Circular Dichroism and Fluorescence Studies of Homogeneous Antibodies to Type III Pneumococcal Polysaccharide†

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ABSTRACT: The near-ultraviolet circular dichroism (CD) of three homogeneous anti-type III pneumococcal antibodies in the absence and the presence of the specific hexasaccharide ligand was studied. In addition recombinations and hybridizations of H and L chains derived from two of these antibodies were carried out and the CD spectra of bound and free reconstituted IgG molecules were measured. The results indicate that the CD spectra of the native antibodies in the 260–310-nm range are very similar in shape and sign and exhibit a positive band at 285 nm. The homologous reconstituted antibody molecules exhibited CD spectra very similar in shape and sign to those of the native antibody molecules although recombinant molecules are no longer stabilized by interchain disulfide bonds. Upon addition of the hexasaccharide ligand, a significant decrease in amplitude of the CD spectra (18–21%) occurred in all three native antibodies and their Fab fragments as well as in the homologous recombinant molecules. No CD spectral changes could be detected upon interaction of the hapten ligand with the heterologous recombinants. All homogeneous antibodies studied exhibited fluorescence quenching upon oligosaccharide binding and a blue shift of the emission maximum. This property allowed the determination of the binding constant of one selected antibody to be made. Taken together, CD and fluorescence spectroscopic data suggest that oligosaccharide ligands induced detectable conformational changes in the Fab fragment of the antibody.

Optical properties such as tryptophanyl fluorescence and circular dichroism (CD) have been successfully used as sensitive parameters for the detection of small conformational changes in macromolecules, such as myeloma proteins or antibodies which bind small ligands (Pollet et al., 1974; Jolley et al., 1973; Holowka et al., 1972). The enhancement or quenching of tryptophanyl emission in immunoglobulin systems has been used to recognize structural differences in or near the binding sites (Jolley et al., 1974) and to measure the stoichiometry of binding (Pollet and Edelhoch, 1973).

CD of human immunoglobulins G (Johnson et al., 1974), E (Dorrington and Bennich, 1973), and of rabbit antibodies and their fragments (Cathou et al., 1968) has been extensively studied. In the 260–310-nm region, CD transitions seen as discrete bands have been shown to be characteristic of the asymmetric environments of aromatic groupings (Beychok, 1967). Indeed, quantitative changes in intrinsic CD transitions of homogeneous anti-type III pneumococcal antibodies on binding of the specific hexasaccharide hapten were observed (Holowka et al., 1972).

Homogeneous antibodies to polysaccharide antigens are well suited for optical investigations because (1) the oligosaccharide ligands to which these antibodies bind are devoid of absorption bands in the relevant spectral region and thus changes observed upon binding can be attributed to changes in the optical activity of the protein itself; (2) a series of oligosaccharides of repeating sequence and of increasing size can be readily prepared (Campbell and Pappenheimer, 1966). The availability of several homogeneous antibodies and of their monovalent Fab fragments provides a convenient system to compare the effects of hapten binding on the behavior of aromatic chromophores which may be affected upon interaction with ligands. The results of such an investigation are presented below.

Experimental Section

Antibody Production. The antibodies against type III pneumococcal polysaccharide, designated BS-1, K-25, and 45-394, were raised according to the method of Kimball et al. (1971), and purified by using a solid immunoadsorbent consisting of a SIII[1]–azo-bovine serum albumin conjugate linked to bromoacetylcellulose (Jaton et al., 1970) and by agarose block electrophoresis as described by Braun and Jaton (1973). Antibody 45-394 disclosed a single band in cellulose acetate electrophoresis and a 3-band isoelectric-focusing pattern consistent with a homogeneous product; its L chain (allotype b_4) appears to have an unreactive N-terminal residue in the Edman degradation (unpublished). Homogeneous antibodies to streptococcal group A polysaccharide used in control experiments were a gift from Dr. D. G. Braun. Fab fragments were prepared by papain digestion and isolated by ion-exchange chromatography (Porter, 1959).

Oligosaccharide Haptens. The preparation of oligosaccharides, di-, tetra-, hexa-, and octasaccharides, obtained from a partial acid hydrolysate of SIII was described by Campbell and Pappenheimer (1966); the oligomers were characterized by paper chromatography (Kimball, 1972).

Recombinant IgG Molecules. Recombinations and hybridizations of H and L chains derived from antibodies BS-1 and K-25 were carried out as described by Huser et al. (1975). Homologous and heterologous recombinants

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[1] The nomenclature of immunoglobulins, fragments, and chains is as recommended in *Bull. W.H.O. 30,* 447 (1964); SIII, soluble type III pneumococcal polysaccharide; PBS, phosphate buffer, 0.02 *M* (pH 7.3), containing 0.15 *M* sodium chloride.

were purified by gel filtration on Sephadex G-150 prior to use.

