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Immunology

Antigen-induced conformational changes in antibodies and their Fab fragments studied by circular polarization of fluorescence

(tryptophan fluorescence/interchain disulfide bonds)

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ABSTRACT **Conformational changes induced in antibody molecules and in their Fab fragments by binding of antigen were investigated by the circular polarization of the fluorescence emitted by the tryptophan residues. This property of the fluorescence is related to the asymmetry, and thus to the conformation and environment, of the emitting chromophore. Changes in the circular polarization of the fluorescence of the antibody were observed upon binding of RNase to anti-RNase, of poly(DL-alanyl)–poly(L-lysine) to anti-poly(D-alanine), and of the "loop" of lysozyme, a monovalent antigenic determinant, to anti-"loop." The spectral changes were observed at different antigen–antibody ratios, including high antigen excess, indicating that they are due to antigen binding and not to aggregation. The circular polarization of fluorescence also detects changes in conformation of the different Fab fragments upon binding of the corresponding antigens. These changes in conformation were, however, markedly different from those observed for the whole antibody molecules, and indicated an interaction between the Fc and Fab fragments in the antibody molecule, and probably a change in the conformation of Fc upon binding of antigen to the antibody. In contrast, the small hapten, phosphorylcholine, did not induce a change in the circular polarization of the fluorescence of its antibody or corresponding Fab fragments. Reduction of the interchain disulfide bonds of the antibodies abolished the antigen-induced spectral changes due to the presence of the Fc portion in the molecule, but not the changes observed in Fab, suggesting that the disulfide bonds at the hinge region of the antibody are required for the transmission of the conformational change from the Fab to the Fc.**

Antibodies generally exhibit two sets of interlinked functions: the specific binding of antigen at the Fv region of the molecule, and those functions shared by all antibodies of the same class, such as binding of complement, triggering of mast cells to release histamine, and, most important, triggering lymphocytes towards differentiation and antibody synthesis or towards tolerance. The latter functions are localized in the Fc part of the molecule, and under physiological conditions come into effect only after antigen binding.

We would like to understand how antigens trigger these events, and whether a "signal" is transmitted from the Fv to the Fc region within the antibody molecule. The structural expression of such a "signal" will presumably be an antigen-induced conformational change in the Fab, which will affect the Fc fragment. It is desirable to find a method that will resolve the changes in these portions of the antibody.

Various attempts to demonstrate such conformational changes [see Metzger (1) for a review] have not yielded unequivocal conclusions, although some of these studies have indicated changes in the flexibility of the molecule (2), its sedimentation coefficient (3), or its volume (4), as a consequence of hapten binding. Other studies, using optical methods such as absorption, fluorescence, and circular dichroism clearly demonstrated changes in the antibody molecules that take place upon binding of hapten, but could be interpreted as changes in the combining site or in its vicinity (5, 6). In this study we have investigated the conformational changes that take place in antibody molecules upon binding of antigens or hapten by the circular polarization of the tryptophan fluorescence of the antibodies because this spectroscopic technique has some pronounced merits.

The circular polarization of luminescence (CPL) of a chromophore is the emission analog of circular dichroism, and is related to the conformation of the electronically excited chromophore in the same way that circular dichroism is related to the conformation of the chromophore in the ground state (7–13). CPL is thus a manifestation of the chirality, or asymmetry, of the molecule in addition to circular dichroism and optical rotatory dispersion. CPL has a pronounced advantage over the other two spectroscopic techniques mentioned, i.e., the advantage of specificity when one studies complex systems. This is because only luminescent chromophores contribute to the CPL of the system, whereas, circular dichroism and optical rotatory dispersion are affected by all chromophores present. In the case of proteins, CPL probes only the region of those tryptophan, and to some extent tyrosine, residues that are both fluorescent and are situated in an asymmetric environment when they are in their electronically excited states (11, 13). The spectral resolution between tyrosines and tryptophans, and among different residues of each kind, is significantly greater in emission than in absorption (14). One may thus expect the CPL of proteins to provide specific information about the systems studied which cannot be deduced from their circular dichroism spectra.

