Conformational Changes Induced in a Homogeneous Anti-Type III Pneumococcal Antibody by Oligosaccharides of Increasing Size†

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ABSTRACT: The circular polarization of luminescence (CPL) emitted by tryptophan residues was used as a sensitive probe for measuring ligand-induced structural changes in a homogeneous type III pneumococcal antibody. A series of oligosaccharide ligands of increasing size derived from type III polysaccharide by partial acid hydrolysis was assayed. Ligand-induced changes in the circular polarization of fluorescence of the antibody were observed for all antigens tested, including tetra-, hexa-, and octasaccharides and a 16-residue oligomer, the largest changes being recorded upon interaction with the intact soluble type III pneumococcal (SIII) polysaccharide. When Fab′ or $F(ab')_2$ fragments were used instead of the antibody IgG for binding of SIII polysaccharide, the extent of conformational changes was decreased. This suggests interactions between Fab and Fc portions in the IgG molecule and subsequent conformational changes in Fc part upon antigen binding. Reduction of interchain disulfide bonds abolished the additional spectral changes attributed to the Fc part but not the changes observed in the Fab part, thus suggesting that the presence of the interchain disulfide bond in the hinge region is required for maximal CPL changes to occur. Small monovalent ligands, i.e., the tetra-, hexa-, and octasaccharides, were capable of inducing CPL changes in the Fab part of the antibody molecule as well as CPL changes attributed to the Fc portion. A multivalent ligand containing about 16 sugar residues appears to be the minimal antigenic size required for triggering conformational changes attributed to the Fc part, similar to those seen in the interaction with the whole polysaccharide antigen.

Circular dichroism (CD), fluorescence spectroscopy, and recently small angle x-ray scattering have been successfully used to demonstrate ligand-induced conformational changes in antibodies and myeloma proteins with known hapten binding activity (Holowka et al., 1972; Pollet et al., 1974; Pilz et al., 1973).

In addition to CD and optical rotatory dispersion, the optical activity of an asymmetric molecule is manifested by circular polarization of luminescence (CPL) provided the asymmetric molecule is fluorescent (Gafni and Steinberg, 1972; Schlessinger and Steinberg, 1972; Steinberg, 1975). CPL is the emission analogue of CD and therefore reflects the optical activity of the chromophore in its electronically excited state, whereas the CD spectrum reflects the asymmetry of the chromophore in the ground state. Since only luminescent chromophores (e.g., tryptophan residues and to a lesser extent tyrosine residues) contribute to the CPL spectrum, this method is more discriminating than CD where all absorbing chromophores contribute to the observed spectrum (Steinberg et al., 1974; Schlessinger et al., 1975a).

CPL studies on antibodies to various proteins or polypeptide antigens have shown large spectral changes in antibody molecules as a result of antigen binding (Givol et al., 1974; Schlessinger et al., 1975b). Evidence for conformational changes not only in the antigen-binding part of the molecule (Fab) but also in the Fc part was provided. The structural changes in Fc were observed in antibodies upon binding of multivalent antigenic molecules, e.g., ribonuclease and multichain poly(DL-alanine) or of a large monovalent determinant as the "loop" of lysozyme. No CPL changes, however, were noted as a consequence of interactions between small haptenic molecules, such as phosphorylcholine and several mouse myeloma proteins endowed with this specificity (Givol et al., 1974; Schlessinger et al., 1975b).

In this work we have studied by CPL spectroscopy the interactions between a rabbit homogeneous antibody to type III pneumococcal polysaccharide and a series of oligosaccharides of increasing size derived from the intact polysaccharide. Since SIII[1] ($[cellobiuronic\ acid]_n$, Heidelberger, 1967) neither absorbs nor fluoresces in the spectral range investigated, CPL changes observed must reflect molecular events occurring in the antibody molecule only.

