AFFINITY LABELING OF ANTI-DINITROPHENYL ANTIBODIES WITH BROMOACETYL DERIVATIVES OF HOMOLOGOUS HAPTENS

Y. Weinstein, M. Wilchek and D. Givol
Departments of Chemical Immunology and Biophysics, The Weizmann Institute of Science, Rehovot, Israel

Received April 30, 1969

Affinity labeling (1) was used extensively by Singer and his colleagues to study the combining sites of antibodies (2). In these studies diazonium affinity labeling reagents were used and in most cases the reagent formed an azo linkage to tyrosine residues at the combining site (2). In an attempt to develop affinity labeling reagents with a reactive group of different chemical specificity we prepared the bromoacetyl derivatives of some dinitrophenyl (DNP) haptens and tested their specific covalent binding to anti-DNP antibodies. We report here the affinity labeling of anti-DNP antibodies by α-N-bromoacetyl, ε,N-DNP-L-lysine and by N-bromoacetyl, N^1-DNP-ethylene diamine. Both reagents are very specific but differ in their efficiency of labeling. More than 95% of the labeling was due to O-alkylation of tyrosine residue in the antibody combining site and after acid hydrolysis O-carboxymethyltyrosine was identified as the labeled residue.

MATERIALS AND METHODS

Rabbit anti-DNP antibodies were isolated from a pooled rabbit serum using a trinitrophenyl-bovine gamma globulin-bromoacetyl cellulose immunoadsorbent as described by Jaton et al. (3), except that the elution of antibodies from the immunoadsorbent was done with 0.1 M acetic acid, pH 2.8. Non immune rabbit IgG was prepared from rabbit serum by chromatography on DEAE-cellulose.

α,N-Bromoacetyl, ε,N-DNP-L-lysine (BADL) was preapred by reacting ε-DNP-lysine hydrochloride (350 mg; 1 mmole) with bromoacetyl succinimide ester (590 mg; 2.5 mmoles) in 40 ml of 75% dioxane, containing 1.5 g of $NaHCO_3$. After 1 hour the solution was extracted with ethyl acetate and was

acidified by the addition of HCl. The product was extracted into ethyl acetate and after removal of the ethyl acetate by rotary evaporation it was crystallized from acetone-water, m.p. 128°. Analysis: Found: C, 38.65; H, 4.06; N, 12.85; Br, 1838. Calc. C, 38.90; H, 3.73; N, 12.96; Br, 18.49. The preparation of N-bromoacetyl, N'-DNP-ethylene diamine (BADE) was performed by reacting mono-DNP-ethylene diamine with bromoacetyl succinimide ester and triethylamine in ethyl acetate in a 1:1:1 molar ratio of reactants. After 1 hour of stirring the unreated DNP-ethylene diamine was extracted with 1 N HCl and the reaction mixture was concentrated by rotary evaporation. The product was crystallized by the addition of ether to the concentrated oily solution, m.p. 138°.

For the preparation of radioactive BADL and BADE the procedure was scaled down 10-fold using 1, ^{14}C-bromoacetic acid (The Radiochemical Center, Amersham). The radioactive BADL and BADE were found by chromatography in butanol: acetic acid: water (4:1:4) to be free of either ^{14}C-bromoacetic acid or of the hapten. The specific radioactivity was 1000 cpm and 890 cpm per mμmole of BADL and BADE, respectively.

The assay for covalent binding of the affinity labeling reagents was performed as follows: the protein (150 μg/ml) was incubated with either BADL or BADE (2 moles/mole of protein) in 0.1 M $NaHCO_3$, pH 9.0, at 37°. At various times a sample of 1 ml was taken and excess ε-DNP-aminocaproic acid (250 moles/mole of protein) was added to compete with the non covalently bound reagent. After 10 more minutes 0.2 ml of 100% trichloroacetic acid (TCA) was added and the protein was allowed to precipitate for 10 minutes at 0°. The suspension was transferred on a 27 mm selectron filter BA 85/0 (Schleicher and Schull), followed by 4 x 5 ml washes with 5% TCA. The filter was dried under an infra red lamp and was transferred to a toluene scintillation liquid in a counting vial. Radioactivity was measured in a Packard Tri-carb liquid scintillation spectrophotometer. Equilibrium dialysis was performed according to Eisen (4), using α,N-[^{3}H]-acetyl ε,N-DNP lysine (3) as hapten. Acid hydrolysates of labeled antibodies were analysed on the long column of the amino acid analyzer to which a Packard Tri-Carb scintillation flow-cell was attached. The following markers were prepared: ε,N-carboxymethyl lysine and O-carboxymethyl tyrosine by reacting polylysine and polytyrosine with ^{14}C-iodoacetic acid at pH 10.5 for 16 hours, followed by dialysis and hydrolysis in 6 N HCl; carboxymethyl derivatives of histidine were prepared as described by Crestfield et al. (5).

