Correlation between the Exposure of Aromatic Chromophores at the Surface of the Fc Domains of Immunoglobulin G and Their Ability to Bind Complement†

David E. Isenman, James R. Ellerson, Robert H. Painter, and Keith J. Dorrington*

ABSTRACT: The recognition that certain biological effector functions associated with the Fc region of human IgG are mediated exclusively by either the $C_\gamma2$ or $C_\gamma3$ domains prompted a study of some of the physical properties of the isolated domains in an attempt to correlate these with functional differentiation. The degree of aromatic chromophore exposure on intact Fc and fragments corresponding to the $C_\gamma2$ and $C_\gamma3$ domains were determined by solvent perturbation difference spectroscopy using 20% ethylene glycol. For the monomeric $C_\gamma2$ fragment one of the two tryptophans and all four of the tyrosines were exposed to solvent. In the pFc′ fragment, which represented a dimer of two intact $C_\gamma3$ domains, an average of 0.4 of the two tryptophans and 3.3 of the five tyrosines per chain were exposed. These data were consistent with the suggested involvement of tryptophan in complement fixation since $C_\gamma2$ binds C1q but pFc′ does not. Several fragments derived from the $C_\gamma3$ region had previously been shown to have differing environments for their aromatic side chains from circular dichroism studies. These fragments have now been shown to exhibit different degrees of chromophore exposure to solvent. Removal of the carboxy-terminal heptapeptide from the intact $C_\gamma3$ domain resulted in a fragment not only showing a greater exposure of aromatic residues but also having the ability to bind C1q. Our data suggest that the structural requirements for C1q binding may be quite commonplace within Fc, but tertiary folding limits their expression except in $C_\gamma2$ in the native molecule. The solvent perturbation observed with Fc was somewhat lower than would have been expected from the results with the isolated domains, suggesting that interdomain interactions may result in burial of aromatic residues.

It is now established that the γ[1] and L chains of IgG are folded into a linear series of compact domains, each domain corresponding to one of the homology regions apparent in the primary sequence (for a recent review, see Cathou and Dorrington, 1975). Previous studies in this laboratory have provided evidence that certain biological effector functions displayed by the Fc fragment of IgG are mediated exclusively by either the $C_\gamma2$ or $C_\gamma3$ domains (Minta and Painter, 1972; Yasmeen et al., 1973, 1976; Ellerson et al., 1976). The recognition that the $C_\gamma2$ and $C_\gamma3$ domains of IgG perform discrete biological functions has prompted us to study some of the physical properties of the isolated domains in an attempt to correlate these with their functional differentiation.

The involvement of aromatic side chains, especially that of tryptophan, in the interaction of C1 with IgG has been suggested previously (Allan and Isliker, 1974; Johnson and Thames, 1976). Bearing in mind that pepsin Fc′ (pFc′), which corresponds to a dimer of the intact $C_\gamma3$ domains, does not bind C1 and that $C_\gamma2$ does (Yasmeen et al., 1976), we wished to determine whether these differences in a biological property are associated with a different degree of exposure of aromatic residues to the solvent in the isolated domains.

Dorrington et al. (1972) have reported differences among the circular dichroism spectra of several proteolytic fragments derived from the $C_\gamma3$ region of IgG, suggesting differing environments for the aromatic chromophores in these fragments. Therefore, we also wished to determine if these differences were the result of differing degrees of exposure of the aromatic residues to solvent in these fragments.

The degree of chromophore exposure was investigated by the techniques of solvent perturbation difference spectroscopy, enzymatic iodination, and acid denaturation difference spectroscopy. The solvent perturbation technique allows one to obtain an estimate of the number of tyrosine and tryptophan residues in a protein which are partially or completely exposed to the solvent.[2] Conversely the acid denaturation technique gives a measure of the number of aromatic chromophores which are not exposed to the solvent in the native state. The technique of enzymatic iodination gives a measure of whether tyrosine and to a lesser extent histidine residues within a protein are sufficiently exposed to be good substrates for enzymatic iodination.

Using these techniques we now present evidence for a direct correlation between a high degree of aromatic chromophore exposure in an immunoglobulin domain and the ability of that domain to bind C1. Our data also indicate that the inability of an immunoglobulin domain to bind C1 may be the result of tertiary folding which keeps the aromatic side chains sequestered from the surface of the molecule.

Materials and Methods

Proteins. Fc was prepared from IgG1 by trypsin digestion using enzyme (Sigma type XI, DCC treated) coupled to Sepharose as described previously (Ellerson et al., 1976). A

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[1] Abbreviations used in this paper: IgG, immunoglobulin G; γ, heavy chain of IgG; L, light chain; CD, circular dichroism; TBS, Tris buffered saline, LPO, lactoperoxidase.

[2] A fractional change implies either that the perturbation involves only a portion of the chromophore or that, on the average, a particular chromophore is completely exposed for only a fraction of the time.

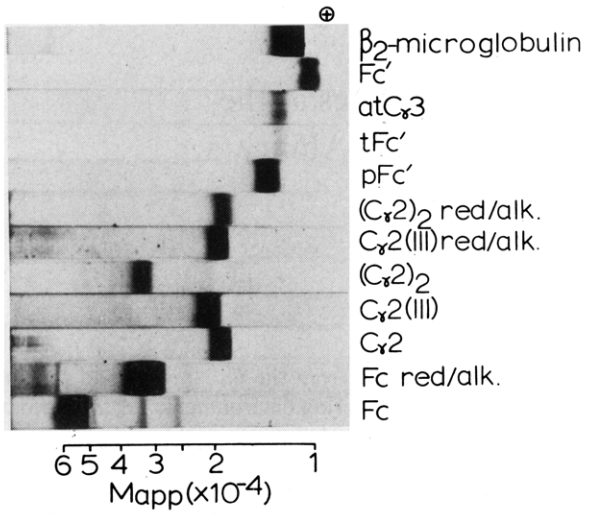

FIGURE 1: Analysis of the proteins used in this study by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were electrophoresed in 11.2% gels in the absence of reducing agent. Refer to text for the details of fragment nomenclature used.

