Properties of the Fd Fragment from Rabbit Immunoglobulin G†

James S. Huston,‡ Ingemar Björk,§ and Charles Tanford*,¶

ABSTRACT: Nearly pure Fd fragments from nonspecific rabbit immunoglobulin G were prepared from reduced and alkylated Fab fragments. Molecular weight measurements in 6 M guanidine hydrochloride showed that Fd polypeptide chains have a molecular weight of 24,000–25,000. Under native conditions the protein is in equilibrium between monomeric and dimeric forms, with a marked heterogeneity of association constants. Chromatographic fractionation leads to separation of the Fd into subpopulations, ranging from largely dimeric to largely monomeric species. The optical rotatory dispersion spectrum of Fd was measured and compared with similar spectra for the polypeptide chains and other fragments of the immunoglobulin molecule. The experimental spectrum for the heavy chain is very similar to that calculated for an equimolar mixture of Fd and Fc, but the experimental spectrum for Fab differs considerably from that calculated for an equimolar mixture of Fd and light chain. This result is in agreement with earlier observations showing that strong complementarity exists between heavy and light chains, and that a significant change in conformation accompanies their association with each other. Limited proteolysis of Fd, using papain, leads to conversion of much of the protein to fragments with mol wt 11,500. These are probably the variable and constant domains of Fd, but experiments to demonstrate this rigorously and to separate the domains have not been carried out. Significant amounts of domain-size fragments could not be obtained under similar conditions from Fab, which is another indication of the marked changes that accompany association between Fd and light chains.

Understanding the structural complexities of immunoglobulins is fundamental to research on many facets of the immune system. Formidable progress has been made in determining their amino acid sequence and the major features of their structure, so that we can now hope to understand the molecular interactions responsible for their native conformation. Our recent approach to this problem has involved the study of isolated heavy and light chains from rabbit IgG[1] (Björk and Tanford, 1971a–c; Painter *et al.*, 1972a,b), which led to this investigation of the amino-terminal half of heavy chain, the Fd fragment.

The physicochemical properties of native rabbit Fd have heretofore been determined only incidental to its initial isolation (Fleischman *et al.*, 1963). Other reports have considered its recombination with light chain (Roholt *et al.*, 1966), and its antigen binding properties, both as the normal fragment (Painter *et al.*, 1972a) and with polyalanyl side chains attached (Jaton *et al.*, 1968). A brief characterization of human Fd' has been published (Heimer, 1966) which suggests that aside from electrophoretic mobility, its properties are basically homogeneous. In contrast, this investigation reveals that rabbit Fd fragments possess distinctly heterogeneous physical characteristics; this behavior in turn suggests refinements to our view of the interactions normally present between light chain and Fd.

Materials and Methods

Rabbit IgG was isolated from 10 l. of pooled serum (Pel-Freez, Rogers, Ark.), according to the procedure of Levy and Sober (1960). Its purity was shown by sedimentation velocity and immunoelectrophoresis using goat antiserum to whole rabbit serium; aliquots of this preparation were stored as 80% ammonium sulfate suspensions at −20°. Fab fragments of IgG were prepared by the papain cleavage and carboxymethylcellulose purification procedures of Porter (1959), with the modification that proteolysis was conducted for 2 hr instead of 16 (Noelken *et al.*, 1965). Mild reduction and alkylation with iodoacetamide of interchain disulfide bonds followed the procedure noted by Björk and Tanford (1971a). Fd was isolated from alkylated Fab by the method of Fleischman *et al.* (1963), whereby chain separation is effected through chromatography on Sephadex G-75 in 1 M propionic acid following a 2-hr incubation of the salt-free Fab in this solvent. Samples of about 100 or 350 mg at initial concentrations of from 15 to 20 mg per ml were applied respectively to G-75 columns with dimensions of 3 × 100 cm or 6 × 115 cm, with the separations obtained similar to that reported by Fleischman *et al.* (1963). Procedures were conducted at 5°, including renaturation of the eluted and pooled Fd by dialysis *vs.* three 6-l. portions of 0.01 M sodium acetate buffer at pH 5.5.

Prior to all physical measurements, Fd preparations were chromatographed on columns of Sephadex G-75 in 0.1 M NaCl + 0.02 M sodium acetate (pH 5.5) at 5°; either individual or pooled fractions from the main eluted peak were utilized in subsequent work. Similar separations were obtained with a variety of column sizes, 2 × 150 cm, 3 × 100 cm, or 5 × 145 cm, with the choice being dictated by the amount of material

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received May 17, 1972.* Supported by Research Grant AM-04576 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

‡ Predoctoral fellow of the National Institutes of Health, U. S. Public Health Service, supported by Grant 5-T01-GM-00233-11. This material was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry, Duke University. Present address: Department of Pharmacology, Stanford University School of Medicine, Stanford, Calif.