It appears that immunoglobulins are amenable to spectral analysis by CPL since their tryptophans are present in small numbers (one to three) in each domain of the macromolecule, and the positions of these tryptophans are homologous between different domains in each molecule and in immunoglobins of different species (15). Moreover, in three-dimensional space they occupy similar positions in each domain (16). Thus, the analysis of the CPL of antibodies makes use of built-in probes in each domain without necessitating the introduction of external fluorescent probes.

In order to be able to ascribe the CPL measurement exclusively to the antibody molecule, the antigen selected for

Abbreviations: CPL, circular polarization of luminescence; A-L, multichain poly(DL-alanyl)–poly(L-lysine); Ab, antibody; Ag, antigen.

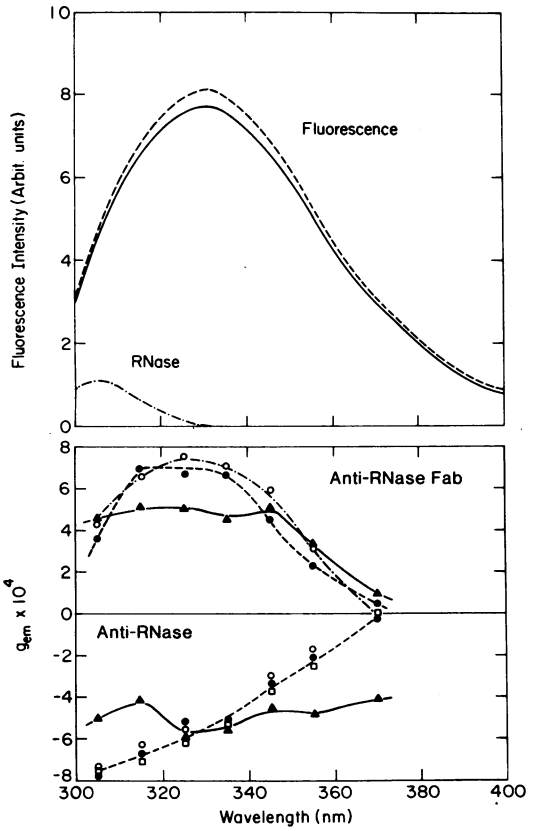

FIG. 1. Spectroscopic data of anti-RNase and its Fab′ fragment in the presence or absence of antigen. *Top:* Fluorescence spectra of anti-RNase, $5 \cdot 10^{-7}$ M (- - -); Fab′, $1.5 \cdot 10^{-6}$ M (—); and RNase, $2.8 \cdot 10^{-7}$ M (- · -). The excitation wavelength was 280 nm. *Bottom:* Emission anisotropy factor, g_{em}, of anti-RNase, anti-RNase Fab′, and poly(alanyl) anti-RNase, in the absence or presence of antigen. (*Lower*) Anti-RNase, $3.5 \cdot 10^{-5}$ M (▲); the same CPL spectrum was obtained also for poly(alanyl) anti-RNase ($5.6 \cdot 10^{-5}$ M). Anti-RNase ($3.5 \cdot 10^{-5}$ M) with RNase ($7.2 \cdot 10^{-5}$ M) at the region of "antigen excess" (□). Poly(alanyl) anti-RNase ($5.6 \cdot 10^{-5}$ M) in the presence of RNase ($7 \cdot 10^{-6}$ M) at the region of "antibody excess" (●). Poly(alanyl) anti-RNase ($5.6 \cdot 10^{-5}$ M) with RNase ($1.4 \cdot 10^{-5}$ M) at the region of "equivalence" (○). (*Upper*) Emission anisotropy factor, g_{em}, of anti-RNase Fab′ ($1.4 \cdot 10^{-4}$ M) (▲), and the Fab′ ($1.4 \cdot 10^{-4}$ M) with RNase ($1.25 \cdot 10^{-5}$ M) (●), and with RNase ($2.5 \cdot 10^{-5}$ M) (○).

the study of the effect of antigen on antibody conformation should not absorb or fluoresce in the emission range of the antibody. Hence, bovine pancreatic ribonuclease, which lacks tryptophan residues, multichain poly(DL-alanyl)–poly(L-lysine) (17) (A–L), and their corresponding antibodies were selected for this study. Antibodies to monovalent determinants, such as phosphorylcholine (18), tetraalanine (19), and the "loop" of lysozyme (20), were also investigated. With the aid of CPL as a spectroscopic tool we present evidence for conformational changes in Fab and Fc induced by antigen binding, and demonstrate the involvement of the interchain disulfide bonds in the transmission of these changes.

