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Studies on the Combining Region of Protein 460, a Mouse γA Immunoglobulin Which Binds Several Haptens. Binding and Reactivity of Two Types of Photoaffinity Labeling Reagents†

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ABSTRACT: Protein 460, a mouse IgA myeloma immunoglobulin which exhibits binding activity toward the 2,4-dinitrophenyl group, was treated with two photoaffinity labeling reagents. These compounds, 2,4-dinitrophenylalanyl diazoketone and 2,4-dinitrophenyl 1-azide, which do not react with protein 460 in the dark but which have relatively high affinities for the protein, can be photolyzed to give reactive intermediates which have the potential of reacting with amino acid residues that contain heteroatoms. Both 2,4-dinitrophenylalanyl diazoketone and 2,4-dinitrophenyl 1-azide competitively inhibit the binding of ε-2,4-dinitrophenyllysine in the dark. After photolysis, the number of moles of diazoketone reagent incorporated was equivalent to the loss of binding sites for ε-2,4-dinitrophenyllysine. Covalent incorporation of the azide reagent also resulted in loss of binding activity toward 2,4-dinitrophenylalanine. The diazoketone reagent attaches predominantly to the light chain, while the azide reagent reacts chiefly with the heavy chain of protein 460.

An important step in understanding antibody specificity is the elucidation of the tertiary structure of a combining site of an immunoglobulin with defined ligand specificity. To complement the recent high-resolution X-ray crystallographic data on homogeneous immunoglobulins with ligand binding activity (Poljak *et al.*, 1972), efforts to identify and locate some of the amino acid residues comprising the antibody combining regions have been attempted with affinity labeling reagents.

Affinity labeling of antibodies was first tried by Wofsy *et al.* in 1962. They employed reagents based on the hapten used to induce the antibody response, which contained reactive groups capable of forming covalent bonds with side chains of amino acid residues. The structural similarity between the reagent and the hapten permits the reagent to be noncovalently bound in the combining region. It has been assumed that when the reagent is bound in the combining site, it reacts

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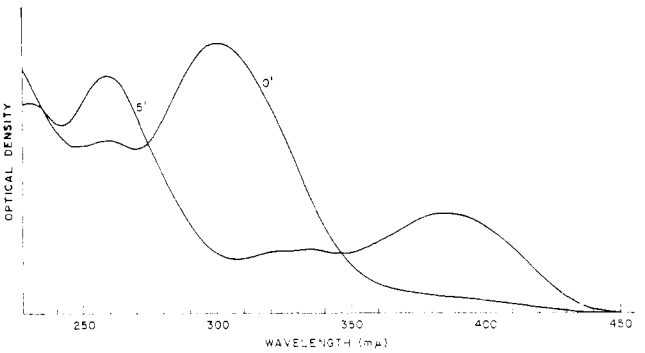

FIGURE 1: Ultraviolet spectra of Dnp-N_3 in ethanol before and after irradiation for 5 min at 300–400 mμ under six General Electric F15T8BL lamps.

preferentially with amino acid residues that are suitably positioned with respect to the reactive group on the reagent.

Affinity reagents with two types of reactive groups, diazonium fluoroborate (Metzger and Potter, 1968; Goetzl and Metzger, 1970; Ray and Cebra, 1972; Martin *et al.*, 1972) and bromoacetyl (Haimovich *et al.*, 1970, 1972; Givol *et al.*, 1971), have been synthesized for the purpose of investigating anti-Dnp[1] myeloma proteins and anti-Dnp antibodies of limited heterogeneity. These reagents have a rather narrow spectrum of reactivity, forming covalent bonds with Tyr, His, Lys, and Cys residues. Photoaffinity reagents which can be activated to give carbenes (Vaughan and Westheimer, 1969; Converse and Richards, 1969) and nitrenes, such as the type designed by Fleet *et al.* (1969) have the potential of inserting into C–O, C–N, C–S, C–H, and N–H bonds of amino acid residues to form covalent linkages between the reagent and the protein. In addition to the broader spectrum of reactivity, the photoaffinity reagents are activated *in situ*. A comparison of the labeling patterns obtained with reagents having different reactive groups should give a more complete picture of the antibody combining site.