§ Present address: The Institute of Medical Chemistry, University of Uppsala, 751 22 Uppsala 1, Sweden.

¶ Research career awardee, National Institutes of Health, U. S. Public Health Service.

[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride. The nomenclature of and the abbreviations for the immunoglobulins and their subunits produced by reduction and proteolysis are those recommended by the World Health Organization (1964).

to be fractionated. A few samples of anti-Dnp Fd prepared from anti-Dnp IgG (kindly donated by Dr. R. G. Painter) were purified on a 1.5 × 100 cm column of Sephadex G-50, in 0.1 M NaCl + 0.02 M sodium acetate at pH 5.5 or 4.5, in order to maximize fractionation of the limited material available. Fd was found stable to storage in dilute salt at pH 5.5 at −20°, and in a few instances was studied after being kept frozen under these conditions.

Sedimentation experiments were conducted on rechromatographed Fd at 20–25° in 0.1 M NaCl (pH 5.5) or in 0.1 M NaCl + 0.02 M sodium acetate at pH 5.5. Sedimentation equilibrium studies utilized several approaches: the high-speed meniscus depletion technique (Yphantis, 1964) was used on a Model E analytical ultracentrifuge equipped with interference optics, while the low-speed method (Schachman and Edelstein, 1966) was conducted on an instrument with the photoelectric scanner system; long-column synthetic boundary runs, following the procedure of Chervenka (1970), were made with both optical systems. Sedimentation velocity was performed with an ultracentrifuge having schlieren optics, except at very low concentrations, where the scanner was utilized. Partial specific volume in dilute salt was calculated for the Fd fragment to equal 0.73 cm^3/g, using the procedure of Cohn and Edsall (1943) and the amino acid composition of Crumpton and Wilkinson (1963); in 6 M Gdn·HCl, an apparent partial specific volume equal to 0.72 cm^3/g was used to correct for preferential salt binding (Hade and Tanford, 1967). Optical rotatory dispersion data were obtained as described by Björk and Tanford (1971a). The Fd extinction coefficient, $E^{1\%}_{280} = 14.4$, was taken from Crumpton and Wilkinson (1963), and it agreed with our determinations based on the dry weights of solutions with known absorbance. Fluorescence quenching of anti-Dnp tryptophan fluorescence by titration with Dnp-aminocaproic acid was conducted according to Eisen and Siskind (1964) on a Turner Spectro 210 spectrofluorometer (G. K. Turner Associates, Palo Alto, Calif.).

Proteolytic cleavage of Fd was performed with 0.5% (w/w) papain (Worthington Biochemical Corp., Freehold, N. J.) in a buffer containing 0.01 M 2-mercaptoethanol, 0.002 M EDTA, and 0.01 M sodium acetate (pH 5.5). In addition, 0.1 M NaCl was present in some experiments without affecting the reaction, but it was found undesirable for cleavage of concentrated protein solutions, as it aids precipitation. The mixture was incubated at 37° for 20 min and then quenched in an ice bath by adding iodoacetamide to a concentration of 0.011 M, keeping the pH at 8.0–8.5 with NaOH; once the pH ceased to change, it was readjusted to 5.5. Protein concentrations for most cleavage reactions were 0.3–0.5 mg/ml, although similar results were obtained at concentrations as high as 20 mg/ml. Yields and molecular weights of polypeptide chains of Fd and its cleavage products were determined by chromatography of the fully reduced and alkylated reaction mixture on a 6% agarose column (1.5 × 90 cm) in 6 M Gdn·HCl + 0.01 M sodium acetate (pH 4.75) (Fish *et al.*, 1969).

Immunodiffusion and immunoelectrophoresis were conducted as described by Björk and Tanford (1971a). A variety of goat antisera were utilized: antiserum to whole rabbit serum was purchased from Schwarz/Mann (Rockville, Md.); anti-Fd serum, obtained by immunization with a sample of pooled Fd, was made for us by Gateway Immunosera Co. (Cahokia, Ill.); anti-Fab serum specific for the Fd determinants of intact Fab fragments was prepared as described by Björk and Tanford (1971a); anti-light-chain serum was kindly donated by Dr. H. N. Eisen.

