# EXTRINSIC COTTON EFFECTS ASSOCIATED WITH AFFINITY LABELED MOPC 315 MYELOMA PROTEIN

G.W. Litman and R.A. Good

Departments of Pathology, Pediatrics, and Microbiology
University of Minnesota, Minneapolis, Minnesota 55455

Received March 9, 1972

SUMMARY. Affinity labeling of MOPC 315 myeloma protein with m-nitro benzene diazonium fluoroborate (MNBDF) resulted in a significant decrease in association constant and number of binding sites expressed for nitrophenol ligands. An absorption spectrum indicative of exclusive formation of diazo tyrosine was observed with over 90% of the affinity label being present on the light chains. Protection of the active site with excess ε-DNP-L-lysine-HCl prevented incorporation of the affinity label. Circular dichroic examination of the labeled protein in the 300nm-600nm spectral region revealed three extrinsic cotton effects to accompany the modification by MNBDF. Denaturation of the modified protein by guanidine-HCl resulted in disappearence of the three CD bands. The usefulness of diazo probes in antibody active site exploration is discussed.

Affinity labeling of immunoglobulins and enzymes has provided considerable information concerning the location and nature of active site amino acid residues (1). Although the identification and location of amino acids in the active site is of fundamental importance, the method, until now, has provided us with little information about the chemical microenvironment of combining site regions. Recently, extrinsic cotton effects have been shown to accompany the covalent modification of several groups of enzymes and plasma proteins by diazonium compounds (2, 3). These extrinsic cotton effects served as accurate indicators of the three dimensional structure in the vicinity of the modified residues. As antibodies have been shown to undergo affinity labeling in their active site(s) with hapten analogs containing reactive diazonium groups, extrinsic cotton effects

could serve as accurate reporters of topographical relationships within the combining site region. To test the possibility of obtaining such an optical probe of the active site region, we used affinity labeling of the highly characterized, homogeneous "antibody-like" protein produced by the number 315 mineral oil induced plasmacytoma (MOPC 315) (4) (5). The results indicate that a distinctive series of extrinsic cotton effects were generated following the formation of a diazo linkage between m-nitro benzene diazonium fluoroborate and a light chain tyrosine residue in the active site of the MOPC 315 protein.

MATERIALS AND METHODS. MOPC 315 was obtained from the ascites fluid of Balb/c mice injected i.p. with homogenized solid tumor cells or tumor cells recovered from the ascites itself. Mice bearing the MOPC 315 tumor, generation 76b, were a gift of Dr. Michael Potter, National Cancer Institute. The ascites fluid from mice bearing the MOPC 315 tumor was partially reduced and alkylated and the myeloma protein was purified by immunoabsorption on columns of Sepharose to which ε-DNP-L-Lysine had been covalently attached (6). The purified myeloma protein was characterized by the methods outlined (6) and found to be homogeneous. Separation of the purified protein into heavy and light chains was effected utilizing the same techniques outlined in (6). m-Nitro benzene diazonium fluoroborate (MNBDF) and N-chloroacetyl-3-(m-nitrophen-ylazo) tyrosine were synthesized as described (7). Affinity labeling with MNBDF was performed at the same ratio of MNBDF/protein and under the same solvent conditions as previously reported (4). "Protection" of the active site against reaction with MNBDF was accomplished by use of ε-DNP-L-Lysine at a ratio of hapten/active site of 250/1. Equilibrium dialysis was performed in the presence of dextran by a standard procedure using [$H^3$] -ε -DNP-L-lysine (New England Nuclear, Waltham Massachusetts). Protein concentrations were estimated by UV absorption at 280nm employing an $E^{1\%}_{1\,cm}$ =14.4 (5). The molecular weight of the MOPC 315 protein was taken to be 150,000 (5). Measurements of circular dichroism were performed with a Durrum-Jasco CD/SP-5

circular dichrometer, thermostatically maintained at 25°C. Solution path lengths varied from .5 cm - 2.5 cm. UV and visible spectra were determined with a Cary 16 recording spectrophotometer.

RESULTS. Employing the solvent and reagent conditions of Potter and Metzger (4), affinity labeling of the MOPC 315 protein was achieved essentially as described. When ε-DNP-L-lysine was used to "protect" the active site of the MOPC 315 protein only a minute formation of azotyrosine occurred. After three hours of reaction, "unprotected" samples analyzed in .15N NaOH exhibited a spectrum in the 300-600 nm region consistent with the exclusive formation of azotyrosine (Fig. 1). The conversion of tyrosine to azotyrosine after three hours was calculated to be 6.75 X $10^6$ mole/liter/optical density unit of protein at 280 nm. Modification of the MOPC 315 protein was accompanied by a 60fold reduction in $K_{ASSN.}$ for $[H^3]$ ε-DNP-L-lysine, and a reduction in number of combining sites/molecule from 1.9 to 1.6 (5,6). Dissociation of the modified