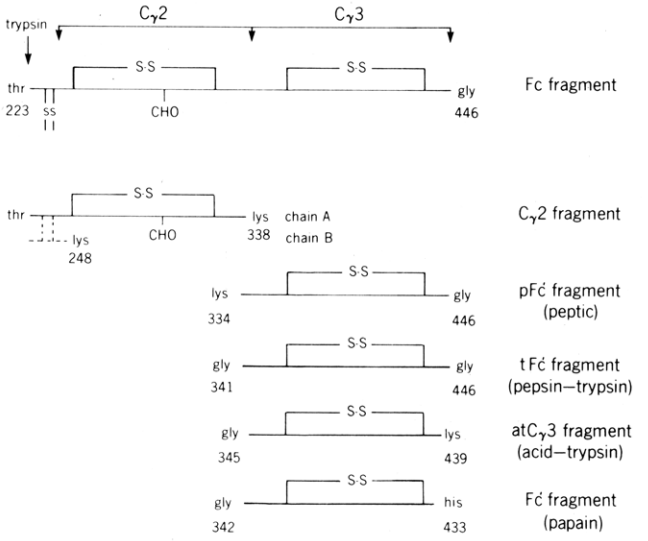

FIGURE 2: Schematic representation of the molecular location of the fragments derived from Fc used in this study.

fragment derived from the $C_\gamma2$ homology region was isolated from trypsin–Sepharose digests of acid-treated Fc as described by Ellerson et al. (1976). This fragment, termed $C_\gamma2$ (III), is composed of two polypeptide chains of unequal mass joined by disulfide bonds: one chain corresponds to the stretch of sequence between Thr-223[3] and Lys-338 and the second to the section between Thr-223 and Lys-248. A monomeric $C_\gamma2$ fragment was prepared by mildly reducing and alkylating $C_\gamma2$ (III) followed by gel filtration on Sephadex G-25 (Ellerson et al., 1976). In addition to $C_\gamma2$, these digestion conditions also produced a fragment derived from the $C_\gamma3$ region referred to in this paper as acid–trypsin $C_\gamma3$ ($atC_\gamma3$). A disulfide-linked dimer of $C_\gamma2$ ($(C_\gamma2)_2$) was also purified from peak II of an acid–trypsin digest of Fc chromatographed on Sephadex G-100 in 1 M acetic acid–25 mM NaCl by the following modification of the procedure previously described by Ellerson et al. (1976). Peak II material in 0.15 M NaCl–10 mM Tris-HCl (pH 7.8) (TBS) was passed through an affinity column of staphylococcal protein A coupled to Sepharose (Pharmacia, Uppsala, Sweden). Since staphylococcal protein A binds intact Fc with high affinity while displaying no affinity for Fc subfragments (Kronvall and Frommel, 1970; Ellerson, unpublished observation), the $(C_\gamma2)_2$ fragment was recovered in the unabsorbed fraction while intact Fc was retained on the column. The small amounts of $C_\gamma2$ monomer and Fc still contaminating the $(C_\gamma2)_2$ were removed by chromatography on Ultrogel AcA 54 (LKB Industries) in TBS. pFc′ was made by incubating Fc with pepsin (Worthington Biochemicals Corp., Freehold, N.J.) at an enzyme to substrate ratio of 1:100 (w/w) for 18 h at 37 °C and pH 4.5. The digestion was terminated by raising the pH to 9.0 and pFc′ was separated from undigested Fc and smaller fragments by gel filtration on Sephadex G-50 equilibrated in TBS. Papain Fc′ was produced by extensive digestion of an IgG1 myeloma protein on a papain–Sepharose column as described previously (Isenman et al., 1975b). Briefly, IgG1 (100 mg) was recycled for 18 h through a column containing 6 ml of Sepharose, to which had been coupled 60 mg of papain (Sigma Chemical Co., St. Louis, Mo.). The buffer contained 10 mM cysteine-HCl to keep the papain in an active form. At the end of the digestion period, the products were washed from the papain–Sepharose column with 1 M NaCl, concentrated in an Amicon ultrafiltration cell, and chromatographed on Ultrogel AcA 54 equilibrated in TBS. The peak eluting at $V_e/V_t = 0.63$ was shown to be Fc′ by immunodiffusion and sodium dodecyl sulfate–gel electrophoresis. Trypsin Fc′ (tFc′) was prepared by digesting pFc′ for 4 h with trypsin (1:50 w/w) followed by chromatography on Sephadex G-50 in TBS (Natvig and Turner, 1971). β_2 microglobulin was prepared from the urine of kidney transplant patients essentially as described by Berggård and Bearn (1968).

The interchain disulfide bonds of Fc, $(C_\gamma2)_2$, and $C_\gamma2$(III) were reduced with 10 mM dithioerythreitol (Sigma) in TBS (pH 8.0) for 60 min at room temperature followed by alkylation with 25 mM iodoacetamide. Excess reagents were removed by exhaustive dialysis against TBS. The purity of all fragments and extent of reduction of reduced fragments were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see Figure 1) according to the method of Fairbanks et al. (1971).

The molecular location of these fragments within Fc has been determined in previous studies (Ellerson et al., 1976; Turner and Bennich, 1968; Natvig, 1971; Turner et al., 1969) and is shown diagrammatically in Figure 2. Molar concentrations of proteins used were determined spectrophotometrically at 280 nm using the $A_{1cm}^{1\%}$ and molecular weight values given in Table I.

Solvent Perturbation Difference Spectroscopy. Difference spectra were recorded with a Cary 118 double-beam spectrophotometer (Varian Inst.) equipped with thermostated cell holders maintained at 25.0 ± 0.1 °C. Full scale expansion (10 in.) of 0.02 A was routinely used. The experimental design involved the use of a matched pair of rectangular tandem cells (Hellma), each having a total pathlength of 0.876 cm. One side of each tandem cell contained 1.0 ml of 40% ethylene glycol in TBS and the other side 1.0 ml of protein solution in TBS. The initial A_{280}^{1cm} of the protein solutions ranged from 0.7 to 1.4.

