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Extrinsic Cotton Effects Characteristic of Specific Hapten-Antibody Interactions

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ABSTRACT **The reversible binding of the haptens 2,4-dinitrophenyllysine (DNP-lysine) and 2,4,6-trinitrophenyllysine (TNP-lysine) to either the nitrophenyl-binding myeloma protein MOPC-315 or to pooled mouse anti-DNP or anti-TNP antibodies produces large and characteristic extrinsic Cotton effects (in the circular dichroic spectra). Despite the similarities in binding characteristics of the three proteins, the circular dichroic spectra produced by the haptens bound to the active sites of these proteins were markedly different. Extrinsic Cotton effects, therefore, provide a powerful new probe of the structure of the reversible complex formed between a hapten and an antibody active site.**

It has recently been demonstrated (1, 2) that the reversible binding of simple haptens to anti-hapten antibodies can give rise to characteristic extrinsic Cotton effects; that is, absorption bands of the hapten that are optically inactive when the hapten is free in solution become optically active when the hapten is bound in the asymmetric environment of the antibody active site. In this paper, we report the circular dichroic (CD) spectra obtained with the haptens DNP-lysine and TNP-lysine upon combination with mouse anti-DNP and anti-TNP antibodies, and with the nitrophenyl-binding protein from mouse myeloma MOPC-315(3). The results show that the binding of a hapten to either homologous antibodies, cross-reacting heterologous antibodies, or the myeloma protein results in readily distinguishable extrinsic CD spectra. Extrinsic Cotton effects, therefore, appear to provide a highly discriminating new technique for studying the structure of the reversible complex formed between a hapten molecule and an antibody active site.

MATERIALS AND METHODS

Mouse anti-DNP and anti-TNP antibodies were raised in Swiss-Webster mice against the multi-haptenic antigens DNP-hemocyanin and TNP-hemocyanin, respectively, by a published procedure (4). The immunized mice were implanted with Ehrlich ascites tumor cells, and the ascites fluids were collected and pooled. The pure antibodies were isolated from the fluids by modifications (4, 5) of the method of Eisen and Siskind (6). MOPC-315 protein was the generous gift of Dr. Herman Eisen. It was in monomeric form, having been mildly reduced and alkylated with ethyleneimine (7). DNP-lysine and TNP-lysine were used previously (2).

CD spectra and absorption spectra were obtained with the J-10 modification of the Durrum-Jasco ORD/UV/CD-5 instrument. The spectra were obtained at room temperature in 0.15 M NaCl—0.01 M sodium phosphate (pH 7.4). The absorbance was kept below 2.0 for all CD measurements. In the spectra shown in this paper, the contribution of the antibody protein itself has been subtracted, so the spectra represent only the contributions of DNP-lysine or TNP-lysine. The concentrations of antibody were determined from an assumed $\epsilon_{278}^{1\%} = 14.2$ and molecular weight of 150,000. The concentrations of stock solutions of DNP-lysine and TNP-lysine were obtained from $\epsilon_{363}^{1\ M} = 1.74 \times 10^4$ and $\epsilon_{348}^{1\ M} = 1.54 \times 10^4$, respectively.

RESULTS

TNP-lysine and DNP-lysine in free solution show no optical activity in the wavelength region from 300–450 nm, but upon binding to MOPC-315, or to the mouse anti-TNP or anti-DNP antibodies, they show pronounced Cotton effects (2) (Figs. 1 and 2). A few CD spectra are shown from the course of a titration experiment with each of the two ligands and each of the three proteins. The spectra followed Beers' Law and intersected at the same cross-over points for the titrations involving MOPC-315, showing that only one species of hapten–protein complex was formed in each titration. For the titrations involving the antibodies, the CD spectra were also largely parallel, although some small changes in spectral shapes were observed (for example, in the titration of anti-TNP antibodies with DNP-lysine in the wavelength region from 400–450 nm, Fig. 2, *middle*). The spectra did not cross the base line for the hapten–antibody systems, and their isosbestic behavior was, therefore, not testable. By and large, however, the spectra appeared to be quite similar when different fractions of the sites of any one antibody preparation were occupied by a given hapten. Anti-DNP and anti-TNP antibodies were readily discriminated from one another, and from MOPC-315, by the CD spectra that developed upon binding a given hapten. The three sets of spectra in