Recent work using CD and fluorescence spectroscopy provided evidence for discrete but significant structural changes in the Fab part of anti-pneumococcal antibodies[1] upon oligosaccharide binding (Jaton et al., 1975). The present CPL study confirms this observation and further demonstrates that in this system small monovalent antigenic determinants may induce additional structural changes in the Fc part of the IgG molecule as well.

Materials and Methods

The antibody against type III pneumococcal polysaccharide, designated 45-394, was raised according to the method of Kimball et al., 1971, and purified by immunoadsorp-

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[1] The nomenclature for immunoglobulin G and its fragments is as recommended in *Bull. W. H. O. 30,* 447 (1964); SIII, soluble type III pneumococcal polysaccharide; CPL, circular polarization of luminescence.

tion and preparative agarose block electrophoresis as described (Braun and Jaton, 1973). The spectrotype revealed by analytical isoelectrofocusing was consistent with a homogeneous product. Pepsin fragment $F(ab')_2$ was prepared according to the method of Nisonoff (1964) and the monovalent fragment Fab' was derived upon reduction of $F(ab')_2$ fragment with 0.005 *M* dithiothreitol followed by alkylation with iodoacetamide. Mild reduction of interchain disulfide bridges in antibody 45-394 was carried out for 30 min at room temperature in the presence of 0.0025 *M* dithiothreitol.

Oligosaccharide Haptens. The preparation and characterization of tetra-, hexa-, and octasaccharides derived from type III pneumococcal polysaccharide has been given elsewhere (Campbell and Pappenheimer, 1966). An oligomer of higher size was isolated by gel filtration on Sephadex G-50 in 0.02 *M* NH_3 (1.5 × 200 cm). Its molecular weight determined by equilibrium sedimentation (Yphantis, 1960), using a calculated value for the partial specific volume of 0.58 (Kratky et al., 1973), was 3000 ± 300, which corresponds approximately to a 16-hexose unit.

Antibodies and oligosaccharides were dissolved in phosphate-buffered saline (0.02 *M* phosphate buffer-0.15 *M* NaCl (pH 7.3)) and stored at 4°C prior to use.

CPL Measurements. The instrument used for measurement of circularly polarized luminescence was built in the Department of Chemical Physics, Weizmann Institute, and has been described (Steinberg and Gafni, 1972). Full details about the modification of the instrument for measuring CPL of proteins were given elsewhere (Steinberg et al., 1974; Schlessinger et al., 1975b). The CPL spectrum is expressed by the emission anisotropy factor g_{em} defined as $g_{em} = 2\Delta f/f$ where $\Delta f/f$ is the extent of circular polarization of the fluorescence (f) defined as positive for left-handed circular polarization (Steinberg, 1975; Steinberg et al., 1974). The estimated uncertainty in the measurements of g_{em} is $\sim 5 \times 10^{-5}$. The excitation wavelength was 275 nm with a resolution of 30 nm. The bandwidth of the analyzing monochromator was 15 nm. All measurements were taken at 22°C. Sample cells of 2-mm light path were used.

Results

The CPL spectra of the homogeneous antibody 45-394 and its Fab' fragment in the absence and the presence of tetra-, hexa-, 16-residue oligosaccharides and of SIII are presented in Figure 1. The emission anisotropy factor of the intact antibody as well as of the Fab' fragment varies across the emission range reflecting heterogeneity of the optical activity of the tryptophan residues which contribute to the emission spectrum of the proteins. The CPL spectrum of the intact antibody is similar in shape and size to that reported for heterogeneous antibody populations (Schlessinger et al., 1975b). Upon addition of oligosaccharide haptens or SIII to either the intact antibody or its Fab' fragment, drastic spectral changes were observed; the largest changes occurred upon binding of SIII to the antibody (range 355-370 nm). SIII was added to the antibody in large "antigen excess" in order to form soluble complexes suitable for CPL measurements. It was indeed shown that CPL changes were independent of the antigen-antibody ratio thus suggesting that they are due to conformational changes in the antibody molecules upon antigen binding rather than to "lattice" formation (Givol et al., 1974; Schlessinger et al., 1975b).