Results

Preparation. The isolation of Fd demands exposures to 1 M propionic acid during chromatography lasting up to about 30 hr; the effect of this treatment was tested in several ways. Pooled Fd from a typical preparation was concentrated immediately after elution in 1 M propionic acid and reapplied to the same column. The resulting elution profile exhibited essentially the same peak position as in the initial chromatography, with some formation of aggregated material, which was however much less than that present in heavy chain after a similar exposure (Björk and Tanford, 1971a); renaturation of this protein gave Fd which was antigenically normal. In another experiment, native Fd was dialyzed *vs.* water, reexposed to 1 M propionic acid for 20 hr, and renatured, with recovery of its usual immunological properties. We found in immunodiffusion experiments that Fd precipitin lines show identity with heavy-chain and native Fab, using either goat antisera directed against free Fd or that in Fab fragments. These studies indicate that Fd structural alterations in 1 M propionic acid are reversible, and that upon renaturation it returns to an immunochemically native protein. Recovery of the native conformation is also indicated by the fact that Fd prepared in the same way from a specific antibody has exactly the same binding isotherm for antigen as native heavy chain (Painter *et al.*, 1972a). Heavy chain, after a similar course of treatment in 1 M propionic acid, has been studied in great detail (Björk and Tanford, 1971a) and found to be undenatured by many criteria.[2]

Simultaneous exposure of Fd to 1 M propionic acid and ionic strength of about 0.1 or higher was found to give largely denatured and aggregated protein, on the basis of immunochemical and chromatographic experiments. A similar result was obtained for heavy chain (Björk and Tanford, 1971a). The renatured protein also tends to aggregate, especially in the presence of 0.1 M NaCl, and for this reason all samples were chromatographed before use. The G-75 elution profile of a typical Fd preparation is presented in Figure 1, showing a symmetrical main peak preceded by two containing aggregate, the larger of which represents its accumulation in the void volume; the amount of aggregate present increases according to the degree of concentration and length of time prior to chromatography.

The yields of Fd after this fractionation ranged from about 40 to 50%, compared to the amount theoretically obtainable from Fab. The loss of material results from conservative pooling of Fd peaks after initial isolation and rechromatography, from nonspecific adsorption to the membrane during concentration, and from aggregation, which may involve slight specificity for a segment of the Fd population.

Purity. Fd which has been isolated from Fab by the procedure of Fleischman *et al.* (1963) contains a small amount of light chain. Typical contamination was in the range of 5–8%, as judged by immunodiffusion against anti-light-chain serum.

[2] The definition of the term "native," when applied to a protein that cannot be isolated in the free state from the native medium, is necessarily indirect. Both heavy chain and Fd recombine with light chain to yield products indistinguishable from native immunoglobulin or the Fab fragment derived from it. A conformational change accompanies this process, but the fact that the recombination and the accompanying conformational change occur rapidly with essentially 100% yield implies that the heavy chain and Fd are in dynamic equllibrium states, responsive to environmental change. The reactions occurring in the association process could be $A \rightleftharpoons B$, $B + L \rightleftharpoons Fab$, where A and B are two different conformations of Fd. The native state of Fd, in the absence of light chains, is the equilibrium mixture of A and B.

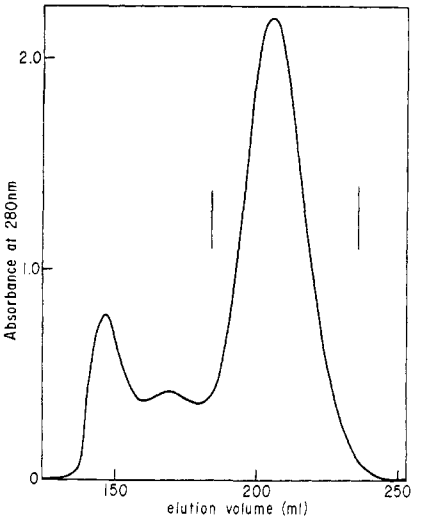

FIGURE 1: Gel chromatography of Fd fragments on Sephadex G-75 in 0.1 M NaCl + 0.02 M sodium acetate buffer at pH 5.5. A total of 59 mg in 3.6 ml of solution was applied to a 2 × 150 cm column; the vertical lines indicate those fractions pooled for subsequent analysis.

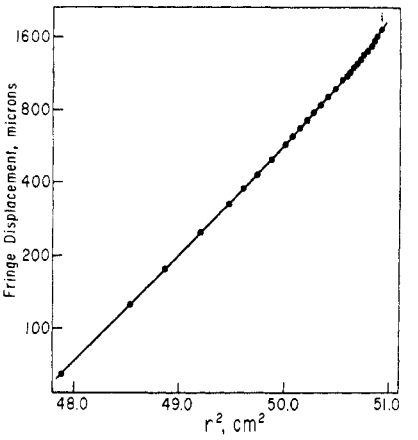

FIGURE 3: Sedimentation equilibrium data for a sample of Fd, pooled after rechromatography under native conditions. Results are plotted semilogarithmically as fringe displacement (ln y) *vs.* the square of the distance from the rotor axis (r^2). Long-column meniscus depletion was conducted using the synthetic boundary technique: 0.190 ml of dialysate (0.1 M NaCl, pH 5.5) was layered over 0.050 ml of protein solution at an Fd concentration of 0.79 mg/ml; the rotor speed was 21,736 rpm and temperature equal to 24°, reached using only the refrigeration. The base of the solution column is indicated by a vertical line.

