AFFINITY LABELING OF ANTIBODIES TO THE p-AZOPHENYLTRIMETHYLAMMONIUM HAPTEN AND A STRUCTURAL RELATIONSHIP AMONG ANTIBODY ACTIVE SITES OF DIFFERENT SPECIFICITIES

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In studies from this laboratory (Wofsy, et al., 1962; Metzger, et al., 1963; Metzger, et al., 1964; Singer, et al., 1965) the method of affinity labeling of the active sites of protein molecules was proposed and investigated experimentally. In this method, a labeling agent is utilized which 1) combines specifically and reversibly with the particular active site; and 2) reacts with one or more amino acid residues in the site to form irreversible covalent linkages. Two anti-hapten antibody (Ab) systems, anti-p-azobenzenearsonate (anti-R) and anti-2,4 dinitrophenyllysyl (anti-DNP), have previously been investigated. To complement these studies with Ab to the negatively charged R and the neutral DNP haptenic determinants, we have now investigated Ab to the positively charged p-azophenyltrimethylammonium (TMA) determinant. The labeling reagent used with the anti-TMA Ab was p-(trimethylammonium)benzenediazonium difluoborate (TDF). As in our previous studies, the diazonium group was the reactive functional group of the labeling reagent, and the formation of covalent and irreversible azo-linkages between the reagent and the Ab was followed spectrophotometrically. The results obtained with all three systems are so closely similar as to suggest some significant generalizations about the composition and structure of Ab active sites.

METHODS

Labeling Reagents and Model Compounds: TDF was made from the corresponding amine, p-aminophenyltrimethylammonium chloride, (Pressman, et al., 1946) by

methods similar to those employed in our earlier studies (Wofsy, et al., 1962). Model azoderivatives were prepared for spectral analysis by reacting TDF to completion in an excess of N-chloracetyl-L-tyrosine or N-acetyl-L-histidine, as in the studies of Tabachnick and Sobotka (1959). P-(arsonic acid)benzenediazonium fluoborate (RDF) was made as previously described (Wofsy, et al., 1962).

Antigens and Antibodies: The immunizing antigen was made by coupling TDF to Keyhole Limpet hemocyanin. The precipitating antigen was prepared by first exhaustively succinylating (Habeeb, et al., 1958; Cherry, 1964) bovine serum albumin with succinic anhydride to increase its net negative charge and its susceptibility to azo-modification, and then reacting it with TDF.

Anti-TMA sera were raised in rabbits by injecting the immunizing antigen in Freund's complete adjuvant into the foot pads, and following this by subsequent intravenous booster injections. The anti-TMA Ab were isolated in a pure state as follows. High titer (> 0.5 mg anti-TMA Ab/ml) sera were pooled; the anti-TMA Ab was specifically precipitated at the equivalence point by the precipitating antigen; after the precipitate was washed, it was dissociated in 0.1 M trimethylphenylammonium chloride in 0.02 M phosphate buffer, pH 7, and the solution was then passed through a DEAE-Sephadex column. The precipitating antigen was firmly bound to the column while the anti-TMA Ab was eluted. The Ab solution was then exhaustively dialyzed. The purified anti-TMA Ab was 60-95% specifically precipitable. Purified anti-R Ab has been previously described (Wofsy, et al., 1962).

Affinity Labeling: Ab solutions at a concentration of about 1.25×10^{-5} M were reacted with 1.5 moles TDF or RDF per mole of Ab. With the former reagent, the reactions were carried out in 0.10 M phosphate buffer, pH 6.0 for 1 hour at 0°C; with the latter, 0.12 M NaCl - 0.17 M borate, pH 8.0, was used under the same conditions. The labeled Ab samples which yielded the data in Table I were denatured in 75% ethanol, were thoroughly washed, were then dissolved in 0.10 M NaOH and the spectra obtained, all essentially as previously described (Wofsy, et al., 1962). For the polypeptide chain

TABLE I. Extinction Coefficients[1] of Anti-TMA and Anti-R Antibodies Reacted with TDF and RDF.

Antibody	Reagent	
	TDF	RDF
Anti-TMA	7.19	1.47
Anti-R	1.59	5.75

[1] Absorption X 10^{-3} per mole of antibody, measured at 490 mμ in 0.1 M NaOH.

separation experiments of figures 1 and 2, the anti-TMA Ab after labeling was dialyzed against an acetate buffer at pH 4.0 to quench the reaction, and then against H_2O, and was then lyophilized. The sample was subsequently reduced with mercaptoethanol, alkylated with iodoacetamide, and the H and L chains fractionated in 1 M propionic acid on G-75 Sephadex according to Fleischman, et al. (1962).

RESULTS

TDF reacts much more extensively with homologous anti-TMA than with anti-R Ab under the same conditions, as shown by the much larger azoderivative absorption of the modified anti-TMA Ab (Table I). Conversely, RDF selectively reacts with anti-R as compared to anti-TMA Ab, as is consistent with previous results (Wofsy, et al., 1962). Separation of the H and L chains of this labeled Ab after reduction and alkylation yields the data of figure 1. The label is present on both chains. The ratio of protein in the H and L fractions (from $A_{280\ m\mu}$) is 72:28, while the ratio of azotyrosine label (from $A_{488\ m\mu}$ in 0.10 N NaOH) is 65:35. The azoderivative spectra of

