Circular Dichroism Study of the Antibody Combining Site†

Dan Inbar, David Givol,* and Jacob Hochman‡

ABSTRACT: The circular dichroism (CD) of the Fv fragment derived from myeloma protein 315 possessing anti-dinitrophenyl (Dnp) activity was studied. The CD spectrum in the far-ultraviolet region exhibits a negative band at 217 nm which is characteristic also of the Fab′ and the 7S monomer, and indicates β structure. In addition a new positive band, characteristic only of Fv (the variable fragment), appears at 233 nm. The 217- and 233-nm bands are not affected by hapten binding. On the other hand, all the CD bands in the region 250–310 are enhanced upon binding of the hapten, N^{ϵ}-Dnp-lysine. The difference spectra in this region of bound and free protein are very similar for Fv, Fab, and the intact protein. Besides this newly observed Cotton effect in the protein absorption range, hapten binding produced large extrinsic Cotton effects in the 310- to 500-nm range, similar to those described earlier by Glaser, M., and Singer, S. J. [(1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2477]. This extrinsic Cotton effect was found to be identical in shape, sign, and magnitude in the 7S monomer and the Fab′ and Fv fragments. It is concluded that the combining site in Fv has the same native features as in the intact protein.

Circular dichroism (CD) is a sensitive probe for analyzing the three-dimensional structure of proteins (Hooker and Schellman, 1970), and has been used to compare the structures of different fragments (Fab and Fc) of immunoglobulins (Cathou *et al.*, 1968) as well as of the V and C domains in the light chain (Bjork *et al.*, 1971). The results of these studies indicated that there is no significant interaction between domains in the immunoglobulin molecule, and that only minimal conformational changes accompany the cleavage of the whole molecule into Fab and Fc fragments or of the light chain into its halves. Each fragment of domain yielded a clearly characteristic CD spectrum reflecting differences in their conformation.

In addition, distinct CD bands may arise upon interaction between antibody and its hapten. This induced optical activity is a consequence of the binding of a symmetric, optically inactive, hapten in the asymmetric environment of the antibody combining site which results in an extrinsic Cotton effect in the absorption region of the haptenic chromophore. This phenomenon, first observed by Conway-Jacobs *et al.* (1970) with an arsanilated periodic polypeptide, was more extensively studied with nitrophenyl haptens and their specific antibodies, as well as the myeloma proteins 315 and 460 which possess anti-dinitrophenyl (anti-Dnp) activity (Glaser and Singer, 1971; Rockey *et al.*, 1971a,b, 1972). The results showed that the extrinsic Cotton effects are highly discriminating between antibody combining sites of similar specificity, and can be used to detect small conformational differences in the active site. Intrinsic Cotton effects in the region of optical absorption of the protein itself were also found recently, upon interaction between anti-S_3 pneumococcal antibodies and the specific hexasaccharide hapten which does not absorb in this region (Holowka *et al.*, 1972).

We have recently reported the preparation of Fv, a fragment composed of the V_H and V_L portions of myeloma protein 315 (Inbar *et al.*, 1972; Hochman *et al.*, 1973). This fragment has a molecular weight of 25,000 as compared with 50,000 for the Fab′ fragment and 150,000 for the 7S monomer of the original myeloma protein. By all criteria of activity so far tested (association constant, number of sites, and kinetics of binding), the Fv fragment is very similar to Fab and the starting 7S monomer (Inbar *et al.*, 1972; Hochman *et al.*, 1972). This suggests that the antibody combining site in the above three preparations is very similar, and its conformation has not been changed upon splitting of the constant portions from Fv. It is, therefore, important to analyze the features of the antibody site in Fv, Fab, and 7S protein by studying the CD of their complex with the hapten N^{ϵ}-Dnp-lysine. We show that by this sensitive technique the conformation of the site in Fv seems identical with that of the intact protein.

We also show that Fv fragment has a unique CD band at 233 nm which is absent in other domains of the molecule.