Immunoelectrophoresis substantiated reports indicating that any light chain present will be recombined with Fd as Fab fragments (Roholt *et al.*, 1966; Björk and Tanford, 1971c). The resulting Fab is a highly stable molecule, which contributes a minor and homogeneous background to the heterogeneous physical properties of Fd.

Mass homogeneity of the Fd with intact disulfides was shown by sedimentation equilibrium studies of several samples in 6 M Gdn·HCl + 0.01 M sodium acetate (pH 5.5); the resulting molecular weight plots were linear, with apparent molecular weights of 24,000–25,000, assuming an apparent partial specific volume of 0.72 cm³/g. However when samples were fully reduced and alkylated, and applied to a calibrated column of 6% agarose in 6 M Gdn·HCl (Fish *et al.*, 1969; Björk and Tanford, 1971a), a minor shoulder on the trailing side of the main 24,500 molecular weight peak was observed (Figure 2), having a weight of about 18,500 and amounting to 5–10% of the total material present. Since slight asymmetry was found on the trailing side of the peak for similarly chromatographed Fab, the smaller polypeptide seems to result from initial papain cleavage of IgG during Fab preparation. For a minor portion of the protein this treatment apparently leads to cleavage at such positions that disulfides attach the smaller peptides to the main chain; at least part of these species seem to be cleaved at only a single point, since the 5700 molecular weight polypeptide shown in the guanidine-agarose elution profile of Figure 2 corresponds to the fragment missing from degraded Fd polypeptide chains. None of the native physical properties of Fd give any indication of these breaks, suggesting that the peptides interact with the main part of the protein to form a normal molecule, such as in the case of ribonuclease S.

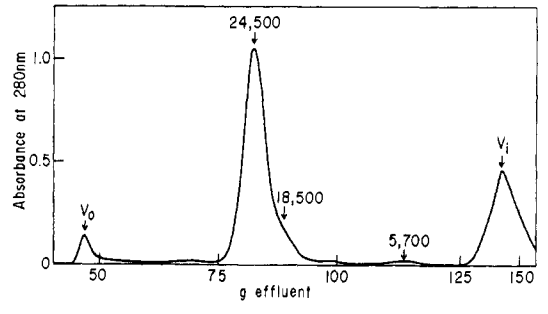

FIGURE 2: Gel chromatography of fully reduced and carboxymethylated Fd fragments in 6 M guanidine hydrochloride; 5 mg was applied to a 1.5 × 90 cm column of Sepharose 6B. The protein had been rechromatographed under native conditions prior to use. Approximate molecular weights (±7%) are noted above peaks.

Molecular Weight. Equilibrium sedimentation of pooled Fd yields the data presented in Figure 3, exhibiting distinct curvature with local weight-average molecular weights ranging from 35,000 near the meniscus to about 49,000 at the base of the solution column. This behavior is indicative of self-association.

To establish whether Fd association is a homogeneous or heterogeneous phenomenon, sedimentation equilibrium was performed on separate fractions of the main G-75 Fd peak. Our rationale was that if all Fd species had the same association constant, identical associative behavior should be found for all fractions, whereas heterogeneity of association would cause dissimilar associative properties for different chromatographic fractions. The results from such an experiment, shown in Figure 4, demonstrate there is a wide variation in apparent weight-average molecular weights and associative behavior for Fd as a function of elution position. This proves that Fd must consist of several species (perhaps many) differing in the equilibrium constant for self-association.

It should be noted that the relative amounts of material with a certain weight-average mass, as indicated by the magnitude of the elution profile, follow essentially a Gaussian distribution. We cannot ascertain from these results whether the whole Fd population accurately follows this same distribution, because there exists the possibility of some specificity in losses during preparation.

Shape. Sediminetation velocity experiments on pooled Fd revealed a single peak, which was somewhat skewed toward the trailing side only at low protein concentrations. A marked