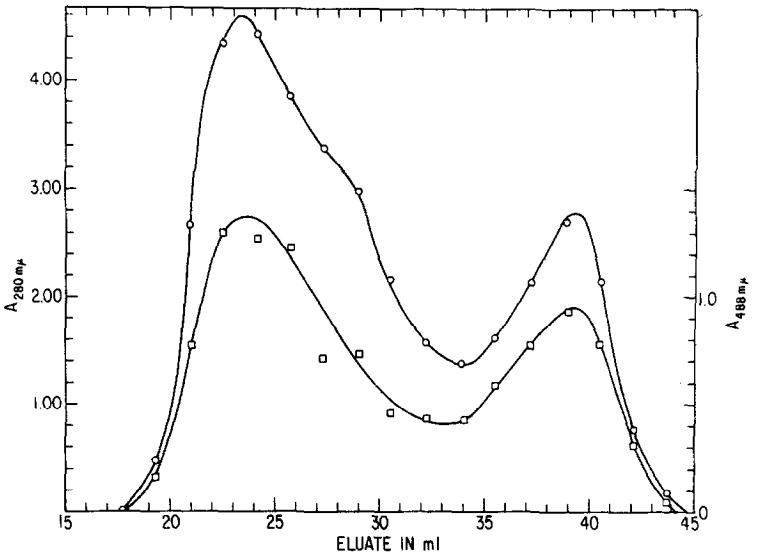

Figure 1. H and L chain fractionation in 1 M propionic acid on G-75 Sephadex of TDF-labeled anti-TMA Ab. The H chain fraction is eluted first. $A_{280m\mu}$ (upper curve) gives the protein concentration of the fraction, $A_{488m\mu}$ (lower curve) the azoderivative concentration.

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the two isolated labeled chain fractions are closely characteristic of azotyrosine, as is demonstrated by comparison with the spectra of model azocompounds (figure 2).

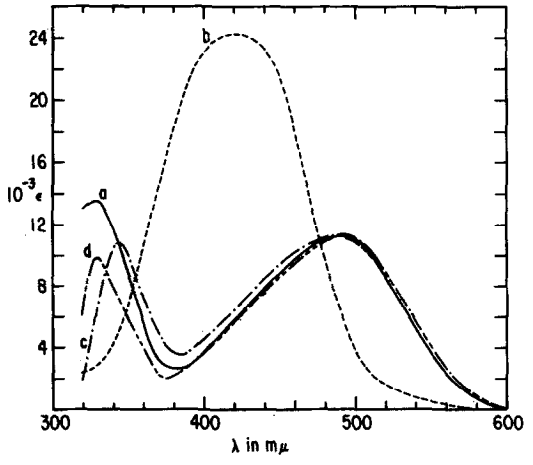

Figure 2. Spectra in 0.1 M NaOH of: a) p-(azophenyltrimethylammonium)-N-chloracetyl-L-tyrosine; b) p-(azophenyltrimethylammonium)-N-acetyl-L-histidine; c) H chain fraction, and d) L chain fraction, of TDF-labeled anti-TMA Ab. The values of ε refer to curves a) and b). Curves c) and d) have been adjusted to the same scale. For the H chain fraction, $A_{490m\mu}$ = 0.087 at a concentration of 1.76 mg protein/ml, and for the L, $A_{490m\mu}$ = 0.137 at 3.15 mg protein/ml.

DISCUSSION

The results prove that the affinity labeling of anti-TMA Ab with TDF is as specific as the labeling of anti-R Ab with RDF. With three different anti-hapten Ab systems: anti-R Ab labeled with RDF (Wofsy, et al., 1962); anti-DNP Ab labeled with p-nitrobenzenediazonium fluoborate (PNBDF) (Metzger, et al., 1963), or m-nitrobenzenediazonium fluoborate (MNBDF) (Singer, et al., 1965); and now anti-TMA Ab labeled with TDF, remarkably similar results have been obtained. In each case, not only are specific azotyrosine linkages formed (whereas with normal γ-globulin, non-specific modification by these diazonium reagents generally produces azohistidine, and perhaps azolysine, as well as azotyrosine linkages (Tabachnick and Sobotka (1959)), but this reaction occurs at greatly accelerated rates. Furthermore the azotyrosine label appears on both H and L chains. By many stringent criteria the H and L chain tyrosine residues that become labeled in anti-R and anti-DNP Ab have been shown to be present in the Ab active sites; this has been discussed elsewhere (Metzger, et al., 1964). The correspondence of the results with TDF and anti-TMA Ab leads to the same conclusion for this system. In all three cases, therefore, both H and L chains participate in the formation of the active sites of Ab by contributing tyrosine, and presumably other, residues to them.

Beyond this, however, it is especially remarkable that the ratio of label (approximately 2:1 on a molar basis) on the H and L chains is so nearly constant, independent of Ab specificity. If two tyrosine residues were randomly positioned in different Ab active sites, they should show wide variations in the relative rates with which they react with an affinity labeling reagent (Wofsy, et al., 1962). This is because of restrictions placed on the equilibrium position of the labeling reagent within an active site. We infer from the constancy of our results, therefore, that the particular H and L chain tyrosines that become labeled are in relatively fixed positions and orientations in the active sites of antibodies of

different specificities. In this connection, the possible analogy with the esterase enzymes is of interest. Although different esterases have different substrate specificities, in their active sites they have identical, or closely similar, tetrapeptide sequences including the critical serine residue (cf. Cohen, et al., 1959). Presumably, elsewhere in the active sites amino acid differences occur which are related to their different substrate specificities. In a similar manner we suggest that there are regions in the active sites of antibody molecules, including the tyrosine residues which become labeled, which are essentially invariant from one antibody to another, while there may be other regions in the sites which change with the specificity of the antibody. Amino acid sequence studies now in progress with affinity-labeled antibodies should help to illuminate these problems.

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