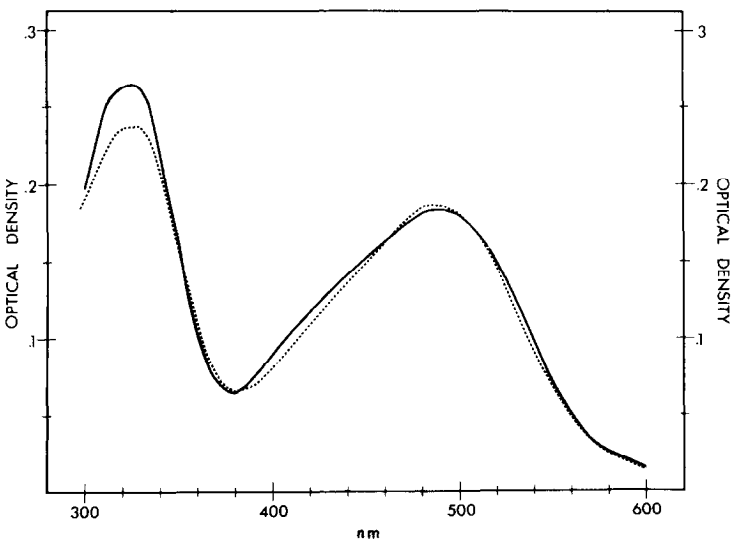


Fig. 1. Absorption spectra of MNBDF modified MOPC 315 myeloma protein (_____) and model compound N-Chloroacetyl-3-(m-nitrophenylazo) tyrosine (---------) determined in .15 N NaOH. Protein concentration is 2.1 mg/ml.

protein into heavy and light chains revealed that over 90% of the affinity label was localized on the light chain. Preliminary analysis of trypsin digests of the modified light chains indicated that a single peptide contained the bulk of the recovered diazonium compound (azo-tyrosine). The size, electrophoretic behavior and amino acid composition of the peptide showed it to be similar to the peptide Goetzl and Metzger reported as being the primary reactant with MNBDF (6). It is unlikely that other residues, e.g. lysine,

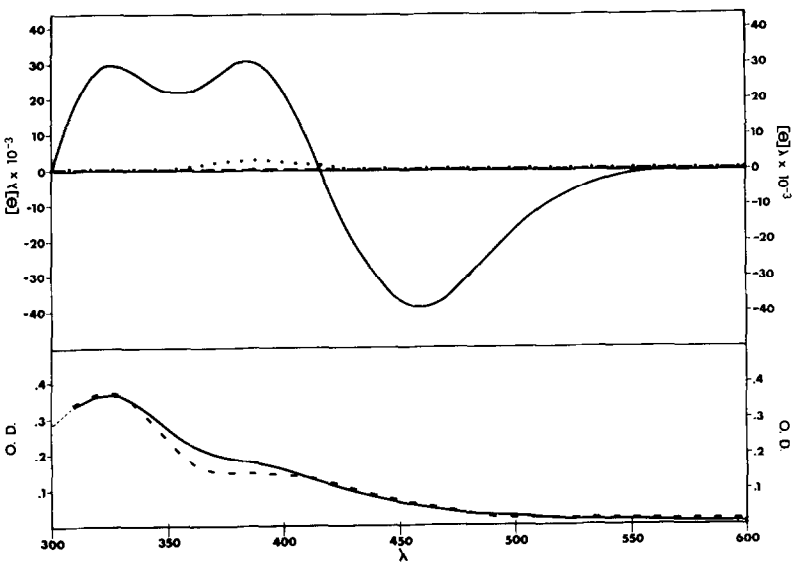


Fig. 2. (TOP) Circular dichroism spectra of MOPC 315 myeloma protein affinity labeled with MNBDF determined in .05 M Tris-HCl, pH 7.5 (_____) unmodified MOPC 315 myeloma protein in .05 M Tris-HCl (.....) and affinity labeled MOPC 315 myeloma protein in 6 M Guanidine-HCl, .05M Tris-HCl (--------) Calculation of $[\theta]_\lambda$ is according to reference (11). Protein concentration is is 2.8 mg/ml in all of the above.

(BOTTOM) Absorption spectra of affinity labeled MOPC 315 protein in .05 M Tris-HCl, pH 7.5 (_____) and in 6M Guanidine - HCl, .05 M Tris-HCl, pH 7.5 (-------). Protein concentration is 2.0 mg/ml in both of the above.

histidine reacted with the diazonium affinity labeling reagent, as no change in the content of these residues was observed between modified and unmodified MOPC 315 preparations.