The tetra-, hexa-, or octasaccharide (curve not shown) induced marked spectral changes very similar to each other.

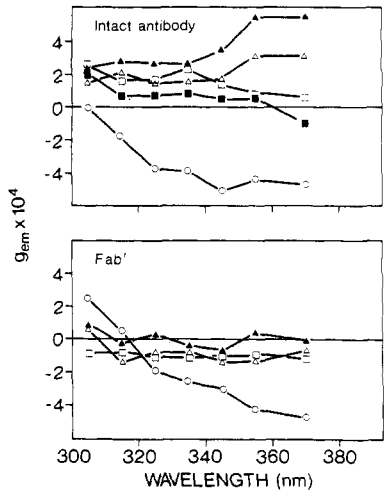

FIGURE 1: CPL spectra of antibody 45-394 and its monovalent Fab' fragment in the absence and presence of SIII polysaccharide and oligosaccharides derived from it. (Upper) Emission anisotropy factor, g_{em}, of antibody 45-394, 5 mg/ml (○); in the presence of tetrasaccharide (□) and of hexasaccharide (■). A similar spectrum was observed upon interaction with an octasaccharide (not shown). Antibody 45-394 in the presence of a 16-hexose unit, 100 μg/ml (△); and of SIII polysaccharide, 500 μg/ml (▲). (Lower) Emission anisotropy factor, g_{em}, of Fab' fragment from antibody 45-394, 5 mg/ml (○); the Fab' fragment in the presence of tetrasaccharide, (□); of the 16-residue oligosaccharide, 100 μg/ml (△); and of SIII polysaccharide, 500 μg/ml (▲). The haptenic oligosaccharides (tetra-, hexa-, and octa-) were used in a tenfold molar excess over the antibody binding site concentration; the multivalent antigens, i.e., the 16-hexose unit and SIII polysaccharide, were added in large "antigen excess", as determined by quantitative precipitin analysis.

In the 345-370-nm range it is noteworthy that a 16-hexose unit induced CPL changes which are intermediate in intensity between those produced by SIII and the shorter oligomers (Figure 1, upper).

The CPL changes recorded for the Fab' fragment were almost indistinguishable in the case of the binding of the tetrasaccharide and of the 16-hexose unit and slightly larger for the intact polysaccharide antigen (Figure 1, lower). It is remarkable that the extent of the CPL changes was enhanced when the Fab' fragment was substituted by its corresponding intact antibody (Figure 1, upper). This suggests that the Fc part in the intact antibody must contribute to the optimal conformational changes observed.

The reduction of the interchain disulfide bridges in the molecule by 0.0025 *M* dithiothreitol did not cause any changes in the CPL spectrum of the free reduced protein as compared to the native one (Figure 2), showing that the microenvironment of the fluorescent tryptophan residues was not affected by reduction of the disulfide bonds. Similarly, the CPL spectrum of the free $F(ab')_2$ fragment was similar to that of the monovalent Fab' fragment suggesting that there are no interactions between the two Fab' fragments in $F(ab')_2$.

The amplitude of CPL changes caused upon interaction of both SIII and the 16-residue oligosaccharide with either the dimeric or the monomeric Fab' fragment was similar for both antigens. However, the additional changes seen upon interaction of the same antigens with the intact IgG molecule in the 345-370 nm range (Figure 1, upper) were not observed using either $F(ab')_2$ (Figure 2, lower), or after reduction of interchain disulfide bridges in the IgG (Figure 2, upper).