In each experiment, a baseline was established prior to mixing over the range 330 to 260 nm at a scanning speed of 0.2 nm/s and a pen period of 5 s. The sample cell was then mixed by repeated inversion and the difference spectrum recorded. Finally the reference cell was mixed and an after mixing

[3] The Eu-numbering system (Edelman et al., 1969) is used throughout this paper.

TABLE I: Values of $A_{1cm,280nm}^{1\%}$ and Molecular Weights Used in This Study.

Fragment	$A_{1cm,280nm}^{1\%}$ [b]	Mol Wt[c]
β_2 microglobulin	16.8	11 700
Fc	13.8	26 500/chain
$(C_\gamma 2)_2$	12.7	14 500/chain
$C_\gamma 2$	12.7	14 500
$C_\gamma 2$ (III)[a]	10.8	17 200
pFc′	15.3	12 000/chain
tFc′	16.2	11 330/chain
at$C_\gamma 3$	15.1	10 900/chain
Fc′	15.8	10 500/chain

[a] This fragment, isolated from peak III following gel filtration of an acid–trypsin digest of Fc on G-100 in acid (see Ellerson et al., 1976), is composed of two polypeptide chains linked by disulfide bonds. One corresponds to the stretch of sequence between Thr-223 and Lys-338 and the other to Thr-223 and Lys-248. [b] The $A_{1cm,280nm}^{1\%}$ values of all species except for β_2 microglobulin and Fc′ were obtained from dry weight determinations (Ellerson, 1976). The values used for the latter two fragments were those of Berggård and Bearn (1968) and Turner et al. (1969), respectively. [c] Molecular weights were calculated from amino acid sequence and carbohydrate data.

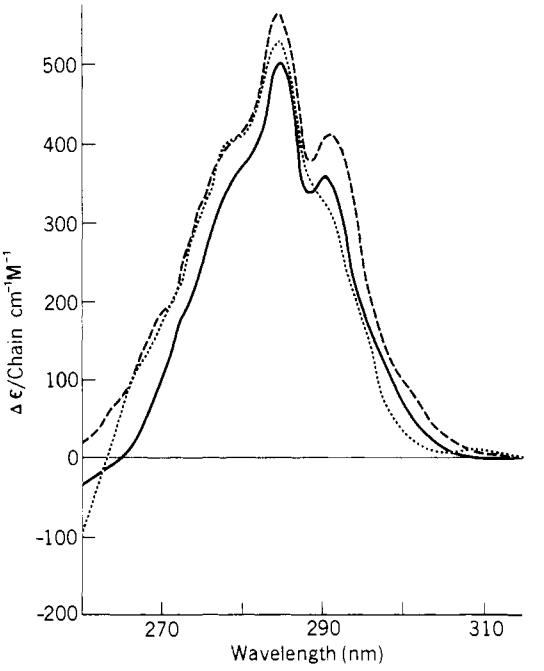

FIGURE 3: Ultraviolet difference spectra generated in the presence of 20% ethylene glycol for $C_\gamma 2$ derived fragments: (- - -) $C_\gamma 2$(III); (—) $C_\gamma 2$; (. . .) $(C_\gamma 2)_2$.

baseline was recorded. The digital output of the instrument was connected to a data logger which stored the data on magnetic tape for subsequent computer analysis. The data were analyzed with the aid of a computer program developed by Dr. D. I. C. Kells, whch averaged the two baselines and calculated the change in molar absorptivity per chain ($\Delta\epsilon$/chain cm^{-1} M^{-1}) of protein produced by solvent perturbation. Data analysis was performed only for experiments in which the before and after mixing baselines were in good agreement, indicating the absence of any significant superimposed protein spectrum contribution resulting from unequal protein concentrations in the sample and reference cells. In order to quantitate the number of tyrosine and tryptophan residues exposed to the perturbant, use was made of the following pair of simultaneous equations (Herskovits and Sorenson, 1969a):

$$\Delta\epsilon_{290\text{-}292}\ (\text{protein}) = a\Delta\epsilon_{290\text{-}292}\ (\text{AcTrpOEt}) + b\Delta\epsilon_{290\text{-}292}\ (\text{AcTyrOEt})$$

$$\Delta\epsilon_{285\text{-}286}\ (\text{protein}) = a\Delta\epsilon_{285\text{-}286}\ (\text{AcTrpOEt}) + b\Delta\epsilon_{285\text{-}286}\ (\text{AcTyrOEt})$$

where a and b represent the number of tryptophan and tyrosine residues exposed per mole of protein and $\Delta\epsilon$ refers to the change in molar extinction coefficient for the protein and the model compounds *N*-acetyltryptophan ethyl ester and *N*-acetyltyrosine ethy ester at the wavelengths indicated. These equations describe a set of overlapping curves whose sum represents the difference spectra peaks at 290–292 and 285–286 nm. This approach has been successfully used (Herskovits and Sorenson, 1968b; Callahan et al., 1973) in determining aromatic chromophore exposure in a number of globular proteins. Tabulated values represent the mean of three or more experiments except in cases where insufficient quantities of fragment were available.

Acid Denaturation. These studies involved an experimental design similar to that used for solvent perturbation. The pH of protein solutions was lowered from 7.8 to 2.4 by mixing equal volumes of a weakly buffered protein solution and a strong glycine-HCl buffer at pH 2.4 in tandem cells. The ionic strength of all solutions was kept constant at 0.2. Protein solutions with absorbances between 0.2 and 0.5 were used and difference spectra were recorded as described above.