Figure 2 illustrates the absorption and CD spectrum at pH 7.5 of the affinity labeled MOPC 315 protein in the range 300-600 nm. The shoulder present at 380 nm seems to be characteristic of all diazonium modified proteins (2). The CD spectrum of the modified protein, however, proved unique with respect to previously published spectra of diazo modified proteins with three ellipticity bands $\lambda$ max=325(+), $\lambda$ max=380(+), and $\lambda$max=460(-) being displayed. Unmodified MOPC 315 protein was largely without optical activity in this spectral region. Similarly, the model compound, N-chloroacetyl-(m-nitrophenyl azo) tyrosine was without optical activity between 300-600nm. When the CD spectrum of the affinity labeled MOPC 315 protein was examined in the presence of 6M guanidine-HCl at pH 7.5, the three extrinsic cotton effects were no longer visible.

DISCUSSION. Affinity labeling techniques have provided valuable information about the chemical nature of antibody and enzyme active sites. One of the major limitations of these techniques, however, has been the somewhat one dimensional nature of the information obtained; i.e., location and identification of the reactive residue(s) within the protein's primary structure. If the affinity labeling approach, with its inherent site specific nature, could be utilized to reflect the chemical microenvironment of the reacted residues, it would be capable of providing far greater information about the active site region than other, less direct, methods of chemical modification. Preliminary analyses of both specifically and nonspecifically diazotized enzymes and serum proteins have emphasized the usefulness of the diazo probe in examination of certain structure-function relationships (8,9).

The active sites of antibodies seemed ideal candidates for topographical exploration by diazo probes. Although the MOPC 315 myeloma protein can not be considered to be an antibody in the classical sense of the term, it

certainly represents a homogeneous IgA immunoglobulin with a rather high ($K_{ASSN.}$ $I \sim X\ 10^{-7}\ M^{-1}$) affinity for dinitrophenyl ligands. Extensive studies of this protein by Goetzl and Metzger have provided complete kinetic analysis of the affinity labeling process and the identification and localization of the modified residue within the primary structure of the protein (6, 10). A single residue, tyrosine 34, located in the variable region of the light chain, was identified as the primary reactant with the MNBDF affinity labeling reagent. Although we have not yet definitively confirmed this location for the reactive tyrosine residue, the virtually identical procedures used, and nearly identical labeling results obtained, are highly suggestive that the reactive tyrosine is the same as had been described previously.

When the absorption spectrum of the affinity labeled MOPC 315 myeloma protein was examined at pH 7.5, a spectrum was observed which was typical of various diazo modified enzymes and plasma proteins. The inability to adequately resolve the spectrum into discreet contributions has been encountered with other diazo modified proteins, and is, partially due to rather broad overlapping of the component chromophoric contributions. Unlike the absorption spectrum, the CD spectrum of the MOPC 315 protein was capable of being resolved into at least three major contributions. The three cotton effects differed in sign, position and ellipticity maxima and minima from other modified proteins reported to date. That the interaction with 6M Guanidine-HCl largely abolished the three CD bands associated with the modified 315 protein emphasized the necessity for three-dimensional structural integrity in definition of the appropriate environment for generation of these extrinsic cotton effects.

Diazo probes promise to provide sensitive indicators of the physico-chemical environment in their immediate region of attachment. Furthermore, they promise to be accurate, covalently attached reporters of perturbations of this environment by second order influences such as temperature, pH, solvent, etc. Such influences would tend to dissociate non-covalent antibody-hapten complexes, another source of extrinsic cotton effects, (11, 12,) rendering their analysis difficult.

We are indebted to Professor D.B. Wetlaufer for the use of the circular dichrometer and to Miss C.L. Finstad for performing the equilibrium dialysis experiments. This work was supported in part by USPHS grants AI-00292 and AI-08677, The National Foundation-March of Dimes, The Graduate School, University of Minnesota and an institutional grant from the American Cancer Society.

REFERENCES

1. Fenton, J.W. and Singer, S.J. (1971). Biochem. 10, 1429.
2. Kagan, H.M. and Vallee, B.L. (1969). Biochem. 8, 4223.
3. Fairclough, G.F. and Vallee, B.L. (1970). Biochem. 9, 4081.
4. Metzger, H. and Potter, M. (1968). Science 162, 1398.
5. Underdown, B.J., Simms, E.S. and Eisen, H.N. (1971). Biochem. 10, 4359.
6. Goetzl, E.J. and Metzger, H. (1970). Biochem. 9, 1267.
7. Traylor, P.S., and Singer, S.J. (1967). Biochem. 6, 881.
8. Fairclough, G.F. and Vallee, B.L. (1971). Biochem. 10, 2470.
9. Johansen, J.T. and Vallee, B.L. (1971). Proc. Nat. Acad. Aci. 68, 2532.
10. Goetzl, E.J. and Metzger, H. (1970). Biochem. 9, 3862.
11. Rockey, J.M., Dorrington, K.J. and Montgomery, P.C. (1971). Nature 232, 192.
12. Glaser, M. and Singer, S.J. (1971). Proc. Nat. Acad. Sci. 68, 2477.