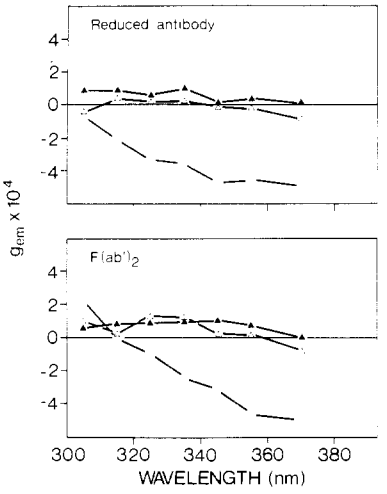

FIGURE 2: The CPL spectra of reduced antibody 45-394 of $F(ab')_2$ fragment in the absence and presence of a 16-residue oligosaccharide and SIII polysaccharide. (Upper) Emission anisotropy factory, g_{em}, of reduced antibody 45-394, 5 mg/ml (○); the reduced antibody in the presence of the 16-hexose unit, 100 μg/ml (△); and the reduced antibody in the presence of SIII polysaccharide, 500 μg/ml (▲). (Lower) Emission anisotropy factor, g_{em}, for fragment $F(ab')_2$ derived from antibody 45-394, 5 mg/ml (○); the $F(ab')_2$ fragment in the presence of 16-hexose unit, 100 μg/ml (△); and the $F(ab')_2$ fragment in the presence of SIII polysaccharide, 500 μg/ml (▲). The amounts of antigens added were in the region of "antigen excess" (see legend of Figure 1).

Discussion

CPL changes monitor macromolecular conformation in the vicinity of tryptophan residues in proteins (Steinberg et al., 1974; Schlessinger and Levitzki, 1974) and therefore indicate structural changes in the environment of those tryptophan residues upon interaction with ligands (Schlessinger et al., 1975a,b).

The results of this study indicate that large CPL changes were observed upon binding of as small haptenic determinants as tetra-, hexa-, and octasaccharides to the antibody as well as to its Fab′ fragment. SIII induced spectral changes both in the intact antibody and its Fab′ fragment, the largest effect being observed with the intact antibody (Figure 1). When the intact antibody was used very similar CPL changes were demonstrated for the tetra-, hexa-, or the octasaccharide, and a larger change was found with the 16-hexose unit, whereas a maximal amplitude was recorded with SIII (Figure 1, upper). The extent of the changes thus appears to be related to the size of the ligands used for binding. The spectral changes observed in the Fab′ and $F(ab')_2$ fragments were smaller with all interacting ligands studied, especially with SIII (Figures 1 and 2, lower), pointing out that the additional spectral changes recorded in the IgG antibody molecule were due to the spectral contribution of the Fc portion of the molecule, or to interactions between Fc and Fab fragments (Schlessinger et al., 1975b).

Upon reduction of the interchain disulfide bonds in the antibody or upon cleavage of the Fc part from the rest of the molecule by pepsin digestion, the additional spectral changes attributed to the Fc fragment vanished (Figure 2); this provides presumptive evidence for the capital role of the interheavy chain disulfide bond in the hinge region. Since the hinge lacks tryptophan residues (Dayhoff, 1972), it is clear that it cannot contribute per se to the spectral changes; the hinge region may, however, be required for the transmission of structural changes to Fc upon antigen binding in the Fab part. Alternatively, interactions between Fab and Fc fragments which are linked by the hinge region might undergo changes subsequently to antigen binding; the interplay between Fab and Fc fragments may be responsible for triggering structural changes in Fc part.

The results of this study are in agreement with those of Schlessinger et al. (1975b) who investigated interactions of heterogeneous antibody populations with various antigens. The CPL spectra of heterogeneous antibodies is weighted average of CPL spectra of the constituents in the mixture. Individual homogeneous antibodies are therefore expected to exhibit different CPL spectra. Experimental evidence shows that this is true (J. Schlessinger and J.-C. Jaton, unpublished).