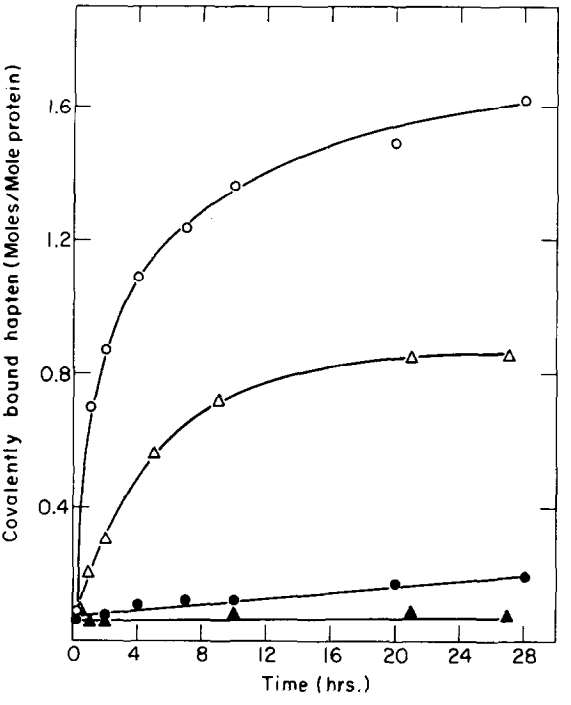

Fig. 1: A comparison of the rate of reaction of BADL and BADE with anti-DNP antibodies and with normal IgG. The molar ratio of reagent to protein was 2:1. ○, BADE with antibody; ● , BADE with IgG; △ , BADL with antibody; ▲ BADL with IgG. For conditions and assay see Materials and Methods.

RESULTS

The kinetics and specificity of the covalent binding of BADL and BADE to anti-DNP antibodies is depicted in Fig. 1. It is shown that practically no labeling occurred with normal IgG, whereas with anti-DNP antibodies 0.9 and 1.6 sites per antibody molecules are labeled by BADL and BADE respectively. This difference might be due to the difference in size of the two reagents (BADE is about 14 Å long and BADL is about 19 Å long), and probably reflects the heterogeneity in size of the combining sites of antibodies. The number of covalently bound hapten molecules did not increase even if the ratio of reagent to antibody was increased 10-fold. Figure 2 shows the pH dependence for the covalent binding of the two reagents. In view of these results, all the experiments reported here were carried out at pH 9.0.

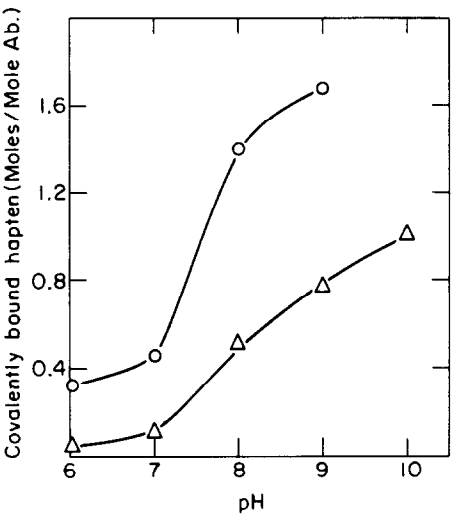

Fig. 2: pH Dependence of covalent binding of BADL and BADE to anti-DNP antibodies. The molar ratio of reagent to antibody was 2:1 and the incubation time 24 hours. ○ , BADE; △ , BADL.

TABLE I: The inactivation of anti-DNP antibodies by affinity labeling. The antibodies were incubated with different amounts of reagents at pH 9.0 for 24 hours and then subjected to equilibrium dialysis against tritiated α,N-acetyl, ϵ, N-DNP-lysine. The dialysis bag contained antibodies (2.5×10^{-7} M) and non-radioactive reagent (1.25×10^{-7} M; 2.5×10^{-7} M and 5×10^{-7} M, according to the amount used in the incubation period). The outside solutions contained radioactive hapten ranging from 2×10^{-7} M to 3×10^{-6} M. The number of binding sites determined for the intact antibodies was 1.47 and the number of residual sites in each of the treated antibodies was calculated in relation to this.