Radiolabeling Techniques. Samples of protein were labeled with ^{125}I using the lactoperoxidase procedure of Marchalonis (1969). To 250 μg of protein in 50 mM phosphate buffer, pH 7.4, were added 200 μCi of carrier-free $Na^{125}I$ (NEN) and lactoperoxidase (Calbiochem, San Diego, Calif.) to give an enzyme-to-substrate ratio of 1:80 (w/w), in a total volume of 55 μl. Iodination was initiated by the addition of 3 μl of 8.8 mM H_2O_2. A total of three such additions were made at 10-min intervals, after which the iodination was terminated by dilution of the reaction mixture with cold phosphate buffer to 0.5 ml. Unreacted $Na^{125}I$ was removed by exhaustive dialysis of the protein against a buffer containing 1 mM cold NaI. Radioactivity was measured using a Nuclear Chicago automatic γ counter.

The terminal sialic acid residues of the carbohydrate moiety in Fc was tritiated by the method of Van Lenten and Ashwell (1971). Radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter, the samples being dispersed in a suitable scintillation cocktail.

Complement Assay. C1 binding by unaggregated proteins was measured by a previously described (Painter et al., 1974; Yasmeen et al., 1976; Isenman et al., 1975a,b) modification of the method of Augener et al. (1971). This assay has been shown by Augener and ourselves to have the same class and subclass specificity as the whole complement-fixation test.

Results

Degree of Chromophore Exposure by Solvent Perturbation. Using 20% ethylene glycol as the solvent perturbant, we examined the degree of chromophore exposure in $Fc_\gamma 1$ and subfragments thereof as shown in Figure 2.

The solvent perturbation difference spectra of the three species of $C_\gamma 2$ fragment are shown in Figure 3. The spectra of $C_\gamma 2$ and $C_\gamma 2$(III) are qualitatively and quantitatively similar. The spectra display three positive (i.e., red shifted) peaks above 270 nm. The peak at 291 nm arises mainly from the pertur-

TABLE II: Summary of Aromatic Chromophore Exposure Determined from Solvent Perturbation.

Protein	No. of Expt.	Total No. of[a] Trp/chain	No. of Trp Exposed/chain (mean ± SD)	Total No. of[a] Tyr/chain	No. of Tyr Exposed/chain (mean ± SD
$C_\gamma 2$	3	2	1.0 ± 0.1	4	3.7 ± 0.35
$C_\gamma 2$ (III)	3	2	1.1 ± 0.16	4	4.0 ± 0.56
$(C_\gamma 2)_2$	1	2	0.9	4	4.1
pFc′	4	2	0.4 ± 0.14	5	3.3 ± 0.26
tFc′	1	2	0.5	5	3.2
$atC_\gamma 3$	4	2	0.9 ± 0.09	5	3.3 ± 0.37
Fc	7	4	1.0 ± 0.24	9	6.8 ± 0.78
Fc reduced/alk	4	4	1.1 ± 0.1	9	6.6 ± 0.15
pFc′ + $C_\gamma 2$[b]		4	1.4	9	7.0
β_2 microglobulin	3	2	1.4 ± 0.15	6	5.4 ± 0.9

[a] Calculated from sequence data. [b] These values represent the algebraic sum of the number of tryptophan and tyrosine residues exposed in the isolated domains.

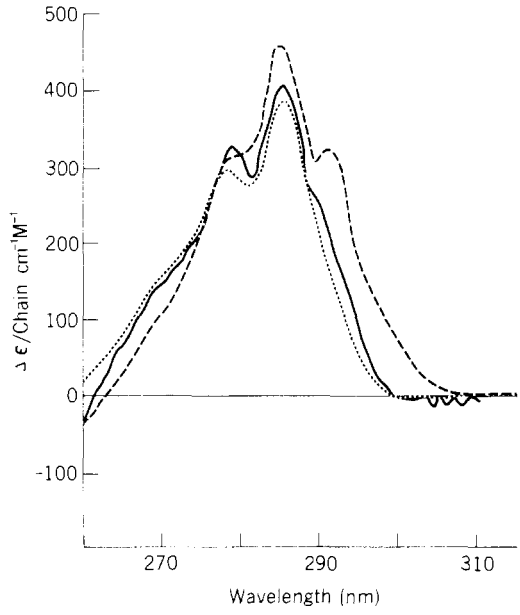

FIGURE 4: Ultraviolet difference spectra generated in the presence of 20% ethylene glycol for $C_\gamma 3$ derived fragments: (. . .) pFc′; (—) tFc′; (- - -) $atC_\gamma 3$.

bation of the indole ring of tryptophan (Donovan, 1969), while that at 284.5 nm is due to the perturbation of the phenolic ring of tyrosine. However, the magnitude of the latter peak is affected by an overlap from the tryptophan peak. The peak at 278 nm also contains contributions from both tyrosine and tryptophan. Also shown in Figure 3 is the spectrum for $(C_\gamma 2)_2$. Although insufficient quantities of this protein were available for replicate experiments and statistical analysis, based on a number of spectra where the both-sides mixed baseline did not meet the criteria outlined in the experimental section, we believe that the small change in shape of the 291-nm peak is probably not significant. Therefore, the exposure of aromatic residues to the solvent appears to be independent of the integrity of interchain disulfide bonds. This conclusion is further supported by the data obtained with Fc (see below). Using the equations and model compound data of Herskovits and Sorenson (1969a), the number of tyrosine and tryptophan residues exposed to the solvent per mole of protein chain was calculated for the three $C_\gamma 2$ derived fragments and are presented in Table II. Approximately 1 out of the 2 tryptophans and all 4 of the tyrosines in the $C_\gamma 2$ domain were found to be accessible to the solvent.

Several fragments related to the $C_\gamma 3$ domain of IgG1 were also examined by this technique. Pepsin Fc′ (pFc′), which is equivalent to a fully intact $C_\gamma 3$ domain since it is generated by a single cleavage in the switch region between the $C_\gamma 2$ and $C_\gamma 3$ domains and is not degraded at the C terminus, displays the solvent perturbation spectrum shown in Figure 4. This spectrum is markedly different from that of $C_\gamma 2$, the region correlated with tryptophan absorbance showing considerably less intensity. The 286-nm peak is also smaller, although this is in part due to a smaller tryptophan contribution to this peak. We have calculated that 0.4 of 2 tryptophans and 3.3 of 5 tyrosines are exposed to the solvent (Table II).