Fluorescence Spectra. Emission spectra were recorded in a Hitachi Perkin-Elmer, Model MPF3 spectrofluorometer equipped with a thermostated cell compartment at 25.0 ± 0.5°C. The excitation was done at 280 nm (band width 4 nm) and the emission spectra were recorded between 290 and 400 nm (width, 10 nm). All spectra were corrected for variation in light source and photomultiplier response. Fluorometric titrations were done by adding increasing amounts of oligosaccharide solutions to 2 ml of the protein solution ($OD_{280\,nm}$ = 0.1). The maximum quenching in emission was reached when no further decrease in emission occurred upon ligand addition. Corrections for dilution were made. The Sips and Scatchard plots as well as the association constants derived therefrom (Eisen, 1964) were evaluated by a computer program.

Circular Dichroism Measurements. These were made with a Jasco J-20 recording spectropolarimeter at room temperature (22°C) and in triplicate. The mean residue ellipticity $[\theta]$ was calculated from the equation

$$[\theta] = \theta M/10cl$$

where θ is the measured ellipticity in degrees, M is the mean residue weight (110), c is the concentration of antibodies in g/ml, and l is the path length of the cell (l = 1 cm). A scanning speed of 2 nm/min and a time constant of 64 sec were used over the wavelength range of 310–260 nm. The average deviation was computed to be ±2.5° cm^2 $dmol^{-1}$. Antibodies were used at a concentration of 0.75–1.0 mg/ml, as determined spectrophotometrically at 280 nm. Antibody–hapten complexes were formed by adding a hexasaccharide solution (2 mg/ml) directly to the cell containing the antibody solution, in a 1.5–2.0-fold molar ratio of hapten to antibody. Corrections for dilution were made. PBS was used as a diluent and also to establish base lines. The hexasaccharide at the highest concentration (2 mg/ml) used exhibited neither absorbancy nor transitions in the wavelength range studied (260–310 nm).

Results

Circular Dichroism Spectra of Anti-Pneumococcal Antibodies. The near-ultraviolet CD spectra of antibodies BS-1, K-25, and 45-394, shown in Figure 1, are similar to each other in shape and sign and exhibit a positive Cotton effect at 285 nm. They differ, however, in that the ellipticity $[\theta]$ of antibodies K-25 and 45-394 is higher than that of antibody BS-1. Upon addition of SIII hexasaccharide ligand to either one of the three antibodies significant CD changes in both shape and magnitude were recorded (Figure 1). A decrease in ellipticity, which is well beyond the limits of experimental error, was observed in the wavelength region 310–260 nm. As a control, no changes in CD spectra of rabbit anti-streptococcal group A antibodies upon addition of SIII hexasaccharide were detected. Difference spectra were computed for antibodies BS-1 and K-25 (Figure 2). The presence of distinctive maxima is apparent. Antibody K-25 exhibits the largest absolute change in ellipticity (15 deg cm^2/dmol). The bands in the difference CD spectra of antibody K-25 at 299 and 291 nm are characteristic of tryptophan (Strickland et al., 1969) and those at 275 and 285 nm are attributed in model compounds to the tyrosyl chromophore (Simmons and Glazer, 1967).

Essentially the same spectral changes were recorded when the Fab fragments derived from the antibodies were used for binding of the hexasaccharide ligand (Figure 3).

Table I: Association Constants of Antibody 45-394 and Its Fab Fragment.

Antibody	Hapten	Q_{max} (%)	K_A[a]	A[b]
IgG	Hexasaccharide	11.1	1.4 ± 1 × 10^5	1.07
IgG	Octasaccharide	11.0	3.0 ± 1 × 10^5	1.00
Fab	Hexasaccharide	18.0	2.4 ± 1 × 10^5	1.06

[a] Association constants determined at 25.0°C (see Materials and Methods for details). [b] Heterogeneity index.

Antibodies BS-1 and K-25 differ from each other in the amino acid sequence of the hypervariable sections of the variable domain (Jaton, 1974, 1975, unpublished). Chain recombinations between these two antibodies generated hybrid molecules endowed with new binding site structures which, however, were unable to interact significantly with the hexasaccharide ligand (Huser et al., 1975). No differences in ellipticity nor in shape could be found when the spectra of the homologous recombinants were compared with those of the native antibodies (Figure 1). The presence of the H chain in the heterologous recombinant appears to play a major role in defining the shape and amplitude of the CD spectrum, since the latter is indistinguishable from that of the native antibody containing the same H chain. As expected, no CD changes could be detected in the hybrid molecules in the presence of the SIII hexasaccharide, in contrast to the CD changes detected in the ligand interactions with both the native antibodies and their corresponding homologous recombinants (Figure 1).