TABLE 1. *Molar ellipticities, $[\theta]$, of DNP-lysine and TNP-lysine bound to mouse antibodies and a mouse myeloma protein*

Protein	DNP-lysine		TNP-lysine	
	λ*, nm	$[\theta]$ †	λ*	$[\theta]$ †
MOPC-315	375	21,000	365	13,500
Anti-TNP	365	12,000	330	5,500
Anti-DNP	365	10,000	365	10,000

* Wavelengths of maxima in CD spectra, see Figs. 1 and 2.

† Calculated from spectra obtained with excess antibody, on the assumption that all of the added hapten was bound to antibody at the concentrations studied. See *Results*.

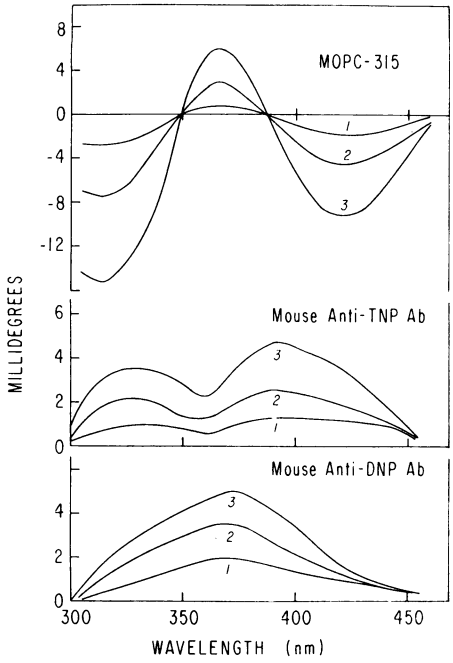

FIG. 1. The circular dischroic spectra produced upon binding of TNP-lysine to (*top*) MOPC-315; (*middle*) mouse anti-TNP antibodies; and (*bottom*) mouse anti-DNP antibodies. The protein concentration in each case was 28 μM. The curves labeled *1*, *2*, and *3* were obtained at different concentrations of added TNP-lysine: 7, 20, and 47 μM, respectively, for the *top set*; 10, 20, and 50 μM for the *middle set*; and 10, 20, and 40 μM for the *bottom set*. At the highest hapten concentration in each set, the binding sites were saturated, since further addition of hapten did not affect the spectra. The spectra for the mixtures containing MOPC-315 were obtained in a cell of 1.0-cm path length, the others were obtained in a 2.0-cm cell.

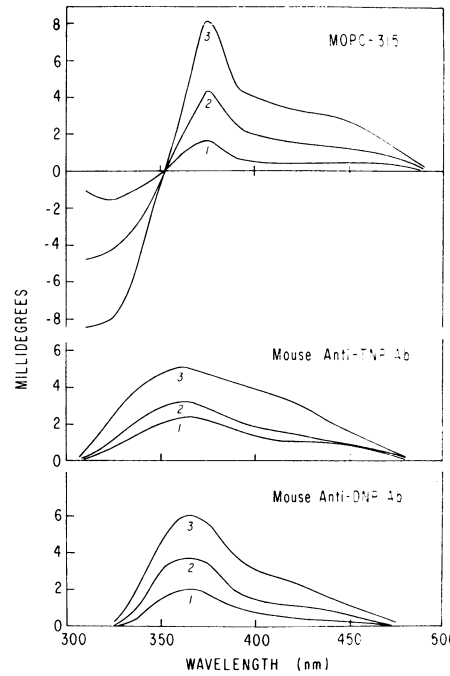

FIG. 2. The circular dichroic spectra produced upon binding of DNP-lysine to (*top*) MOPC-315; (*middle*) mouse anti-TNP antibodies; (*bottom*) mouse anti-DNP antibodies. The protein concentration in each case was 28 μM. The curves labeled *1*, *2*, and *3* were obtained at different concentrations of added DNP-lysine: 8, 20, and 64 μM, respectively, for the *top set*; 10, 20, and 40 μM, respectively, for both the *middle* and *bottom sets*. At the highest hapten concentration in each set, the binding sites were saturated, since further addition of hapten did not affect the spectra. The spectra for MOPC-315 mixtures were obtained in a 1.0-cm cell, the others in a 2.0-cm cell.