Recalling that the circular dichroism spectra of pFc′ and tFc′ were significantly different from that of papain Fc′, suggesting a different environment for the aromatics of the latter fragment (Dorrington et al., 1972), we decided to examine the possibility that the spectral changes were correlated with differences in the degree of exposure of aromatic residues to the solvent. Since the papain Fc′ fragment lacks Tyr-436 from its C-terminal end, we made use of the newly characterized $atC_\gamma 3$ fragment (Ellerson et al., 1976) which contains the same number of tyrosines as pFc′ (C terminus of $atC_\gamma 3$ is Lys-439) and has a CD spectrum virtually identical with that of Fc′ (Ellerson et al., 1976). The solvent perturbation spectra of tFc′ and $atC_\gamma 3$ are shown in Figure 4. While the spectrum obtained for tFc′ is very similar in shape and intensity to that of pFc′, the spectrum obtained for $atC_\gamma 3$ is significantly different, especially in the region of tryptophan absorbance. Indeed the $atC_\gamma 3$ difference spectrum closely resembles that of $C_\gamma 2$. We have calculated that 0.9 of 2 tryptophans and 3.3 of 5 tyrosines (Table II) in $atC_\gamma 3$ are seen by the perturbant. Thus the altered environment of the aromatic chromophores observed in the CD spectrum for $atC_\gamma 3$ relative to pFc′ may be attributed to the greater degree of exposure of these chromophores to the solvent.

Figure 5 shows the solvent perturbation spectra of native and mildly reduced and alkylated Fc fragment together with a spectrum calculated by summing the spectra of $C_\gamma 2$ and pFc′. All three spectra are qualitatively similar and, while the spectra of Fc and mildly reduced Fc were of very similar intensity, the additive spectrum was significantly larger (see Table II).

Finally we examined the degree of chromophore exposure in β_2 microglobulin, a protein resembling a free immunoglobulin domain. A very large solvent perturbation spectrum

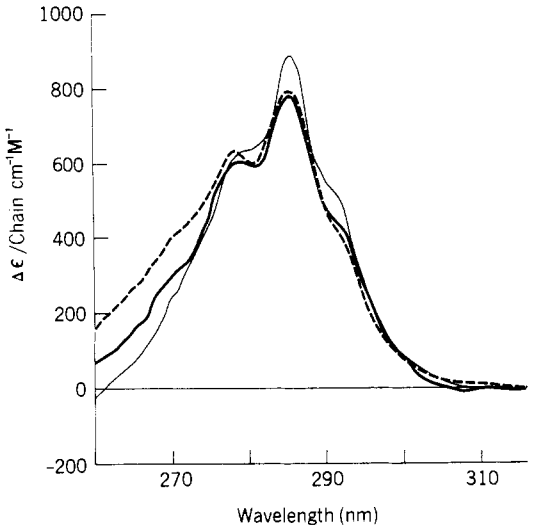

FIGURE 5: Ultraviolet difference spectra generated in the presence of 20% ethylene glycol for Fc (- - -) and mildly reduced and alkylated Fc (—) compared with a theoretical spectrum calculated by algebraically summing the spectra of $C_\gamma 2$ and pFc′ (—).

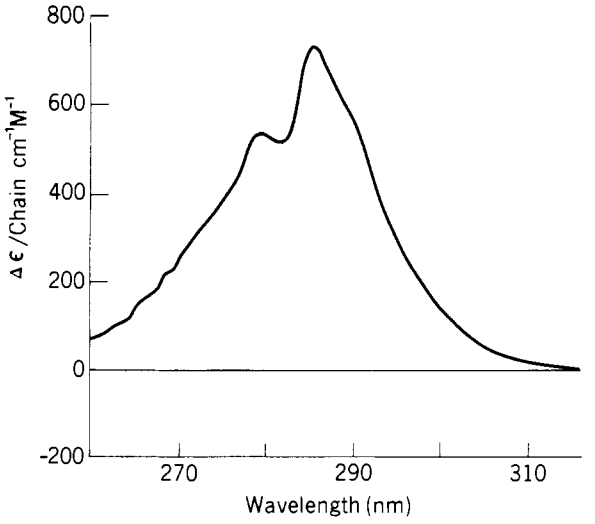

FIGURE 6: Ultraviolet difference spectrum generated in the presence of 20% ethylene glycol for β_2 microglobulin.

was obtained for this protein (Figure 6), indicating 1.4 of 2 tryptophans and all 6 tyrosines are exposed to the solvent (Table II).

The Distribution of Radioiodinated Tyrosines in the Domains of Fc. The number of chromophores seen by a solvent perturbant is dependent upon the molecular radius of the perturbant used. It is thus possible to detect by this technique chromophores which are sufficiently removed from the surface so as not to be able to interact with large molecules such as other proteins yet are close enough to be reached by a relatively small molecule such as ethylene glycol. Lactoperoxidase-catalyzed radioiodination was therefore used as a probe to determine whether tyrosines deemed to be exposed by the solvent perturbation technique were sufficiently exposed so as to be good substrates for LPO-catalyzed iodination.