MATERIALS AND METHODS

Antigens and Antibodies. RNase and phosphorylcholine were purchased from Sigma. Multichain polyalanine (A–L) of 200,000 molecular weight was a gift of Mrs. M. Schwartz. The "loop" peptide of lysozyme and rabbit anti-"loop" antibodies (20) were a gift of Dr. R. Arnon. The loop peptide was treated with *N*-bromosuccinimide (21) to modify its tryptophans, and the peptide was separated from the reagents on Sephadex G-25. The recovered peptide did not show any fluorescence. Rabbit antiserum to poly(D-alanyl) diphtheria toxoid (19) was obtained from Mr. A. Licht, and the mouse myeloma protein McPC 603, which binds phosphorylcholine (18), was a gift of Dr. M. Potter. Rabbit anti-RNase and anti-poly(D-alanine) antibodies were purified on RNase-Sepharose and (A–L)-Sepharose, respectively. The absorbed antibodies were eluted from the immunoadsorbent with 0.1 M acetic acid. $(Fab')_2$ was prepared by pepsin digestion of the antibodies at pH 4.5 according to Nisonoff (22), and Fab′ was obtained by reduction of this preparation with 5 mM dithiothreitol followed by alkylation with iodoacetamide. Papain digestion was performed according to Porter (23) in 1 mM dithiothreitol. Poly(DL-alanyl) anti-RNase antibodies were prepared as described by Fuchs and Sela (24). These modified antibodies do not precipitate with the antigen, but exhibit full binding capacity, as measured by inhibition of antigen–antibody precipitation (24, 25). The CPL of RNase–anti-RNase complexes was measured at antigen excess (Ab/Ag = 0.5 mol/mol), where soluble complexes prevail, whereas measurements at antibody excess (Ab/Ag = 8 mol/mol) and equivalence (Ab/Ag = 4 mol/mol) were performed with poly(alanyl) anti-RNase (25).

Fluorescence and CPL Measurements. Corrected fluorescence spectra were obtained on a Hitachi-Perkin Elmer spectrofluorometer model MPF-3. The instrument for the measurement of CPL was built in our Institute and has been described elsewhere (26, 27). Sample cells of 1.0 or 2.0 mm light path were used. The circular polarization of luminescence is expressed by the emission anisotropy factor, g_{em}, defined as $g_{em} = 2\Delta f/f$, where Δf is the intensity of the circularly polarized part of the fluorescence (defined positive for left-handed circular polarization) and f is the total intensity of the fluorescence light (10, 11). The excitation light was selected by a Bausch and Lomb High Intensity monochromator set at 275 nm, with a band pass of 30 nm, and then passed through a chlorine filter (27) (Ophthos Instruments) to remove stray light. The fluorescence light was monochromated by a Jarell Ash double monochromator (model 82-410) at a spectral resolution of 15 nm. The experimental error in the measurement of g_{em} was $\pm 5 \times 10^{-5}$.

Protein solutions were in 0.15 M NaCl/0.01 M sodium phosphate (pH 7.4) except for McPC 603 protein where 0.1 M Tris·HCl (pH 8) was used.

RESULTS

Conformational Changes in Antibody Molecules upon Binding of Antigen. The fluorescence spectra of anti-RNase and of the Fab′ fragment of this antibody are shown in Fig. 1. The emission spectra of these two proteins are very similar in shape. In contrast, the CPL spectra of anti-RNase and its Fab′ fragment are very different from each other, g_{em} of the intact immunoglobulin being negative and that of Fab′ being positive throughout the emission spectrum. The CPL spectrum of poly(alanyl) anti-RNase was very similar to that of the unmodified antibody, indicating a negligible effect of the poly(alanylation) on the protein conformation, at least in the vicinity of the tryptophan residues. This antibody preparation was used to measure CPL of soluble antigen–antibody complexes at various ratios, whereas unmodified antibodies were used at antigen excess.