Fig. 1, for example, are very different, showing that the TNP-lysine is bound in a distinguishably different local environment in the active sites of the three different proteins. The molar ellipticities, $[\theta]$, for the DNP and TNP groups at the maxima of the different CD spectra are given in Table 1. These were calculated from the spectra obtained with hapten–antibody mixtures containing excess antibody. At the concentrations of antibody (28 μM) and of hapten (about 10 μM) used, essentially all the hapten was bound. By this procedure, the precise number of binding sites available in the antibody solutions was not required for the calculations of $[\theta]$.

DISCUSSION

Inherently symmetric chromophores can be made optically active if they are placed in an asymmetric environment. In this case, DNP-lysine and TNP-lysine exhibit no Cotton effects by themselves in the visible-wavelength region. However, when these haptens are bound to an antibody molecule, Cotton effects are generated in the wavelength region of the absorption bands (2). These Cotton effects reflect the interactions between the transition electric and magnetic dipole moments of the haptens and the transition moments of amino-acid residues in and surrounding the binding sites (8). These interactions depend on the relative orientations of these transition moments, and the distances between the groups involved. Extrinsic Cotton effects should, therefore, provide a sensitive probe of the structure of antibody sites. The experiments reported here demonstrate that this is true. MOPC-315 is a myeloma protein that has a high binding affinity for nitrophenyl ligands ($K = 2 \times 10^7$ M^{-1} for DNP-lysine) (3) and appears to be homogeneous with regard to structure and binding properties. By contrast, anti-DNP and anti-TNP antibodies that have been raised and isolated by conventional procedures are grossly heterogeneous. The CD spectra obtained when different proportions of the available sites on MOPC 315 were occupied by a given hapten are consistent with the existence of a single type of binding site. At low concentrations of hapten, when the hapten was practically all bound, the CD signal was proportional to the hapten concentration; at higher concentrations, when the sites were nearly saturated, the CD signal reached a plateau (all the spectra are not shown). Also, the spectra at various concentrations of hapten showed isosbestic points on the baseline, which indicates that only one type of protein binding site was present.

With pooled heterogeneous antibody preparations, the initial amounts of hapten added occupy the binding sites of highest affinity and, subsequently, further hapten binds to sites of decreasing affinity. When different fractions of the sites of anti-DNP or anti-TNP antibodies were occupied by a given hapten, the shapes of the CD spectra changed slightly and the CD signal at a given wavelength did not appear to be exactly proportional to the concentration of bound hapten.

This reflects the heterogeneity of the binding sites in any one antibody preparation. However, such small differences should not obscure the remarkable fact that the CD spectra obtained in the course of a hapten–antibody titration were closely similar, which suggests a structural similarity of the different sites in a heterogeneous population of antibody molecules. Otherwise, if the sites were structurally very diverse, the contributions to the CD spectrum from all the hapten-site interactions in a given antibody preparation might be of different signs and magnitudes, and might largely cancel one another out. On the contrary, however, the CD spectra for the hapten–antibody systems showed molar ellipticities (Table 1) of the same order of magnitude as for the hapten–MOPC 315 mixture.

The existence of a nearly parallel set of CD spectra for a hapten bound to different sites in a given antibody preparation is particularly striking in view of the marked differences in the spectra for a hapten bound to either mouse anti-DNP, mouse anti-TNP, or MOPC-315 active sites. Different mouse anti-DNP sites, therefore, appear to be structurally similar, and likewise anti-TNP sites appear structurally similar, but there is no indication that there is any significant overlapping fraction of anti-DNP and anti-TNP sites that are structurally alike. In other words, despite the fact that extensive cross-reaction of anti-DNP and anti-TNP antibodies occurs, and the antibody sites are therefore related by their specific affinity, the two kinds of sites are structurally distinct. This phenomenon was first discovered, and has since been extensively investigated, by Eisen, Little, and their colleagues (9–13) with anti-DNP and anti-TNP antibodies raised in rabbits to the antigens DNP- and TNP-bovine γ-globulin. In their studies, the anti-DNP and anti-TNP antibodies were distinguished by their different intrinsic fluorescences and by the fractional quenching of that fluorescence on binding a hapten. Even the few per cent of anti-hapten antibodies that bound most strongly to the *heterologous* hapten was found, when fractionated, to have the spectral properties characteristic of the bulk of the antibodies from which it was derived.