Materials and Methods

Protein 315 and its pepsin produced Fab′ and Fv were prepared as described (Inbar *et al.*, 1971; Hochman *et al.*, 1973). Protein concentration was determined from absorbance at 280 nm employing extinction coefficients $E_{1\text{ cm}}^{1\text{ mg/ml}}$ of 1.4, 1.4, and 1.5 and molecular weights of 153,000, 50,000, and 25,000 for the 7S monomer, Fab′, and Fv, respectively.

Circular dichroic spectra were recorded on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichrometer at 27°. Protein solutions with optical density up to 1.5 in 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4) were used for measurements. The hapten N^{ϵ}-2,4-Dnp-lysine was added in 10-fold molar excess over the amount of protein, for measurement of the CD of the complex antibody–hapten.

Fused quartz cells with path lengths of 0.1, 0.2, and 1 cm were used for measurements between 500 and 215 nm. Circular dichroic data are presented in terms of molar ellipticity $(\theta)_\lambda$ in deg·cm²·dmol⁻¹ for the range 250–500 nm and as mean residue ellipticity $[\theta]$ in deg·cm²·dmol⁻¹ for the range 215–250 nm, taking 110 as the average residue weight for the three proteins. Each CD spectrum was repeated at least three times with different preparations.

† From the Department of Chemical Immunology, Weizmann Institute of Science, Rehovot, Israel. *Received May 30, 1973.* This work was supported in part by a grant from the Israel National Science Foundation and Grant No. 4 R01 AI 11453-01 from the National Institutes of Health.

‡ From the Department of Zoology, Hebrew University, Jerusalem, Israel.

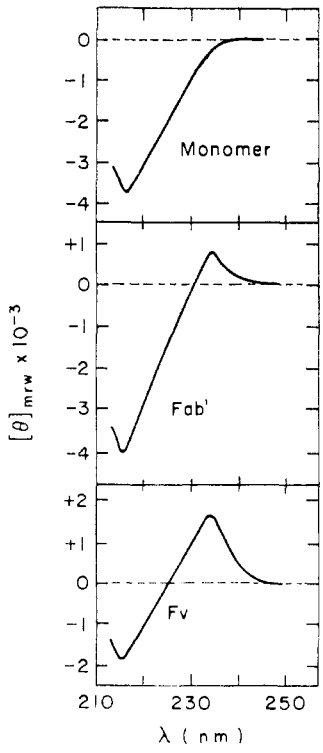

FIGURE 1: CD spectra of protein 315 monomer, Fab', and Fv in the far-ultraviolet region. Results are expressed as mean residue ellipticity $[\theta]_{mrw}$ in deg·cm²·dmol⁻¹ based on a mean residue weight of 110.

Results

The circular dichroic spectra of protein 315 monomer, of the Fab' and of the Fv fragments in the region of amide bond absorption are illustrated in Figure 1. All three proteins show a negative band at about 217 nm with mean residue ellipticity of 3.9×10^3, 4.0×10^3, and 1.9×10^3 deg·cm²·dmol⁻¹ for the 7S monomer, Fab', and Fv, respectively. A characteristic feature of the present system is the appearance of a distinct positive circular dichroic band at 233 nm. This band is absent from the CD spectrum of the 7S monomer, possibly because of its being masked by the negative contribution of the Fc fragment derived from this IgA protein. The Fab' fragment shows the 233-nm band with a mean residue ellipticity of $+0.8 \times 10^3$ deg·cm²·dmol⁻¹ which is half the value present in the CD spectrum of Fv ($+1.6 \times 10^3$ deg·cm²·dmol⁻¹). Since Fv is half the size of Fab, the doubling of the ellipticity in Fv, relative to Fab, suggests that the 233-nm band is a property of Fv of protein 315, and not of its constant domains. An interesting feature is the fact that the negative peak in the 217-nm region has about the same mean residue ellipticity for the monomer and for the Fab' (4.0×10^3 deg·cm²·dmol⁻¹), whereas the Fv peak at 217 nm has an ellipticity of approximately half that value (-1.9×10^3 deg·cm²·dmol⁻¹). It should be stated here that no difference either in the shape or magnitude of the circular dichroic spectrum of Fv in the region 215–250 nm could be detected upon binding of the hapten Dnp-lysine to the protein.