The binding of RNase to anti-RNase or to its Fab′ fragment resulted in significant, but different changes in the

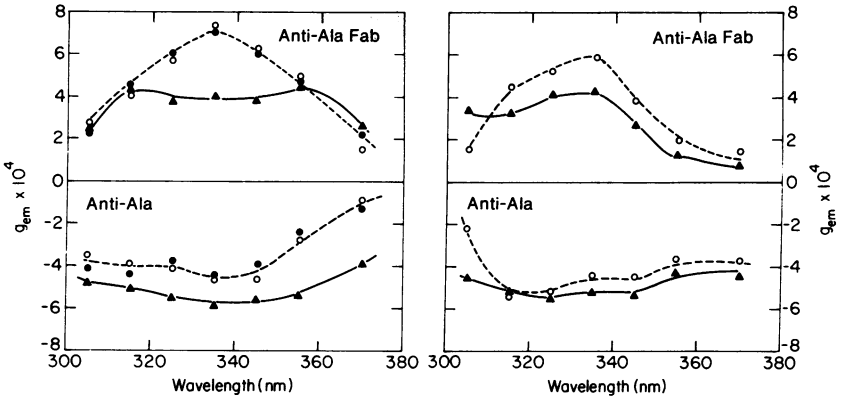

FIG. 2. Emission anisotropy factor, g_{em}, of anti-poly(D-alanine) and its Fab' in the absence or presence of A–L or tetra-D-Ala. *Lower left:* Anti-poly(D-alanine), 6.5 mg/ml (▲), and anti-poly(D-alanine) with 2.4 mg/ml of A–L (●) or with 3.5 mg/ml of A–L (○). Both concentrations are in the region of antigen excess. *Upper left:* Anti-poly(Ala) Fab', 6 mg/ml (▲), and the Fab' with 0.7 mg/ml of A–L (●), or 1 mg/ml of A–L (○). *Lower right:* Anti-poly(Ala) ($4\cdot10^{-5}$ M) (▲), and anti-poly(Ala) ($4\cdot10^{-5}$ M) with $5\cdot10^{-4}$ M tetra-D-Ala (○). *Upper right:* Anti-poly-(Ala) Fab' (10^{-4} M) (▲) and anti-poly(Ala) Fab' (10^{-4} M) with tetra-D-Ala ($5\cdot10^{-4}$ M) (○).

CPL spectra of the tryptophan fluorescence of the antibodies (see Fig. 1). The changes in g_{em} of Fab' occur in the spectral range of 315–345 nm, whereas those of the intact antibody occur in the spectral ranges of 305–320 nm and 350–370 nm. The changes in the CPL spectra are little dependent on the antigen–antibody ratios, suggesting that the conformational changes responsible for the changes in CPL upon antigen binding are not due to aggregation but to antigen binding *per se;* sedimentation velocity analysis of the antigen-antibody complexes formed at antigen excess (Ab/Ag = 0.5 M/M) indicated the presence of antibody monomers (Ab_1Ag_2) and dimers (Ab_2Ag_1) only (25).

Fig. 2 describes the CPL spectra of anti-poly(D-alanine) antibody, of its Fab' fragment, and of these proteins when bound to their corresponding antigen A–L. These spectra, including the changes that take place upon antigen binding, are roughly similar to those observed for anti-RNase antibodies. It may be noted that A–L is not fluorescent at all, while RNase is fluorescent below 320 nm (Fig. 1). Thus, the changes observed in g_{em} cannot originate in the antigen in any range of the spectrum in the case of A–L/anti-poly-(alanine), or in the spectral range above 320 nm in the case of RNase/anti-RNase.

From the above results it is obvious that the conformational changes in the intact antibody molecules that take place upon binding of the antigen, as reflected in the changes in the CPL spectra, cannot be accounted for by the changes taking place in the isolated corresponding Fab fragment upon antigen binding. We have also demonstrated that upon papain cleavage of anti-RNase antibody in the complex with RNase the changes observed in the CPL spectrum of the intact antibody disappear. Similarly, the addition of RNase to the papain digest of the antibody does not cause the CPL changes observed with the intact antibody (25), indicating again that the changes in the CPL spectrum of the antibody upon binding of antigen do not take place when the Fc is disconnected from the Fab fragments.