Reagent	Mole reagent/mole Ab	Moles of Ab sites lost %
-	-	0
BADL	0.5	35
"	1.0	67
"	2.0	70
BADE	0.5	35
"	1.0	53
"	2.0	74
ϵ-DNP lysine	0.5	16
" "	1.0	14
" "	2.0	20

In oder to establish that the labeling is in the antibody combining site it is necessary to demonstrate loss of antibody activity in addition to specific labeling. Anti-DNP antibodies (150 μg/ml in 0.02 M $NaHCO_3$, pH 9.0) were reacted with radioactive BADL, BADE or ε-DNP-lysine in different molar ratios. After 24 hours at 37° the solutions were diluted four-fold with 0.1 M phosphate buffer, pH 7.0, and the number of residual antibody sites was determined by equilibrium dialysis against α,N-[^{3}H]-acetyl ε,N-DNP lysine at five different concentrations of radioactive hapten. Table I shows that the number of antibody sites by the reaction with affinity labeling reagents increases with the amount of reagent used, reaching 70% loss of sites when 2 moles/mole antibody were used. On the other hand, the number of sites lost due to reaction with ε-DNP-lysine is small and due only to competition between the radioactive and non-radioactive haptens.

If the specific labeling is indeed in the combining site of the antibody, there should be protection by hapten against labeling with the reagent. As is shown in Table II, ε-DNP-lysine inhibits the covalent binding of BADL and BADE to antibodies and the protection is proportional to the amount of hapten used. In order to determine the labeled residue in the antibody, the labeled antibody was

TABLE II: Protection by hapten against affinity labeling. Antibodies were incubated with ε,N-DNP lysine for 1 hour, and then affinity labeling reagent (2 moles/mole of antibody) was added. The amount of covalently bound hapten was determined after 24 hours.

ε-DNP-lysine moles/moles Ab	BADL		BADE	
	Bound	Inhibition	Bound	Inhibition
	mole/mole Ab	%	mole/mole Ab	%
0	0.76	-	1.6	-
2	0.64	16	1.25	22
10	0.36	53	0.84	48
20	0.24	68	0.62	61

precipitated with trichloroacetic acid, and the precipitate was washed with acetone, dried and hydrolysed with 6 N HCl. The analysis of such hydrolysate on the long column of the amino acid analyser shows that in both cases over 95% of the labeled residue is O-carboxymethyl tyrosine (Fig. 3).