Fluorescence Changes Associated with Binding of Oligosaccharides to Antibody 45-394. Fluorescence spectra of antibody 45-394 were measured in the presence and the absence of hapten oligomers of increasing size. Upon addition of increasing concentration of hexa- or octasaccharide (10^{-3}–10^{-2} M), a significant blue shift of 7–8 nm in the emission maximum (340 to 332 nm) of the antibody was recorded, together with a pronounced quenching of fluorescence. Only a slight decrease in fluorescence intensity was observed upon binding of the disaccharide. No effect was observed when anti-streptococcal antibodies were used with the same haptens.

Titrations of antibody 45-394 and its Fab fragments with the hexa- or the octasaccharide were carried out. Given the antibody concentration, the fraction of antibody sites occupied (r) and the free hapten concentration (C), the association constant K_A can be obtained from the Scatchard plot. The results are summarized in Table I where it can be seen that the association constant, K_A, is 2 ± 1 × 10^5 M^{-1} for both the intact antibody and its Fab fragment, thus indicating the independence of the two binding sites. Homogeneity of ligand binding is expressed by the heterogeneity index which is close to unity.

Discussion

The near-ultraviolet CD spectra of three homogeneous antibodies to type III pneumococcal polysaccharide, BS-1, K-25, and 45-394, and of some reconstituted antibodies from isolated H and L chains were analyzed. They are very similar in sign and shape but differ in the magnitude of the ellipticity (Figure 1), thus reflecting the involvement of more optically active chromophores interacting with the

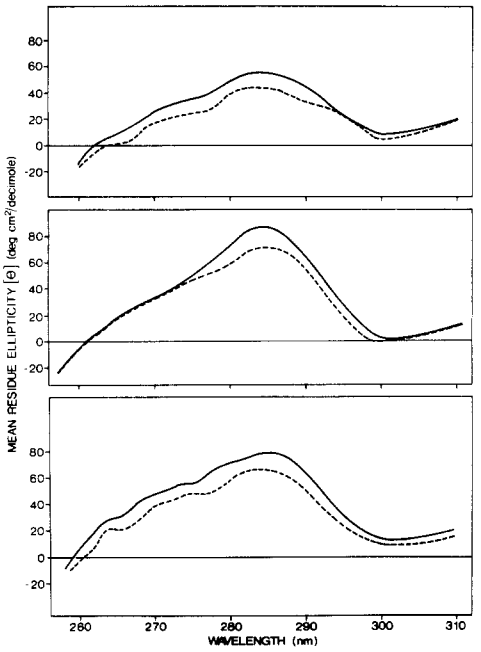

FIGURE 1: CD spectra of anti-SIII pneumococcal antibodies. (—) Free antibody; (- - -) antibody in the presence of hexasaccharide in a 1.5:1 molar hapten to site ratio. Free and bound homologous recombinant molecules exhibited CD spectra indistinguishable to those of the corresponding native antibodies. Antibody BS-1 (top); K-25 (middle); and 45-394 (bottom).

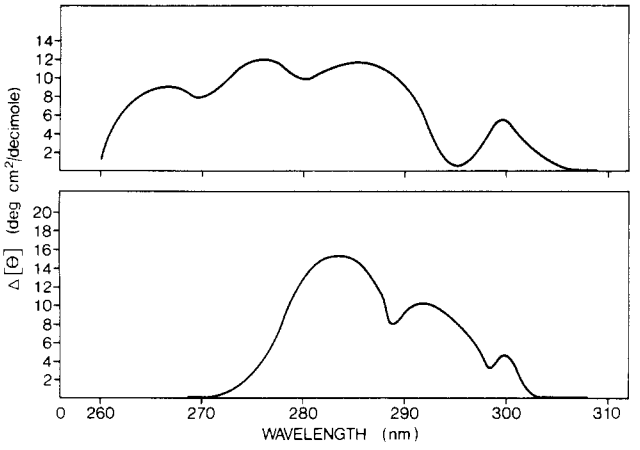

FIGURE 2: Difference spectra between CD spectrum of free antibody and CD spectrum of the bound antibody. (Top) Antibody BS-1; (bottom) antibody K-25.

hapten in antibody K-25 than in antibody BS-1, a suggestion in agreement with amino acid sequence data of their V domains (Jaton, 1974, 1975, unpublished). The CD spectra of the Fab fragments derived from the three homogeneous antibodies are very similar in sign, shape, and magnitude to those of the parent intact molecules (Figure 3). This implies that the contribution of the Fc fragment to the CD spectrum of the intact antibodies in the wavelength range 260–310 nm must be very small or even slightly negative, an observation in accord with previous results (Cathou et al., 1968).