Conformational Changes in Antibody Molecules upon Binding of Hapten. Three different systems were analyzed for the effect of monovalent hapten or monovalent antigenic determinant on the CPL spectra of the corresponding antibodies. In Fig. 3 it is shown that the binding of the small hapten phosphorylcholine to the mouse IgA produced by McPC 603 does not change the CPL of the Fab' or of the intact antibody (Fig. 3). Measurement of CPL of anti-poly(D-alanine) antibodies upon binding of the hapten tetra-D-alanine (Fig. 2) reveals some changes in the Fab' but no significant change in the intact antibody. On the other hand, the

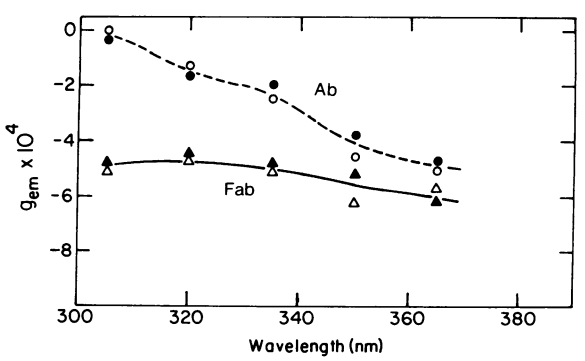

FIG. 3. The CPL spectrum of protein McPC 603 and its Fab' in the absence or presence of phosphorylcholine. Intact protein ($9.6\cdot10^{-5}$ M) (○), and intact protein ($9.6\cdot10^{-5}$ M) with phosphorylcholine ($3\cdot10^{-4}$ M) (●). Fab' of protein McPC 603 ($1.4\cdot10^{-4}$ M) (△) and Fab' with phosphorylcholine ($5.2\cdot10^{-4}$ M) (▲).

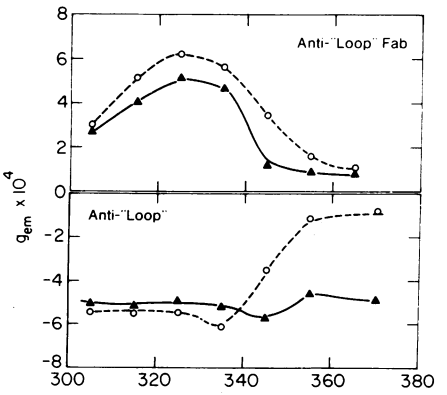

FIG. 4. The CPL spectrum of anti-"loop" and its Fab' in the absence and presence of the "loop" region of lysozyme. *Lower:* Emission anisotropy factor, g_{em}, of anti-"loop" ($5\cdot10^{-5}$ M) (▲), and anti-"loop" ($5\cdot10^{-5}$ M) with "loop" (10^{-4} M) (○). *Upper:* g_{em} of anti-"loop" Fab' (10^{-4} M) (▲), and anti-"loop" Fab' (10^{-4} M) with "loop" (10^{-4} M) (○). The "loop" peptide was oxidized with *N*-bromosuccinimide to modify its tryptophan residue.

"loop" peptide of lysozyme, a single antigenic region composed of 24 amino acids (20), did induce changes in the CPL spectra of both Fab' and the intact anti-"loop" antibody (Fig. 4). These changes are similar to those described above for other antigen–antibody systems (Figs. 1 and 2) and provide direct evidence that the binding of a monovalent antigenic determinant is capable of inducing in its corresponding intact antibody conformational changes similar to those observed in the antibodies to multivalent antigens, the requirement presumably being that the determinant is big enough.