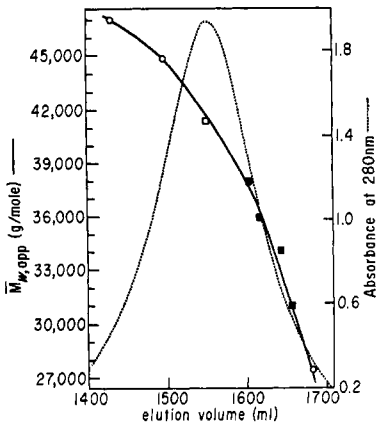

FIGURE 4: The solid line shows the variation in apparent weight-average molecular weight ($\overline{M}_{w,app}$) for Fd as a function of its elution position; the elution profile for Fd fractions, to which molecular weights apply, is superimposed as a dotted line. Sedimentation equilibrium data: filled symbols indicate low-speed equilibrium measurements using a uv scanner optical system, while open symbols refer to high-speed meniscus depletion experiments studied with interference optics; squares indicate that a given ln y *vs.* r^2 plot is virtually linear, while circles represent distinctly curved plots whose tangent for data above 0.93 mg/ml was used to calculate $\overline{M}_{w,app}$. Gel chromatography data: the sample of Fd (308 mg in 20 ml) was applied to a 5 × 145 cm column of Sephadex G-75 in a buffer containing 0.1 M NaCl + 0.02 M sodum acetate, pH 5.5; fractions were collected at 15-min intervals and had a volume of 13.2 ml each.

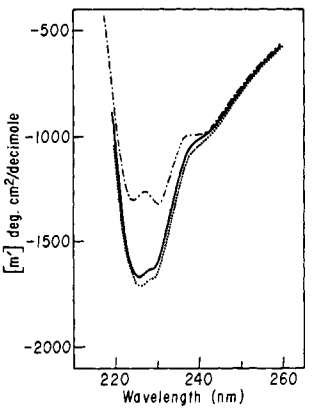

FIGURE 6: Optical rotatory dispersion spectra of rabbit IgG (pH 7.0, dashed line), Fd fragment (pH 5.5, solid line), and Fd corrected for 15% Fab contamination (dotted line) at 25–27° in 0.1 M NaCl.

concentration dependence was found for its $s_{20,w}$, as shown in Figure 5; such distinct curvature is typical of rapidly equilibrating self-association equilibria, based on the theory of Gilbert (1955, 1959) which has been recently summarized by Cann (1970). The curve asymptotically approaches a normal regression curve for a globular protein above a concentration of 5 mg/ml and the limiting sedimentation coefficient is that expected for a dimer. This result, together with that obtained by sedimentation equilibrium, indicates that Fd self-association is a dimerization reaction.

Sedimentation coefficients were also determined for very slightly associating Fd species, isolated by gel chromatography. The data points are noted in Figure 5 to give an $s_{20,w}$ of about 2.2S at less than 0.2 mg/ml, where equilibrium sedimentation on the same material gave a monomer molecular weight. This value is essentially the same as the $s^0_{20,w}$ for light-chain monomer which has been found to equal 2.23 S (Björk and Tanford, 1971b).

We observed that elution profiles for Fd and Fab are centered at the same volume upon chromatography on Sephadex G-75 columns, although the Fd peak tends to be somewhat broader than that for an equal amount of Fab. This behavior agrees with dimeric Fd being the limiting species in its self-association, while suggesting that its shape may be slightly less compact than that of the Fab fragment, since the most dimeric fractions elute slightly ahead of the Fab peak. Since the monomeric fractions elute beyond the Fab peak, the comparatively broad Fd elution profile appears to result from the different shapes of its limiting dimer and monomer and the equilibria between them.

Optical Rotatory Dispersion (ORD). The average ORD spectrum of several pooled Fd samples is given in Figure 6, together with a curve corrected for 15% Fab contamination and the spectrum for rabbit IgG; the corrected spectrum is slightly more negative in reduced mean residue rotation than the experimental curve, but virtually identical in shape. Spectral features are much the same for Fd and IgG, namely double

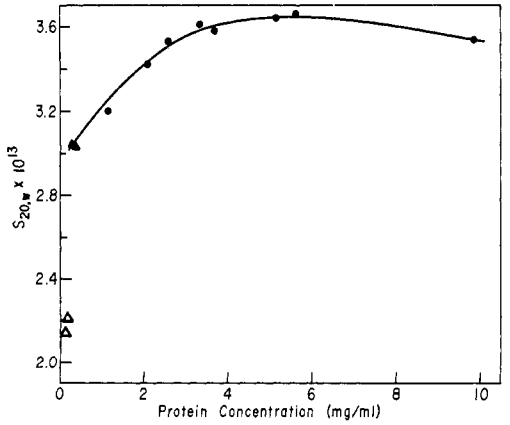

FIGURE 5: Sedimentation coefficients of pooled rechromatographed Fd at different protein concentrations (filled symbols) and also for a low association constant fraction which is monomeric at the concentrations used (open triangles). Circles refer to data collected with schlieren optics, while triangles indicate data collection with the uv scanner.