The CD spectrum of native Fv in the near-uv region (250–320 nm) is characterized (Figure 2) by two negative peaks at 293 and 286 nm, and a negative shoulder at about 280–282 nm. Three positive peaks are located at 270, 264, and 258 nm. This circular dichroic pattern closely resembles in shape those of the whole protein monomer and its Fab' fragment (unpublished data). The free hapten (Figure 2) does not show any optical activity over the investigated wavelength range (and this is taken as base line for all calculations).

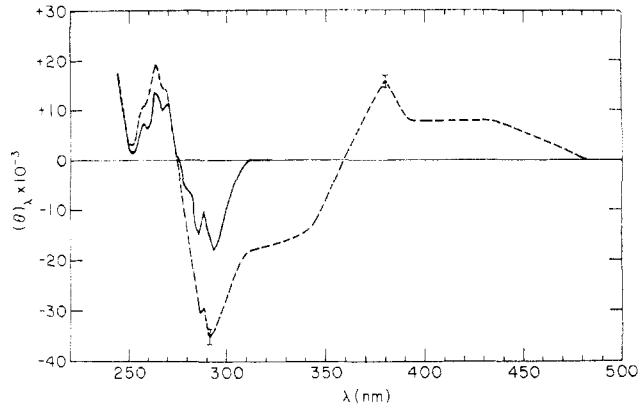

FIGURE 2: Near-ultraviolet and visible CD spectra of native Fv (solid line) and Fv bound to Dnp-lysine (dashed line). Dnp-lysine in buffer (horizontal line) shows no ellipticity throughout the entire spectra. Ellipticity $[\theta]$ is based on moles of active site.

Binding of the hapten Dnp-lysine to Fv generated distinct extrinsic circular dichroism bands (Figure 2); a broad positive band centered near 410–420 nm, a sharper positive band at 380 nm and a broad negative band extending into the protein CD region are generated upon that interaction. These bands (in the region 310–500 nm) characterize exclusively the optical activity of the now asymmetric Dnp-lysine moiety (Rockey *et al.*, 1971a,b). Binding of hapten to Fv also caused enhancement of the existing spectrum in the 280- to 310-nm region but did not generate new bands, nor did it change the sign or the positions of the maxima of the various bands.

Difference CD spectra of the 7S monomer, the Fab' and Fv in the 245- to 500-nm region were calculated by subtracting the circular dichroic spectrum of the native protein from the induced spectra of the protein–hapten complexes (Figure 3). All three difference spectra are very similar in their signs, shapes and magnitudes in the region 270–500. There exists, however, a difference in the region 250–270 nm. The difference CD spectrum in this region, although similar in sign and shape for all three proteins, is different in its magnitude, as it is smallest for Fv.

Discussion

Measurement of circular dichroism is a very sensitive tool for the investigation of the conformation of the antibody combining site and antibody–hapten complex. It has been demonstrated (Glaser and Singer, 1971) that the extrinsic Cotton effects observed are different for antibodies from different sources in spite of very close specificity (*e.g.*, anti-Dnp from rabbit, mouse, and myeloma proteins). Our studies compare the CD properties of active fragments of decreasing molecular weights derived from the same protein. The striking similarity in sign, shape, and magnitude of the difference CD spectra of Fv, Fab', and the 7S monomer (Figure 3) clearly demonstrates that the native features of the Dnp-lysine binding site, situated in the variable portion of protein 315 molecule, are preserved throughout the fragmentation procedure. Since it is very likely that the induced optical activity is due to the binding of the hapten to the antibody combining site, and since Fv contains only variable domains, the present results support previous data (Hochman *et al.*, 1973) that the constant domains of the molecule do not contribute significantly either to