Role of Interchain Disulfide Bonds in Conformational Changes Induced in Antibodies by Antigens. As shown above, the changes that take place in the CPL spectrum upon binding of antigen are markedly different in the case of intact antibody and in the corresponding Fab fragments. To gain further information about the factors involved in the extra conformational changes that occur in intact antibodies, we followed the changes in the CPL spectrum of anti-RNase induced by RNase, upon mild reduction of the interchain disulfide bonds of the antibody. As shown in Fig. 5, the changes induced by RNase in intact anti-RNase and in anti-RNase that has been reduced in 2.5 mM dithiothreitol are significantly different. As a matter of fact, the changes induced in the reduced antibody may be accounted for by the changes that take place in the Fab fragments when separated from the rest of the molecule*. Hence, the additional conformational changes observed in the whole antibody upon antigen binding require the interchain disulfide bonds to be intact. To rule out the possibility that the reduction affected the antigen, the experiment was repeated with antibody that was reduced, alkylated, and dialyzed, and then reacted with the antigen. The observed CPL spectra were very similar to those shown in Fig. 5 for the reduced antigen–antibody mixture. It should be noted that reduction of the antigen-$(Fab')_2$ complexes to antigen-Fab' has only a small effect on the CPL spectrum; furthermore, this effect is not in the spectral ranges 300–320 nm and 345–380 nm (Fig. 5). Hence, the Fc fragment must be involved in the changes observed upon reduction of the total antibody.

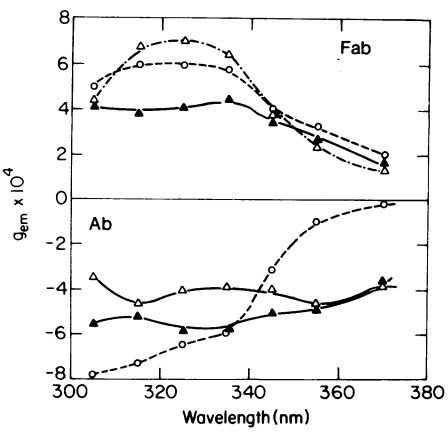

FIG. 5. Effect of reduction of disulfide bonds in anti-RNase on changes in its CPL upon binding of antigen. *Lower:* Emission anisotropy factor, g_{em}, of anti-RNase ($3 \cdot 10^{-5}$ M) (▲); anti-RNase ($3 \cdot 10^{-5}$ M) with RNase ($6 \cdot 10^{-5}$ M) (○); and anti-RNase with RNase in the presence of 2.5 mM dithiothreitol (△). Reduction with dithiothreitol concentrations lower than 2.5 mM resulted in spectra intermediate between (○) and (△), whereas higher dithiothreitol concentrations resulted in the same spectrum as with 2.5 mM (△). *Upper:* g_{em} spectrum of $(Fab')_2$ ($4 \cdot 10^{-5}$ M) (▲); $(Fab')_2$ with RNase ($8 \cdot 10^{-5}$ M) (△); and $(Fab')_2$ with RNase in the presence of 2,5 mM dithiothreitol (○).

DISCUSSION

For a collection of identical chromophores the emission anisotropy factor, g_{em}, is expected, as a rule, to be approximately constant across the emission band (10, 11). Deviations from such behavior may reflect heterogeneity in the emitting chromophores, various subgroups of them having different emission spectra and different asymmetry. In the present study the Fab fragments and the antigen–antibody complexes showed variable g_{em} factors across the emission bands. This may be due to the multitude of tryptophan residues in each molecule and to the heterogeneity of the population of antibody molecules. In all cases studied, the antibodies exhibited markedly different CPL spectra from their corresponding Fab fragments. These results reflect the contribution of the Fc fragments to the CPL of the intact antibodies through their tryptophan fluorescence, and possibly indirectly by modifying to a greater or lesser extent the conformation of the Fab fragments as compared to the conformation of the Fab when free.

Upon binding of RNase, A–L, and the lysozyme "loop" to the corresponding antibodies, the CPL, and hence conformation, of the antibody molecules was found to change markedly. The conformational changes observed in the different antigen-antibody systems, although not identical, seem to be very similar, suggesting that they may arise mainly in the constant portions of the antibody molecules. In contrast, the binding of the hapten tetra-D-alanine to anti-poly(D-alanine) and of phosphorylcholine to McPC 603 led to little, if any, change in the CPL of the proteins. It thus seems that smaller determinants fail to bring about the changes, as followed by CPL, in the antibody molecules that determinants in bigger moieties cause upon binding to the corresponding antibody molecules.

The fact that a monovalent antigenic determinant, like the "loop," can induce CPL changes in the antibody strongly suggest that the conformational changes result from the mere binding of the determinant in a monomeric antigen–antibody complex. This does not exclude the possibility of changing the relative spatial position of the two Fab fragments within the antibody molecule as a consequence of binding a monovalent determinant. However, if this occurs, the experiment shows that it is not necessarily a result of steric constraints imposed by a polyvalent antigen.