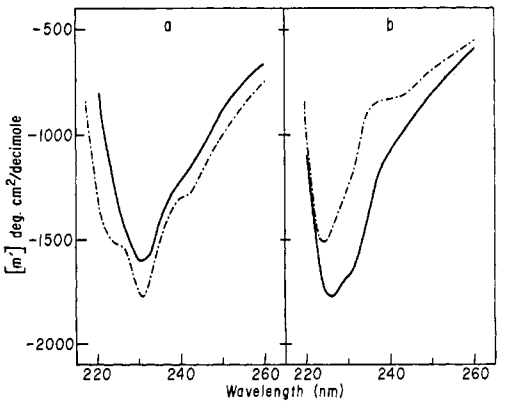

FIGURE 7: Optical rotatory dispersion spectra: (a) for rabbit heavy chain, as found experimentally in 0.01 M NaCl, pH 5.5 (dashed line) and as calculated for an equimolar mixture of Fd and Fc (solid line); (b) for rabbit Fab fragment, as observed experimentally (dashed line) and as calculated for an equimolar mixture of Fd and light chain dimer (solid line).

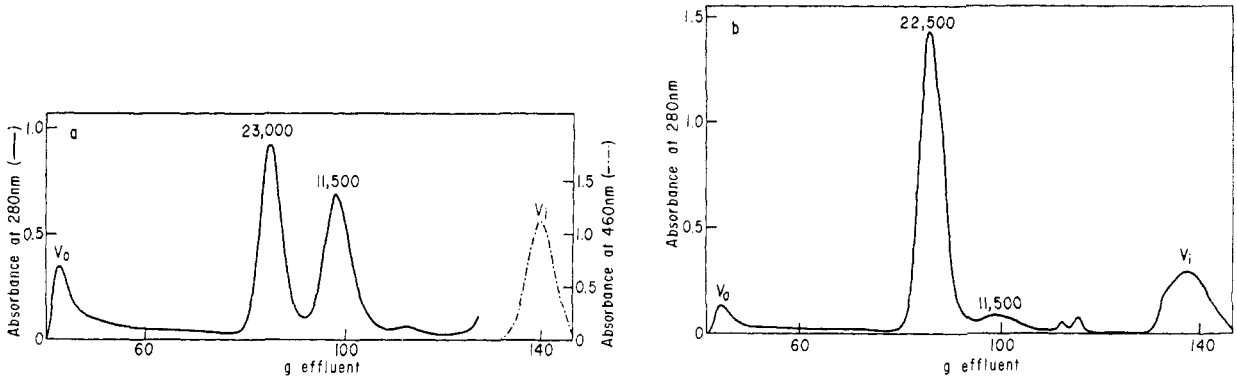

FIGURE 8: Gel chromatography of fully reduced and carboxymethylated papain digests of (a) 13.4 mg of Fd and (b) 11.8 mg of Fab, on 6% agarose in 6 M guanidine hydrochloride. The samples were treated with 0.5% papain for 20 min; the reaction was then quenched with iodoacetamide and the digests were quantitatively applied to the column (1.5 × 90 cm) after reduction and alkylation. The V_i peak for (a) was determined by the elution position of Dnp-valine, whose absorbance was read at 460 nm; Blue Dextran was used to determine the void volume, V_0, for all experiments.

minima between 225 and 230 nm and a shoulder around 240 nm, but the fragment has a distinctly greater levorotation than the whole immunoglobulin throughout this range.

In addition to studies on pooled Fd, individual chromatographic fractions were also monitored by ORD. The results of such experiments suggest there is an increase of levorotation as a function of decreasing weight-average molecular weight. A pool of less associated, trailing Fd fractions was found to give a similar increase as its concentration was decreased from 2.6 to 0.03 mg per ml, which indicates that these ORD changes depend at least partially upon the degree of Fd association. This same ORD behavior was observed for light-chain monomer by Painter *et al.* (1972a).

The ORD data given in Figure 7a show that the heavy-chain spectrum obtained by Björk and Tanford (1971a) is almost the same as that calculated for an equimolar mixture of Fd and Fc. The Fd spectrum used for the calculation is the corrected spectrum of Figure 6; the Fc spectrum is taken from Steiner and Lowey (1966). The resolution obtained in the latter work is relatively poor: for example, the double minimum in the parent IgG spectrum was not resolved. This probably accounts for the failure to observe the shoulder at 225 nm in the calculated spectrum. The small difference in magnitude between the two curves is typical of differences between different preparations of the same protein in the IgG system. The essential similarity of the two curves, as well as the identity of Fd and heavy chain shown by immunodiffusion studies, indicates that Fd conformation is substantially the same in isolated fragments and in heavy chain, insofar as these methods can determine.