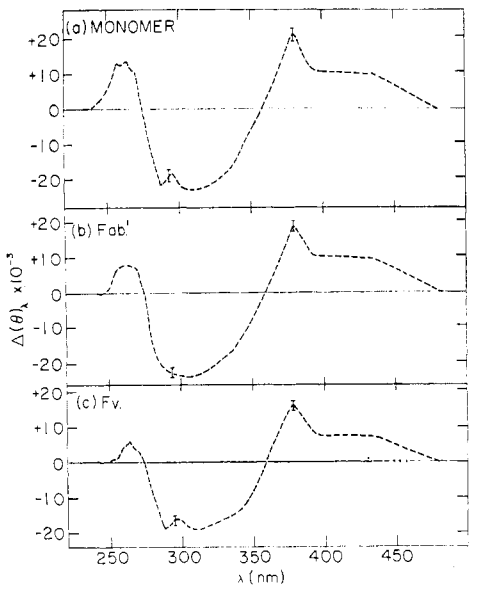

FIGURE 3: Difference CD spectra in the near-ultraviolet and visible region of MOPC-315 7S monomer, Fab', and Fv. Results are expressed as change in molar ellipticity (based on moles of active site) $\Delta(\theta)_\lambda$ in deg·cm²·dmol⁻¹.

the conformation or to the binding affinity of the combining site.

The CD spectrum in the far-uv region contains a negative band at 217 nm which is present in the monomer, Fab', and Fv derived from the MOPC 315 protein. This band is due to β structure and has previously been shown to be characteristic of all immunoglobulins (Litman *et al.*, 1970b), of Fab and Fc fragments of IgG (Cathou *et al.*, 1968), and of heavy and light chains (Bjork and Tanford, 1971a,b; Litman *et al.*, 1970a).

The most striking feature in the far-uv circular dichroic spectrum of Fv is the appearance of a new positive band at 233 nm. This band has been previously shown to appear in spectra of Fab (Cathou *et al.*, 1968; Litman *et al.*, 1970a) and of the variable portion of Bence-Jones λ chain (Bjork *et al.*, 1971). Our results clearly show that this band is a unique feature of the Fv fragment in protein 315. This band is present in Fab but its magnitude is only half of the ellipticity exhibited by Fv and, therefore, it may be a contribution of Fv to the Fab CD spectrum. It is likely that the positive band at 233 nm is due to a chromophore moiety in an asymmetric environment and previous reports assigned this band in proteins to tyrosine (Cathou *et al.*, 1968; Bewley *et al.*, 1972). It remains to be seen whether such a band will appear in variable fragments of other immunoglobulins. It is notable that neither the 233-nm band nor the 217-nm band is affected at all by hapten binding.

Our results in the far-uv region (210–250 nm) are in accord with the results of other investigators who failed to detect conformational changes in the antibody molecules in the presence of ligand (Ashman *et al.*, 1971; Steiner and Lowey, 1966; Metzger, 1970). On the other hand, quantitative changes in the CD region of the near-uv (250–310 nm) do occur upon hapten binding. These results are difficult to interpret because of the absorption of the Dnp group in this region, but it is not impossible that they reflect some structural changes upon binding of hapten, similar to those reported by Holowaka *et al.* (1972). However, since such changes are very similar in Fv, Fab, and 7S monomer, our results suggest that the structural changes (if they exist) are confined to the Fv region only.

An additional feature of the present system deserving comment is the decreasing magnitude of the difference spectra in the range 250–270 nm (Figure 3), being smallest for Fv and largest for the 7S monomer. These changes, although small in magnitude, may indicate some sort of interaction between the combining site and the rest of the molecule upon binding of the specific hapten.

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