unit cells, it is understandable that the diffraction patterns were relatively weak. However, it is not clear why the crystals were so resistant to radiation damage.

Intensity Differences in the Diffraction Patterns of the IgG2 and $F(ab')_2$ Proteins. Inspection of the diffraction patterns in Figure 2 showed only small differences in intensities of corresponding reflections from the two crystals. The comparison of diffractometer data verified this conclusion. After data reduction the value of $\Sigma|\Delta F|/\Sigma F$ (R_F) for the two crystals was 0.155 for 409 pairs of matched and scaled reflections (ΔF is the difference in the structure factor amplitudes for pairs of reflections from the IgG2 and $F(ab')_2$ proteins, and F is the structure factor amplitude for the parent IgG2 molecule).

The observed differences were of the same order of magnitude as those of the heavy-atom derivatives used to solve the structure of the Mcg Bence-Jones dimer. At 6-Å resolution for example, the R_F values ranged from 0.12 to 0.23 for six mercury derivatives (Edmundson et al., 1972; Ely et al., 1973; Schiffer et al., 1973).

Some differences in intensity were expected because of the differences in cell dimensions of the IgG2 and $F(ab')_2$ crystals (i.e., 1–1.5% in the a and b dimensions and 2–2.5% in c). Crick & Magdoff (1956) estimated that changes in cell dimensions of 0.5% would alter the average intensities by about 15% in the 3-Å sphere. Such intensity differences increase with $\sin\theta/\lambda$ and are of less serious concern in low resolution comparisons as in the present studies.

In x-ray analysis of proteins, electron dense atoms ("heavy" atoms) are diffused into crystals of native protein to give derivatives which do not alter the crystal packing. The diffraction patterns of the derivatives show intensity changes which can be estimated by the equation given below (Crick & Magdoff, 1956). We used this equation to calculate the expected fractional change in intensity when the Fc region was formally treated as a heavy atom added to an $F(ab')_2$ fragment to give an IgG2 molecule. If I_1 and I_2 are diffracted intensities from $F(ab')_2$ and IgG2 crystals, the fractional change in intensity ($\phi\Delta I$) is defined as

$$\phi\Delta I = \langle (I_1 - I_2)^2 \rangle^{1/2} / \langle I_1 \rangle$$

For acentric data $\phi\Delta I$ is approximately equal to $\sqrt{2}\sqrt{\langle I_3 \rangle / \langle I_1 \rangle}$, where $\langle I_3 \rangle$ is the mean intensity for an Fc region. The latter contains about half as many atoms as the $F(ab')_2$ protein. If the Fc region contributes fully to the diffraction pattern of the IgG2 crystal, $\sqrt{\langle I_3 \rangle / \langle I_1 \rangle} \sim \sqrt{1/2}$ and the expected fractional change in intensity is ~1. However, the value calculated from 409 pairs of measured intensities of comparable reflections from the $F(ab')_2$ and IgG2 crystals was only 0.61.

The combined evidence strongly indicates that the Fc region of the Zie IgG2 protein does not significantly contribute to the diffraction pattern. Furthermore, the Fab′ regions probably have closely similar conformations in the $F(ab')_2$ fragment and its parent IgG2 protein. The latter conclusion does not support the proposal of an allosteric model for antigen triggering of effector functions in antibodies (Huber et al., 1976).[2]

Since the crystals are practically isomorphous, the space occupied by the Fc regions in the IgG2 crystal probably contains solvent in the $F(ab')_2$ crystal. The principal packing forces in the IgG2 crystal presumably are the same as those in the $F(ab')_2$ crystal and obviously do not restrain the Fc regions from assuming more than one orientation. This capacity for independence of motion of the Fab and Fc arms will be accentuated in solution.