The CD spectra of the homologous recombinants from antibodies BS-1 and K-25 were indistinguishable from those of the native antibodies (Figure 1), although the recombinant molecules were no longer stabilized by interchain disulfide bonds. The hybrid recombinants in which

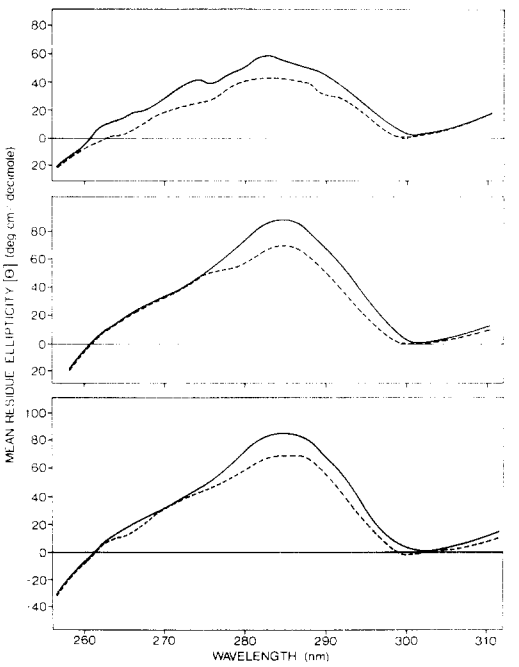

FIGURE 3: CD spectra of Fab fragments of anti-SIII pneumococcal antibodies. (—) Free Fab fragment; (- - -) Fab fragment in the presence of hexasaccharide in a 2.0:1 molar hapten to site ratio. Fab BS-1 (top); K-25 (middle); and 45-394 (bottom).

the H chain was drawn from antibody BS-1 (or K-25) exhibited a CD spectrum almost identical with that of the native antibody BS-1 (or K-25, respectively). This observation suggests that the characteristic features of the CD spectrum of this homogeneous pneumococcal antibody is largely determined by its constituent H chain.

A significant decrease in ellipticity upon addition of SIII hexasaccharide to antibodies 45-394, BS-1, and K-25 and their homologous recombinant molecules was observed (Figure 1). The calculated CD difference spectra for antibodies BS-1 and K-25 (free antibody–bound antibody) differ in shape, magnitude, and number of peaks obtained (Figure 2), indicating structural differences between the two antibodies. This finding is in agreement with the amino acid sequence data of their V domains (Jaton, 1974, 1975, unpublished). The same spectral changes persisted after cleavage of the Fc fragment from the intact IgG molecule (Figure 3), indicating that the spectral changes occurred in the Fab part as a result of hapten binding. These spectral differences result from changes in the asymmetric environment of the chromophores, which may be caused by small conformational changes upon hapten binding or merely by the interaction between the aromatic chromophores and the asymmetric hapten (Conway-Jacobs et al., 1970; Glaser and Singer, 1971; Holowka et al., 1972). No CD changes were noted on addition of hapten to the heterologous recombinants, which differ from the homologous recombinants mainly in the first and third hypervariable sections of the V domain (Jaton, 1975, unpublished). This was expected in view of the very weak binding activity of these hybrid molecules (Huser et al., 1975).

The quenching of the fluorescence intensity of antibody 45-394 (Table I) upon hapten binding was greater for the Fab fragment (Q_{max} 18%) than for the intact protein (Q_{max} 11%); this results mainly from the relative contribution of the Fab and Fc fragments to the fluorescence of the whole Ig and rules out to a large extent the possibility of spectral

changes of the tryptophan residues in Fc fragment (Jolley et al., 1973; Pollet and Edelhoch, 1973). It is noteworthy that the emission maximum of antibody 45-394 was blue-shifted (7–8 nm) upon oligosaccharide binding. This may imply that the tryptophanyl residues residing in polar environment became preferentially quenched by decreased exposure to solvent, e.g., by direct hapten shielding (Pollet and Edelhoch, 1973). On the sole basis of fluorescence data, it is difficult to interpret fluorescence quenching and polarity changes of tryptophanyl residues as ligand-induced modification in the antibody conformation. However, the CD studies described above with the same antibody as well as an investigation by circular polarization of luminescence (see accompanying paper (Jaton et al., 1975)) point out that significant structural changes occur in the antigen-binding fragment subsequent to interactions with haptens.

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