The binding of small haptenic determinants (up to the size of an octasaccharide) to the intact antibody and to the corresponding Fab′ fragment produced comparable but quantitatively different changes in g_{em}. It should be pointed out that since the Fc moiety contributes significantly to the total fluorescence intensity and if indeed only the Fab′ fragment underwent a conformational change, the corresponding change in g_{em}, i.e., $2\Delta f/f$, of the intact antibody should be smaller compared to the change seen in the isolated Fab′ (see footnote in Schlessinger et al., 1975b) which is contrary to the observation. This implies that an additional spectral change due to the Fc moiety occurred in the intact antibody. The tetra- and the hexasaccharide, for example, caused CPL changes in the 310–340-nm region, which are larger in the intact antibody than in the Fab′ (Figure 1). The same argument also applies to the results concerning the effect of reduction of the disulfide bond in the hinge region on CPL changes. If one compares in the 310–340-nm region the effect of the binding of the 16-residue oligosaccharide, before and after reduction of the antibody, quantitative differences in CPL changes can be seen (Figures 1 and 2).

In the 345–370-nm region, only SIII and to a lesser extent the 16-residue oligosaccharide were able to trigger large spectral changes attributed to the Fc part. Since the disaccharide hapten (cellobiuronic acid) is the smallest unit known to bind effectively to the anti-polysaccharide antibodies (Speyer et al., 1973), the 16-residue oligomer can be considered as a multivalent ligand.

Ultracentrifugation analysis of this 16-residue unit–antibody complex formed in large hapten excess under the conditions used for CPL measurements revealed a major peak ($s_{20,w}$, 6.9) with a shoulder ($s_{20,w}$ = 8.5). This indicates the presence of mainly monomers and probably of some dimers of antibody. This would imply that the conformational changes attributed to the Fc moiety reflect hapten–antibody interactions rather than the effect of cross-linking between antibody molecules.

Of special interest is the correlation between possible structural changes in Fc fragment upon binding of oligosaccharide antigens and the fixation of complement by the immune complexes. Results of such an investigation will appear elsewhere (manuscript in preparation).

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A Cell Free System from HeLa Cells Active in Initiation of Protein Synthesis†

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ABSTRACT: A cell free system programmed by endogenous mRNA and active in initiation of protein synthesis has been obtained from HeLa cells by adding 25-100 μM hemin to the medium used to homogenize the cells. Hemin stabilizes the initiation activity of the extract, which otherwise decays rapidly even at 0°C. The role of hemin in promoting initiation has been examined by fractionating the extracts into ribosomes and postribosomal supernatant (S150). An extract prepared without hemin or the S150 obtained from this extract inhibits protein synthesis of the extract containing hemin by about 30%. The ribosomes prepared from extracts containing hemin are active in initiation of protein synthesis, whereas the ribosomes obtained from the extracts prepared without hemin show little or no initiation. These results have suggested that addition of hemin prevents the formation of an inhibitor of initiation in the S150 and at the same time protects from inactivation an initiation factor associated with ribosomes or ribosomal subunits. Addition of 2 mM GTP to HeLa extracts stabilizes the initiation activity, though to a smaller degree than hemin. The effects of hemin and GTP are not additive, suggesting that they may act on the same target molecule, though possibly by different mechanisms. The mechanism of action of GTP is discussed in view of similar observations made in the rabbit reticulocyte cell free system.

In order to study translational control mechanisms in a cell free system it is necessary to have available a cell extract active in initiation of new polypeptide chains. A very active cell free system can be obtained from rabbit reticulocytes and several features of the regulation of α and β globin synthesis have been studied in this system (Lodish, 1974). However, it has been difficult to obtain initiation in vitro with extracts of HeLa cells, due to a rapid loss of activity during cell fractionation (Reichman and Penman, 1973). This problem has partially been overcome by preparing extracts by high-speed techniques (Goldstein et al., 1974). These extracts cannot, however, be fractionated by conventional biochemical techniques and cannot be frozen without loss of the ability to initiate new polypeptides. This is somewhat unfortunate, since there is a wealth of observations on the regulation of protein synthesis in HeLa cells which have been made by Penman and his associates

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