Unlike the Fc arm, the hinge region of the Kol IgG1 protein (Colman et al., 1976) was clearly defined in the electron density map. These authors proposed that the flexibility of the molecule was mediated through the segments of the hinge region on the carboxyl side of the two interheavy chain disulfide bonds. The Zie IgG2 protein has four interheavy chain linkages in the amino part of the hinge region. Such highly structured segments can be considered as spacers between the Fab and Fc arms, effectively allowing greater freedom of movement of the Fc about the flexible regions.

[2] A major prediction based on this theory includes the stiffening of an immunoglobulin when antigen is bound. This stiffening can be visualized as a closing of the angle between the pseudodiad axes relating the V and C dimeric modules of the Fab regions. Reported values for such angles range from 170° (extended conformation) for the Kol IgG1 protein (Colman et al., 1976) to 113° (closed conformation) in the Mcg Bence-Jones dimer (Schiffer et al., 1973). Release of an Fab fragment by proteolysis was predicted to result in a change from the extended to the closed conformation, as indicated by the value of the "Fab angle." As Silverton et al. (1978) pointed out, however, the finding of an Fab angle at an intermediate value of 146° in the intact Dob IgG1 protein argues against the allosteric model. In a more stringent test of their hypothesis, Huber and his colleagues are currently determining the structure of the Kol Fab fragment for comparison with the parent IgG1 molecule.

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Characterization and Affinity Labeling of Nucleotide Binding Sites of Bacterial Plasma Membrane Adenosine Triphosphatase (F_1)†

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ABSTRACT: 6-[(3-Carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate (Nbs^{6}ITP), a chemically reactive ATP analogue, has been used to label nucleotide binding sites of the plasma membrane ATPase (F_1) from *Micrococcus* species ATCC 398. Nbs^{6}ITP is not hydrolyzed by the ATPase. Binding of the ATP analogue to the enzyme slowly (within hours) produces an irreversible inhibition of the ATPase which shows a pseudo-first-order kinetics. This "long-term" inhibition is preceded by a reversible "short-term" competitive inhibition. The K_m value for the "long-term" inhibition and the K_i value for the competitive inhibition are identical, indicating that the binding of Nbs^{6}ITP occurs at the same site(s) in both cases. Nbs^{6}ITP has been labeled with ^{32}P in the β and γ positions to elucidate the stoichiometry of nucleotide binding. The degree of inhibition of the enzyme is stoichiometrically related to the number of nucleotides bound. One-hundred percent inhibition is correlated by extrapolation with the incorporation into the F_1 complex of about six nucleotides which are not released by gel filtration in glycerol containing buffer. These "firmly" bound nucleotides are to some extent covalently bound to the enzyme as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. At 80% inhibition 1.7 of a total of 4.8 bound nucleotides are covalently bound. Covalent binding of Nbs^{6}ITP to F_1 occurs exclusively at the β subunit(s) of the enzyme. A cysteinyl residue is not involved in this reaction.

Membrane-bound ATPases[1] from mitochondria, chloroplasts, and bacteria are enzymes of very complex structure and function. The F_1 part of this protein complex can be solubilized in dilute buffers. It consists of up to five different subunits depending on the conditions of the preparation. The F_0 part of the ATPase complex is a hydrophobic integral membrane protein, which can only be solubilized in the presence of detergents. If bound to intact vesicular membranes and linked to energy-producing electron transport reactions, the ATPase is able to synthesize ATP from ADP and P_i. Conversely, the energy of ATP hydrolysis can be used for promotion of energy-dependent reactions such as active transport (Tsuchiya & Rosen, 1975).

Because the site of ATP synthesis or hydrolysis is the F_1 factor, the nucleotide binding sites of F_1 are of central interest for the elucidation of the complex enzyme mechanism. There

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[1] Abbreviations used: F_1 ATPase, adenosinetriphosphatase (EC 3.6.1.3); Dnps^{6}ITP, 6-[(2,4-dinitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate; Nbs^{6}ITP, 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate; P_i, inorganic phosphate; Nbf-Cl, 4-chloro-7-nitrobenzofurazan.

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