In contrast, Figure 7b shows that the experimental ORD curve for Fab (Steiner and Lowey, 1966) differs markedly from the spectrum calculated for an equimolar mixture of Fd and light-chain dimers. An even greater disparity would have been observed had the calculated curve been based on Fd and light-chain monomers. It is evident that association between Fd and light chain to form Fab results in considerable rearrangement of the structures of one or both constituent chains. This result is similar to what has been observed previously from a comparison of heavy- and light-chain ORD spectra with that of native or reconstituted IgG (Dorrington *et al.*, 1967; Björk and Tanford, 1971c).

Fluorescence Quenching. The relationship between Fd association and its hapten binding was pursued in a preparation of anti-Dnp Fd, identical with that used by Painter *et al.* (1972a). These workers found that pooled anti-Dnp Fd has essentially the same binding affinity and number of sites as the corresponding heavy-chain dimers, as determined in equilibrium dialysis measurements of pooled material. Under the conditions of high protein concentration required for these experiments, effectively all the Fd present would exist as dimers, according to the sedimentation velocity data cited previously; thus it is understandable that only one binding site per dimer was found, as with heavy-chain dimer (Björk and Tanford, 1971a). Fluorescence quenching can, however, be conducted at much lower concentrations where the Fd subpopulation with low association constant will be essentially all monomer.

We conducted fluorescence quenching titrations with Dnp-aminocaproic acid to compare hapten binding ability for G-75 fractions of largely dimerized anti-Dnp Fd from the leading side of the peak with slightly associated trailing fractions; sedimentation equilibrium measurements were made to ensure that the latter were mostly monomeric species. The dimeric fraction was found to exhibit the same quenching behavior found for pooled anti-Dnp Fd by Painter (1971); unfortunately, inconsistent results were obtained for monomeric Fd, quenching in one instance being only slightly above the nonspecific Fd control, while close to the dimer curve in another. Stocks of the antibody fragment were exhausted at this point, and the experiment could not be repeated

Proteolytic Cleavage of Fd. When purified Fd was incubated with 0.5% papain at 37° for 20 min, substantial cleavage of the protein took place. The product was denatured, fully reduced, carboxymethylated, and applied to a calibrated guanidine–agarose column. About 40% of the protein was degraded to small peptides eluting in the internal volume peak. The remainder, as shown in Figure 8a, consisted about equally of undegraded Fd chains and of polypeptide chains of mol wt 11,500. No polypeptides of intermediate size were detectable. This result is similar to that obtained by Karlsson *et al.* (1969) upon proteolysis of human light chains, which they suggest reflects the initial cleavage of light chain into constant and variable halves, followed by degradation to smaller peptides. It is probable that the same explanation applies here, and that the 11,500 molecular weight peak of Figure 8a contains the variable and constant portions of Fd.

Chromatography of the cleavage products under native conditions yielded only a small peak with mol wt 11,500, but enough material was obtained for preliminary physical stud-

ies. Sedimentation equilibrium measurement yielded a curved plot, with molecular weight 11,500 near the upper meniscus and a weight-average molecular weight of 16,000 near the base of the column, where the protein concentration was 0.65 mg/ml. Sedimentation–velocity experiments using an ultracentrifuge equipped with a photoelectric scanner gave an $s_{20,w}$ of 1.66 S, at concentrations of 0.05 and 0.08 mg per ml, where, according to the sedimentation equilibrium results, the products exist entirely in the "monomeric" form with mol wt 11,500. The frictional ratio obtained from this result is 1.11, indicating that this species is compact and globular. The results are almost identical with those obtained by Karlsson *et al.* (1969), for the cleavage product of human light chains, and it seems highly probable that the product obtained by us consists of a mixture of the variable and constant domains of the Fd fragment, in compact, globular, presumably native-like form. The sedimentation equilibrium results show that the domains (if that is what they are) have, like Fd itself, a tendency to aggregate, and this accounts in part for the low yield obtained from chromatography under native conditions. Some of the cleavage product elutes with monomeric Fd, and, indeed, the Fd peak in this experiment was found to be skewed on its trailing side.

Figure 8b shows the result of a control experiment in which Fab was subjected to the same treatment. Only a very small peak is observed at a molecular weight of 11,500. Even this amount of domain-size fragment probably did not arise from the experiment, as a small peak at this location is usually already present in the original Fab, *i.e.*, it probably results from the cleavage of highly susceptible Fd or light chains during the preparation of Fab. In any event, the association between Fd and light chains to form Fab clearly protects the Fd fragment from significant further proteolysis.

Immunochemical Studies. Ouchterlony analysis of Fd using goat antiserum prepared against pooled Fd fragments gave a dark outer precipitin line and a less pronounced inner one, both of which show identity with the Fab line; these results agree with the immunodiffusion data presented by Fleischman *et al.* (1963). We found that the outer and inner precipitin lines, respectively, show identity with dimeric and monomeric Fd fractions. In contrast to these data, the use of goat antiserum prepared against intact Fab fragments but specific for Fd determinants leads to only single precipitin lines with identity between pooled Fd, its G-75 fractions, and Fab.