In all cases studied, the changes in the CPL spectra of the antibodies induced by binding of antigen cannot be accounted for, even qualitatively, by changes in the Fab fragments that take place when the latter are separated from the rest of the molecule. In fact, the changes in CPL of the antibodies and of their Fab fragments that take place upon antigen binding occur at different spectral regions. This is not likely to be due to Fab–Fab interactions in the antibody molecule since $(Fab')_2$ does not behave at all like the intact antibody

* It may be recalled that $g_{em} = \Delta f/(f/2)$. Let us designate f_{ab} and f_γ as the fluorescence intensities of the Fab fragments and the total antibody molecules, respectively. If the change in the circularly polarized component, $\Delta(\Delta f)$, of the emitted light is due exclusively to the Fab fragments, the changes in g_{em} upon binding of antigen will be $\Delta(\Delta f)/(f_{ab}/2)$ if only Fab fragments are present, and $\Delta(\Delta f)/(f_\gamma/2)$ if the intact antibody is present. Since f_{ab} is approximately equal to $\frac{2}{3} f_\gamma$, the changes in g_{em} induced by the antigen should be $\frac{3}{2}$ as much for isolated Fab fragments as for the intact antibody. This is indeed the case within experimental error ($5 \cdot 10^{-5}$), as is seen from Fig. 5.

but rather similarly to Fab′ (Fig. 5). This clearly points to the Fc fragment as responsible for the extra conformational changes that take place in the intact antibody, compared to those that take place in the isolated Fab fragments, upon antigen binding. The Fc fragment thus seems to undergo a change in conformation upon binding of antigen to the Fab fragments. Another possibility is that in the intact antibody the Fc imposes constraints on the Fab fragments so that the latter behave differently upon antigen binding compared to when they are free. It should be noted, however, that since the interaction of Fab and Fc is reciprocal, the latter explanation of the data makes it also very probable that a change in Fab will result in a change in Fc. We are, therefore, led to the conclusion that binding of antigen to antibody leads eventually to changes in conformation in the Fc fragment as well as in the Fab fragments. It is noteworthy that using low angle x-ray scattering, binding of tetra-D-alanine to anti-poly(D-alanine) antibodies was found to yield a volume change in the antibody molecule (4), but not in the Fab fragment (28).

The question as to the mechanism by which the "signal" is transmitted from the Fab fragments to the Fc fragment upon antigen binding is not easy to answer at this stage. The data presented show that the interchain disulfide bonds are most probably involved. The changes shown in Fig. 5 level off at 2.5 mM dithiothreitol, at which concentration reduction of the inter-heavy-chain disulfide bonds was found to be complete (30). Thus, reduction of this bond seems to block the transmission of the signal (see Fig. 5). It is of much interest in this connection that reduction of the disulfide bonds was found to diminish complement fixation (31) or antibody-mediated cytotoxicity of lymphocytes (32), although it has no effect on the capacity of antigen binding.

X-ray analysis showed that the peptide chains of the antibody are comprised of separate domains with an overall cylindrical shape which are linked in a fixed angle between them (16, 29, 33). Thus, in the Fab′ the angle between V_L (the variable portion of light chain) and C_L (the constant portion of light chain) domain is 100–110°, whereas between V_H (the variable portion of heavy chain) and C_H1 (the first constant portion of heavy chain) the angle is 80–85° (16). On the other hand, x-ray analysis of light chain dimer showed that one monomer behaves as normal light chain whereas the other monomer appears to play the role of the heavy chain, having an angle of 70° between V_L and C_L (33). This suggests that light chain and probably also heavy chain can be present in at least two different conformations. It is possible that if a large antigenic determinant occupies the entire binding site, extending over the tips of both V_L and V_H, it may force a slight change in the angle between the domains in each chain. This may result in contraction or expansion of the entire Fab which will be transmitted through the hinge region to the Fc portion. For this process to occur it is apparently necessary that the whole area of the binding site be occupied. The change in conformation in the Fc fragment is probably a necessary, though not always sufficient, condition for triggering the functions of Fc.

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