Fc radioiodinated by the LPO method was digested with pepsin and, following inactivation of the enzyme, the digest was chromatographed on Sephadex G-50 (Figure 7). The most striking feature of this profile is the low level of iodine incorporation into the pFc′ fragment compared with the peptides derived from the $C_\gamma 2$ region. In a separate experiment we chromatographed a peptic digest of Fc in which the terminal sialic acid residue of the carbohydrate chain had been radiolabeled with tritium, thereby enabling us to identify which of the $C_\gamma 2$-derived peptides were glycopeptides. The tritiated glycopeptide eluting a $V_e/V_t = 0.68$ corresponded to one of the peptides which showed high iodine content. This result is consistent with this portion of the protein chain being on the surface of the molecule.[4]

To determine whether the scant iodine labeling of the $C_\gamma 3$ was an inherent property of this domain rather than the result of cis interactions with $C_\gamma 2$ leading to steric blockage of tyrosine residues, we radioiodinated isolated pFc′ and $C_\gamma 2$

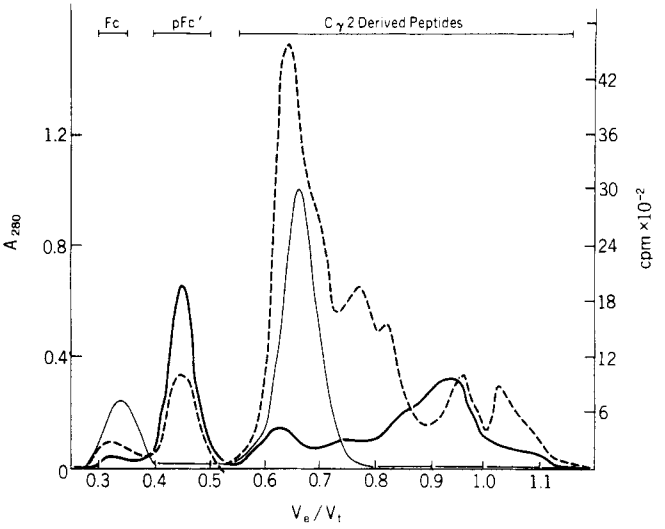

FIGURE 7: Elution profile from a Sephadex G-50 column of pepsin-digested Fc. In one experiment Fc was radiolabeled with iodine by the lactoperoxidase procedure prior to pepsin digestion (- - -). In a separate experiment Fc was radiolabeled with tritium on the terminal sialic acid residue of the carbohydrate chain prior to pepsin digestion (—). The A_{280} profile is also shown (—).

TABLE III: The Extent of Iodine Incorporation into Fc and Its Subfragments in the Presence of Lactoperoxidase.

Fragment	^{125}I cpm/mg
Fc	6.8×10^8
$C_\gamma 2$	5.4×10^8
pFc′	4.5×10^6
at$C_\gamma 3$	5.4×10^8

fragments. The results clearly show that the extent of iodine incorporation into intact Fc is almost entirely accounted for by the $C_\gamma 2$ domain and that the differential labeling of pFc′ and $C_\gamma 2$ occurs regardless of whether they are present together in intact Fc or in the isolated state (Table III). Thus, although ethylene glycol which has a Stokes radius of only 4.4 Å was able to detect 3 of the 5 tyrosines per chain of pFc′, these residues were either not reactive or not sufficiently exposed to

[4] Previously Yasmeen et al. (1976) had reported that the terminal sialic acid residue of the carbohydrate chain in Fc could not be removed by neuraminidase, suggesting that the carbohydrate was buried within the molecule. We have subsequently shown that the concentration of Fc used at that time was insufficient for it to be bound by the neuraminidase which has a K_m value of about 10^{-3} M. When millimolar concentrations of Fc are incubated with neuraminidase, the terminal sialic acid is indeed released, thereby further demonstrating that the carbohydrate is exposed to the solvent.

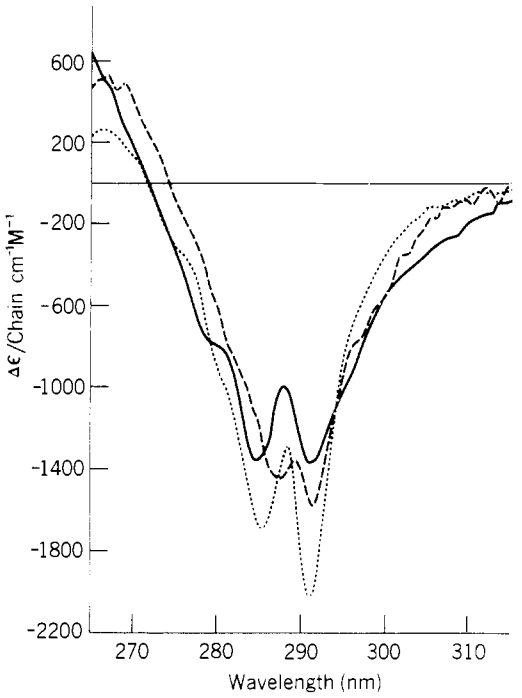

FIGURE 8: Ultraviolet difference spectrum generated for $C_\gamma 2$ (—), pFc' (. . .), and $atC_\gamma 3$ (- - -) when the pH was lowered from 7.8 to 2.4.

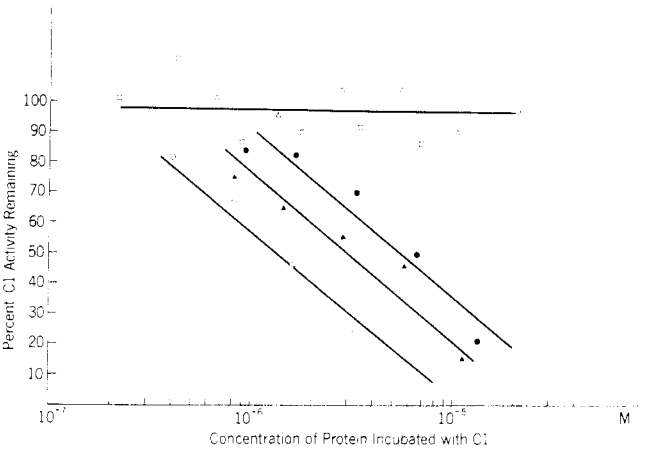

FIGURE 9: C1-binding activity of several fragments derived from the $C_\gamma 3$ region compared with $C_\gamma 2$: pFc' (△), tFc' (□), Fc' (▲), $atC_\gamma 3$ (○), $C_\gamma 2$ (●).

be good substrates for lactoperoxidase. In the latter case, these residues probably would not be accessible to other macromolecules such as C1q. From this point of view, it is of interest that the tyrosines of the $C_\gamma 2$ domain are detected by both methods.