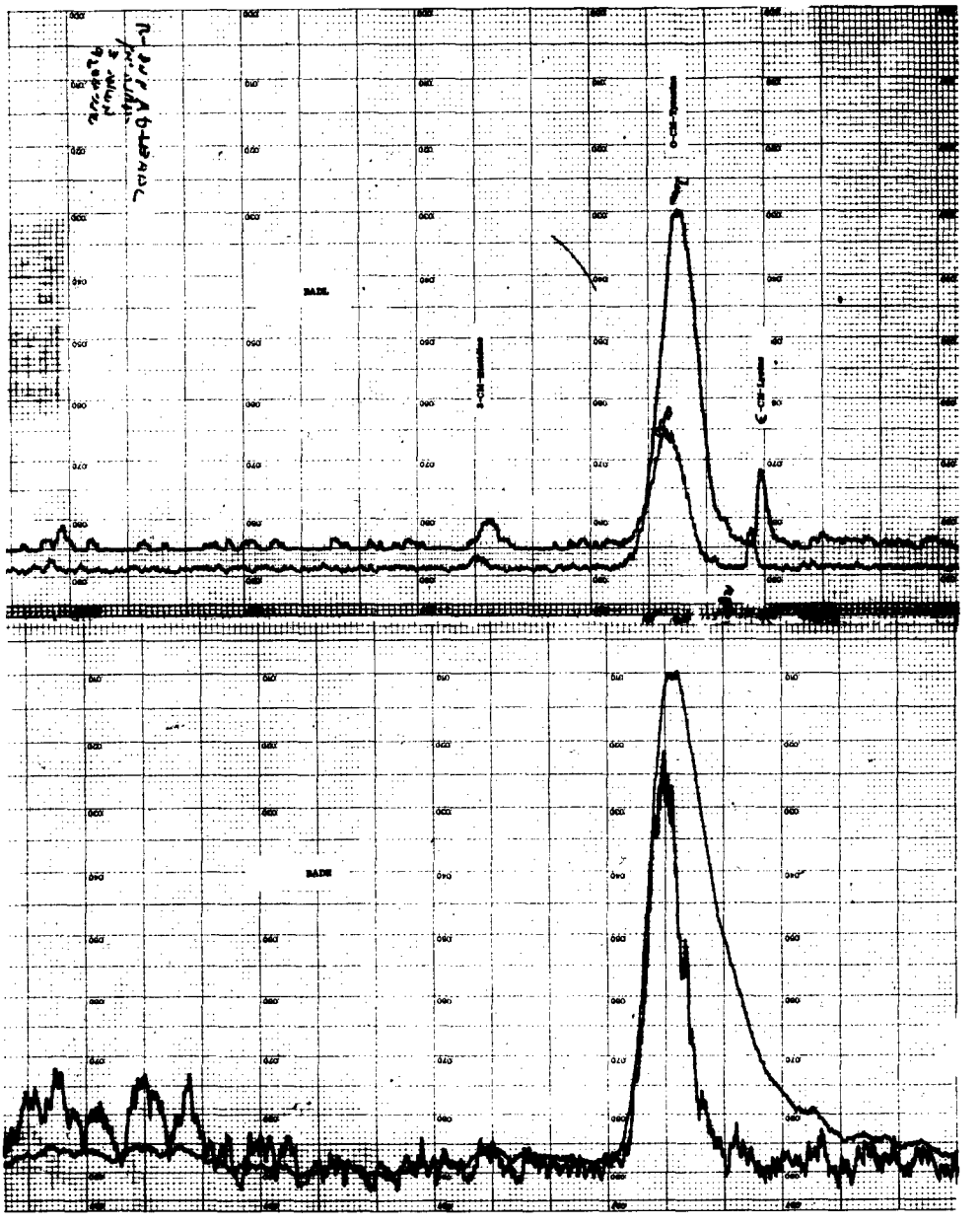

Fig. 3: Analysis of the radioactive residues in labeled anti-DNP antibodies. The labeled protein (1 mg) was precipitated with trichloroacetic acid, washed with acetone, dried and hydrolysed with 6 N HCl. The hydrolysate, containing between 6000 and 10,000 cpm, was applied to the long column of the amino acid analyzer and the radioactivity was detected in a Packard scintillation flow cell. Top - antibody labeled with BADL. Bottom - antibody labeled with BADE.

DISCUSSION

The carboxymethylation of the side chains of cysteine, histidine, methionine and lysine in proteins is readily effected by bromoacetate or other α-halo acids (6). On the other hand, modification of the phenolic group of tyrosine by bromoacetate is much slower and incomplete (7). However by affinity labeling reagents tyrosine at the active site can be alkylated in high yields as was demonstrated by Plummer and Lawson on carboxypeptidase B (8) and by Cuatrecasas et al. (9) on Staphylococcus nuclease. The results reported here demonstrated that in anti-DNP antibodies the residue modified by BADL or BADE is tyrosine, yielding after acid hydrolysis O-carboxymethyl tyrosine. The high yield of this modification (1.6 residues per divalent antibody in the case of BADE) indicates that if the labeling reagent is of the correct size, the tyrosine in the combining site is quantitatively modified under relatively mild conditions. Preliminary work on anti-DNP antibodies labeled with BADL showed that the label is distributed between the heavy chain and the light chain in a ratio similar to that reported by Singer et al. for the labeling of anti-DNP antibodies with diazonium reagent (2). It is thus possible that in both cases the same tyrosine is labeled by either diazonium or bromoacetyl derivatives of haptens. The reaction with the bromoacetyl derivatives of haptens fulfil the criteria of affinity labeling by being specific to the homologous antibodies, inhibited by the hapten and by causing inactivation of the antibody concomitant with covalent binding.

The stability and the ease of preparing bromoacetyl derivatives of haptens and the facile identification of the labeled residue are important features of this type of affinity labeling reagents.

REFERENCES

1. Wofsy, L., Metzger, H. and Singer, S.J., Biochemistry 1, 1031 (1962).
2. Singer, S.J., Slobin, L.I., Thorpe, N.O. and Fenton, J.W., II, Cold Spring Harbor Symposia XXXII, 99, (1967).
3. Jaton, J.-C., Klinman, N.R., Givol, D. and Sela, M., Biochemistry 7, 4185 (1968).
4. Eisen, H.N., Meth. Med. Res. 10, 115 (1964).

5. Crestfield, A.M., Stein, W.H. and Moore, S., J. Biol. Chem. 238, 2413 (1963).
6. Gurd, F., in Methods in Enzymology XI, 532 (1967).
7. Korman, S. and Clark, T.H., J. Biol. Chem. 221, 133 (1956).
8. Plummer, T.H., and Lawson, W.B., J. Biol. Chem. 241, 1648 (1966).
9. Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B., J. Biol. Chem., in press.