Our results, therefore, demonstrate the usefulness of extrinsic Cotton effects as a sensitive probe of the structures of antibody active sites and of hapten–antibody complexes. This sensitivity is also reflected in the fact that DNP-lysine and TNP-lysine complexes of *rabbit* anti-DNP and anti-TNP antibodies give extrinsic Cotton effects that are markedly different from those reported here with the mouse antibodies. These results, and others dealing with the binding of a series of DNP-haptens to anti-DNP antibodies, and the effects of temperature and other variables on the structure of hapten–antibody complexes, will be reported elsewhere (Glaser, M., and S. J. Singer, in preparation). Extrinsic Cotton effects may, therefore, provide the means to detect any small conformational changes produced in the active sites by perturbations of the antibody molecule.

The complexes formed between haptens and MOPC-315 give rise to very different CD spectra than are found with the corresponding hapten–antibody complexes. This must mean that MOPC-315, although it has a large affinity for DNP- and TNP-haptens (3), has an active site that is structurally different from the active sites of the bulk of elicited mouse anti-DNP or anti-TNP antibodies. Homogeneous hapten-binding myeloma proteins are currently under intensive chemical and structural investigation on the tacit assumption that they are representative of the corresponding anti-hapten antibodies that are elicited upon immunization. On the other hand, our results suggest that the fine structural details of the active sites of MOPC-315 are not likely to be representative of the sites of the *majority* of elicited mouse anti-DNP and anti-TNP antibodies. In order to find a myeloma protein that is likely to be more representative of elicited anti-hapten antibodies, a useful criterion would be the similarity of the extrinsic Cotton effects exhibited by their hapten complexes.

As this manuscript was being prepared for submission, the extrinsic Cotton effects accompanying the binding of DNP-lysine to MOPC-315 were reported (14). No studies with antibodies were reported, however. This work was supported by USPHS Grant AI-06659.

1. Conway-Jacobs, A., B. Schechter, and M. Sela, *Biochemistry*, **9**, 4870 (1970).
2. Reid, V., M. Glaser, R. Kennett, and S. J. Singer, *Proc. Nat. Acad. Sci. USA*, **68**, 1184 (1971).
3. Eisen, H. N., E. S. Simms, and M. Potter, *Biochemistry*, **7**, 4126 (1968).
4. Thorpe, N. O., and S. J. Singer, *Biochemistry*, **8**, 4523 (1969).
5. Kennel, S. J., and S. J. Singer, *Immunochemistry*, **7**, 235 (1970).
6. Eisen, H. N., and G. W. Siskind, *Biochemistry*, **3**, 996 (1964).
7. Underdown, B. J., E. S. Simms, and H. N. Eisen, *Biochemistry*, in press.
8. Schellman, J. A., *Accounts Chem. Res.*, **1**, 144 (1968).
9. Little, J. R., and H. N. Eisen, *Biochemistry*, **5**, 3385 (1966).
10. Steiner, L. A., and H. N. Eisen, *J. Exp. Med.*, **126**, 1185 (1967).
11. Little, J. R., and H. N. Eisen, *Biochemistry*, **7**, 711 (1968).
12. Little, J. R., and H. N. Eisen, *J. Exp. Med.*, **129**, 247 (1969).
13. Eisen, H. N., J. R. Little, L. A. Steiner, E. A. Simms, and W. Gray, *Is. J. Med. Sci.*, **5**, 338 (1969).
14. Rockey, J. M., K. J. Dorrington, and P. C. Montgomery, *Nature*, **232**, 192 (1971).