When $atC_\gamma 3$ was iodinated by the LPO method, the degree of iodine incoporation was identical with that observed for $C_\gamma 2$ (Table III). Thus the increased chromophore exposure of this fragment relative to pFc' detected by solvent perturbation is also apparent in the enzymatic iodination experiments.

Acid Denaturation Difference Spectroscopy. Acid denaturation difference spectroscopy was also used as a conformational probe. The difference spectra obtained when samples of $C_\gamma 2$, pFc', and $atC_\gamma 3$ were partially unfolded by adjusting the pH from 7.8 to 2.4 are shown in Figure 8. The intensity of the indole peak at 291–294 nm was decreased in the spectra of $C_\gamma 2$ and $atC_\gamma 3$ relative to pFc', suggesting that in the latter fragment fewer tryptophan residues were initially exposed to the solvent in the native state. The fact that the 291–294-nm peaks of $C_\gamma 2$ and $atC_\gamma 3$ were of similar magnitude was consistent with the solvent perturbation and enzymatic iodination data indicating similar degrees of tryptophan exposure in these two fragments. When papain Fc' and tFc' were examined by this technique, Fc' was found to give a difference spectrum very similar to that obtained for $atC_\gamma 3$ whereas tFc' gave a spectrum similar to pFc'. These findings therefore were also in accord with the results we have obtained by solvent perturbation.

Complement Binding Studies. There is now general agreement among several laboratories, including our own, that the complement binding site of IgG is located in the $C_\gamma 2$ domain (Kehoe and Fougereau, 1969; Utsumi, 1969; Connell and Porter, 1971; Ellerson et al., 1972; Yasmeen et al., 1976; Ovary et al., 1976). Furthermore, Yasmeen et al. (1976) have shown that the C1-binding activity of the whole molecule can be fully accounted for by the isolated $C_\gamma 2$ region, while intact $C_\gamma 3$ is inactive in all systems tested. The different degree of chromophore exposure observed in $atC_\gamma 3$ relative to pFc' and tFc' prompted us to reexamine the C1 binding activity of these fragments in the fluid-phase C1 assay described by Augener et al. (1971). The results of such a C1 inhibition assay are shown in Figure 9. While both pFc' and tFc' did not interact with C1q, $atC_\gamma 3$ was at least as active as $C_\gamma 2$ in binding C1q and the dose–response curves were parallel for the two fragments.

Recalling that the CD spectrum of papain Fc' was virtually identical with that of $atC_\gamma 3$ (Ellerson et al., 1976) suggesting that these two fragments have similar conformations, we decided to test the ability of papain Fc' to interact with complement and found that it was also active in binding C1q (see Figure 9). Thus the four $C_\gamma 3$ derived fragments, pFc', tFc', Fc', and $atC_\gamma 3$, can be divided into two groups based on their ability to interact with C1q in this assay. The group which reacts with C1q (Fc' and $atC_\gamma 3$) also shows increased chromophore exposure.

Discussion

Our solvent perturbation studies have demonstrated the presence of one exposed tryptophan per chain of Fc from human igG1. Furthermore, this exposed tryptophan was shown to be located within the $C_\gamma 2$ homology region, the Fc domain responsible for the binding of the first component of complement (Yasmeen et al., 1976). By each of three criteria, solvent perturbation, enzymatic iodination, and acid denaturation, we have consistently found a greater degree of aromatic chromophore exposure in the $C_\gamma 2$ domain than in the intact $C_\gamma 3$ domain. Considering the highly conserved folding patterns of Ig domains, this departure is interesting and may be associated with the known lack of intrinsic affinity between $C_\gamma 2$ domains (Ellerson et al., 1976; Michaelsen et al., 1975; Deisenhofer et al., 1976). The fact that the solvent perturbation spectrum obtained by algebraically summing the spectra of $C_\gamma 2$ and pFc' was slightly larger than that of intact or mildly reduced and alkylated Fc may indicate cis interdomain interactions leading to the partial burial of aromatic side chains in the $C_\gamma 2$–$C_\gamma 3$ contact regions. These results are in accord with the findings of Ellerson et al. (1976) that the CD spectral features of Fc are not entirely reproduced when the spectra of $C_\gamma 2$ and pFc' are algebraically summed. These CD differences suggest that the environments of certain aromatic chromophores are modified in Fc when compared with the isolated domains.

The presence of an exposed tryptophan in Fc, $C_\gamma 2$, and β_2

microglobulin, all of which are able to bind C1, is in accord with the chemical modification studies of Cohen and Becker (1968) and more recently of Allen and Isliker (1974). The latter workers demonstrated that most of the complement-binding activity of rabbit IgG or Fc was lost under conditions where 1–2 tryptophans per molecule were modified. Since the modifications were done under relatively mild conditions, it is reasonable to conclude that only the exposed tryptophan residues were attacked and that these are involved in C1 binding.

The results of the present study taken together with those of Dorrington et al. (1972) clearly demonstrate that the conformation of the $C_\gamma3$ domain is not substantially altered by the removal of eight residues from the NH_2 terminus. On the other hand, the conformation of this domain is greatly affected by the additional removal of as few as seven residues from the COOH terminus. $C_\gamma3$ fragments, prepared by acid–trypsin or extended papain proteolysis, which lack the C terminus show a marked increase in chromophore exposure relative to pFc′; the extent of chromophore exposure in at$C_\gamma3$ approaches that observed in $C_\gamma2$. In view of the evidence suggesting the involvement of tryptophan in the C1 binding site, it was gratifying to find that, while pFc′ was not active in the C1 assay, at$C_\gamma3$ with its more exposed tryptophan was able to bind C1. The failure of other workers (Turner et al., 1969; Utsumi, 1969; Yasmeen et al., 1976) to demonstrate complement fixation by various Fc′ fragments from human and rabbit IgG may be a reflection of the assay used.