The dissimilar behavior of these two antisera must reflect differences in determinants on Fd as it exists free and in combination with light chain. This could be a consequence of Fab antiserum recognizing only conformational features unique to Fd when self-associated or when combined with light chain. Another possible explanation is that antibodies specific for Fd monomers might be directed against surfaces which lie between chains in the dimer or Fab, and these determinants can only be immunogenic on slightly associating Fd species.

Immunodiffusion was also performed on the 11,500-dalton cleavage product, using goat anti-Fd serum and anti-Fab serum with specificity for Fd determinants. The product reacted with anti-Fd serum and showed partial identity with whole Fd fragments, whereas no precipitin lines were observable with anti-Fab serum. It is significant that the product preserved some determinants found on the intact Fd. The unreactivity with the anti-Fab serum may simply reflect the loss of features from the interdomain region; it may also result from inability of the antiserum to recognize features of the Fd surface which were covered by light chain in the Fab used for immunization. This behavior is similar to loss of the Inv marker on a C_κ domain upon cleavage of its parent light chain, which has been reported by Solomon *et al.* (1970).

Discussion

The data we have presented reveal that Fd fragments from rabbit IgG possess strikingly heterogeneous physicochemical properties. This cannot be attributed to the presence of denatured species because chromatography was used to remove them, but reflects intrinsic heterogeneity of the protein. Its heterogeneous behavior is thus a manifestation of sequence variation, which can be unequivocally attributed to residue changes in the variable region of Fd. This conclusion arises from amino acid sequence studies of rabbit Fd, which prove that both allotypic changes related to genetic differences, and idiotypic alterations involved with hapten binding properties are situated only in the variable half of the fragment (Mole *et al.*, 1971). Current views of immunoglobulin structure (Edelman and Gall, 1969; Dorrington and Tanford, 1970) predict that the constant and variable regions of Fd should exist as compact, globular domains within the intact molecule, and this is consistent with the results of the cleavage experiments given above. The range of Fd association constants must stem from interactions mediated by the variable domains.

Studies in this laboratory by Painter *et al.* (1972a) showed that the unitary free energy of hapten binding to anti-Dnp IgG is equal to its sum for isolated light chain and heavy chain or Fd dimer. This indicates that the binding site remains intact for these chains whether free as dimers or associated in IgG. Using fluorescence quenching experiments we have attempted to ascertain if such binding sites can exist on monomeric Fd. While we find that highly dimeric fractions exhibit the same binding properties shown by pooled Fd, monomeric fractions have not given satisfactory results; the extent to which integrity of the hapten binding site is maintained in monomeric Fd remains an open question.

While Fd association yields a broad equilibrium between stable monomer and dimer, rabbit light chain exists in two distinct populations, 60% being monomers and 40% dimers, which respectively exhibit a slight tendency to associate or dissociate (Björk and Tanford, 1971b). Both types of light chain recombine with heavy chains under native conditions to produce a fully normal immunoglobulin (Björk and Tanford, 1971c); since our ORD and immunodiffusion data indicate that the Fd portion of heavy chain is virtually identical with the isolated fragment, the latter may also be considered able to reconstitute native Fab when it reassociates with light chain. These Fd–light-chain interactions yield a stable molecule with homogeneous physical properties, and the chains are inseparable even in 0.01 M HCl, which disrupts the Fc dimer (Palmer *et al.*, 1963). The association between Fd and light chain differs so drastically from their self-association that we are forced to conclude they possess unique complementarity for each other. The conformational change indicated by ORD data to occur upon chain recombination is probably involved with these highly specific interactions. They would be expected to exist between both pairs of domains, although they may well be greatest between constant domains.

The 6-Å resolutions crystallographic structure of human myeloma Fab′ (New) indicates close proximity of constant domains and of variable domains (Poljak *et al.*, 1972), but lacks the resolution needed to reveal complementarity between them. On the basis of their interpretation of Fab tertiary and quaternary structure these investigators predict formation

of very stable Fd and light-chain dimers; as we have shown, such is not the case for rabbit chains.

It may be noted in conclusion that the ready cleavage of Fd by papain suggests the possibility of preparing the variable and constant domains of Fd in stable, globular form. The preparation of the corresponding fragments from human light chains has been described by Solomon and McLaughlin (1969) and by Karlsson *et al.* (1969), and their ability to adopt their native conformations independently of each other has been demonstrated (Björk *et al.*, 1971; Karlsson *et al.*, 1972). A preliminary report, indicating the possible isolation of the variable domain from the heavy chain of a human cryoglobulin, has recently appeared (Dammaco *et al.*, 1972).

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