The apparently crucial seven amino acid chain segment missing from the C terminus at$C_\gamma3$ has the sequence Ser-Leu-Ser-Leu-Ser-Pro-Gly. The nature of the residues involved and the repetitive nature of the sequence suggested that this section might be involved in the formation of a β sheet. X-ray crystallographic studies by Poljak et al. (1974) have shown that in Fab′ (New) the regions of C_L and $C_\gamma1$ which are homologous to this section are involved in β-sheet formation. Edmundson et al. (1975) have also reported that the homologous residues of the C_L regions of a Bence–Jones dimer are involved in β-sheet formation. By extrapolation to the $C_\gamma3$ domain, it is probable that either Ser-440 or Leu-441 is involved via hydrogen bonding in the formation of a β sheet. Therefore, the removal of residues 440 to 446 may result in the decreased interaction of the chain segment 432–439 with the remainder of the putative β sheet. As a result of these decreased interactions, Tyr-436 may become exposed to the solvent. Such an exposure would explain the loss of optical activity associated with tyrosine transitions (234 and 272 nm) when the CD spectra of pFc′ and at$C_\gamma3$ are compared. Exposure of Tyr-436 would account for the higher level of enzymatic iodination observed for at$C_\gamma3$ relative to pFc′. The decreased stability of the β sheet may also alter the environment of Phe-423 and Trp-381 with a resultant change in optical activity. Our observation that at$C_\gamma3$ has more tryptophan exposed to the solvent than is the case for pFc′ and tFc′ would tend to support the hypothesis that the β sheet is perturbed when chain segment 432–447 is altered or removed.

That both papain Fc′ and at$C_\gamma3$ exist as high affinity noncovalent dimers, even though they both have an altered β sheet, suggests that this particular β sheet is not involved in the noncovalent association of Fc and $C_\gamma3$ fragments. By analogy with known C_L and $C_\gamma1$ structure (Poljak et al., 1974; Edmundson et al., 1975), it seems probable that this altered β sheet is composed of three antiparallel extended chain segments and that the interdomain contact between $C_\gamma3$ monomers involves the apposition of a four-chain β layer from each monomer. This view implies that either the three-chain β sheet is not modified sufficiently to disturb the conformation of the four-chain-segment β sheet or that the two sheets are relatively independent.

The recent paper by Deisenhofer et al. (1976) lends credence to the above arguments. These workers found electron density for the chain segment His-433 to Leu-441, while no satisfactory density was visible for the remainder of the chain to Gly-446. This suggests that Leu-441 may serve to stabilize the corner of a β sheet while the remainder of the carboxyl terminal of the chain is mobile. From their x-ray crystallographic data, these workers concluded that the $C_\gamma3$ dimer resembles the $C_\gamma1$–C_L dimer found in Fab with four-chain sheets forming the interdomain surfaces. Thus Trp-381, Phe-423, and Tyr-436 are all located in the same three-chain β sheet near the outer surface of the domain and away from the contact regions. If we are correct in concluding that it is Trp-381 which becomes exposed to the solvent when intact $C_\gamma3$ is cleaved to give Fc′ or at$C_\gamma3$, it is noteworthy that this tryptophan occupies the homologous position to Trp-227 of the $C_\gamma2$ domain and it is this latter residue which Allen and Isliker (1974) have implicated as being part of the C1-binding site. Upon tracing the chains in the preliminary x-ray model of Fc (Deisenhoffer et al., 1976) Trp-277 appears to be located in an extremity of the molecule, while the remaining three tryptophans on each Fc chain appear to be buried.

On the basis of the currently available evidence regarding the mechanism of C1 binding by IgG, we propose that there is a requirement for hydrophobic residues, especially tryptophan, to be on the surface of immunoglobulin domains which are capable of binding C1. For example the amphipathic molecule 1,4-diaminobutane is able to bind C1q albeit with an affinity 10^4 times weaker than Fc (Yasmeen et al., 1976). Similarly, small synthetic peptides corresponding to the regions around Trp-277 of IgG1 have recently been shown to bind C1 (Johnson and Thames, 1976); however, as was the case with 1,4-diaminobutane, the affinity was quite low. We have previously shown that totally reduced and alkylated β_2 microglobulin retains the ability to bind C1 even though the molecule was in an unfolded state as judged by spectral criteria (Isenman et al., 1975a). This observation suggested that a C1-binding site could be generated by amino acids which are near neighbors in the primary structure. Total reduction of pFc′ also yields an unfolded molecule (unpublished observation) with the ability to bind C1 (Yasmeen et al., 1976). Presumably sequences homologous to those which interact with C1 and $C_\gamma2$ are exposed to the solvent upon total reduction of the pFc′ fragment. While the observed affinity of these reduced fragments is of the same order as Fc or $C_\gamma2$, this is in part due to the tendency of the reduced species to aggregate, thereby increasing their avidity for C1. Thus it may be that the chemical features required for C1 binding are fairly commonplace and are indeed present in all immunoglobulin domains but are suppressed as a result of tertiary folding that prevents the necessary residues from being exposed to the surface. Thus by cleaving intact $C_\gamma3$ to yield at$C_\gamma3$ or Fc′, the tertiary structure is sufficiently modified so as to expose the hydrophobic residues required for the formation of a C1-binding site. On the other hand, the tertiary structure of the $C_\gamma2$ domain may have specifically evolved in a manner such that the domain is in a stable configuration while having a large proportion of its aromatic side chains completely exposed to the solvent. Thus it is this domain which is responsible for the binding of C1 by native IgG. More detailed crystallographic structures of Fc and some of the $C_\gamma3$ derived fragments will be required to confirm the

above hypothesis. Clearly, however, our data sound a cautionary note to those workers who may attempt to isolate or synthesize complement-fixing peptides in order to more closely define the residues responsible for this effector function in the Fc.

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