This paper describes the synthesis and photolysis of Dnp-N_3. The affinity and reactivity of protein 460 with Dnp-N_3 and Dnp-AD have been investigated. The scope and limitations of affinity labeling are discussed. In an accompanying paper we describe the labeling patterns of the light chains of protein 460 reacted with Dnp-N_3 and Dnp-AD (Hew *et al.*, 1973).

Experimental Section and Results

Synthesis of Tritiated 2,4-Dinitrophenyl 1-Azide ([^{3}H]Dnp-N_3). [^{3}H]Dnp-N_3 was synthesized by direct displacement of the fluorine atom from 1-fluoro-2,4-dinitrobenzene (N_2ph-F) according to the general method of Grieco and Mason (1967). [^{3}H]N_2ph-F (New England Nuclear Corp., NET-363) (0.00772 mg, 1 mCi) was diluted with 14.7 mg of N_2phF and dissolved in 0.25 ml of dimethylformamide. Sodium azide (10 mg) was added and the mixture was incubated with stirring in the dark at room temperature for 2 hr. The mixture was dried *in vacuo* and purified by one-dimensional thin-layer chromatography with chloroform on polyamide plates (Chen-Ching Trading Co., Ltd., Taiwan). Before application of the sample, the polyamide plates were prechromatographed with chloroform. After application of the sample, the plate was exposed to ammonia gas for 5 sec to convert unreacted N_2phF to 2,4-dinitroaniline. [^{3}H]Dnp-N_3 runs with the solvent front while the R_F of dinitroaniline is 0.5. The product is eluted from the chromatogram with chloroform and stored either as a dried powder or in ethanol solution at −10°. [^{3}H]Dnp-N_3 prepared in this manner had a specific radioactivity of 1×10^{13} cpm/mol. The melting point of 65° agreed with that of a previously reported synthesis by a different route (Noelting and Michez, 1893). High-resolution mass spectroscopy gave a high-intensity M − N_2 ion peak at *m/e* 181.01252 corresponding to the composition $C_6H_3N_3O_4$ (calcd 181.01236). The infrared spectrum of synthesized [^{3}H]-Dnp-N_3 gave an N_3 stretching band at 2145 cm^{-1} indicating that the azide, rather than the corresponding 4-nitrobenzo-1,2-furazan *n*-oxide, was present. The nuclear magnetic resonance (nmr) spectrum in $CDCl_3$ showed two doublets at 7.55 and 8.47 ppm. There was also a singlet at 8.79 ppm. These data are consistent with the structure of 2,4-dinitrophenyl 1-azide.

Photolysis of [^{3}H]Dnp-N_3. Although little is known about the reactivity of [^{3}H]Dnp-N_3 with proteins, it has been shown that aryl azides decompose to nitrenes on photolysis (Knowles, 1972) and such nitrenes may be expected to insert into C—S, C—H, C=O, CH, or N—H bonds in a manner analogous to that of carbenes. Nitrenes may also undergo concerted cyclization and loss of nitrogen to the 4-nitrobenzo-1,2-furazan *n*-oxide (Berry, 1970).

Figure 1 shows the absorption spectrum of [^{3}H]Dnp-N_3 in ethanol. The extinction coefficient at 302 mμ was 11,500. An identical spectrum was obtained in 0.2 M Tris-HCl–0.075 M NaCl (pH 8.0) buffer. Irradiation at 300–500 mμ for 5 min causes the spectral changes shown in Figure 1. Further irradiation results in no further spectral changes. When kept in the absence of light, [^{3}H]Dnp-N_3 dissolved in 0.2 M Tris-HCl–0.075 M NaCl buffer is stable as judged by the lack of spectral changes.

Purification of Protein 460. Protein 460 was purified as the 7S monomer from the serum and ascitic fluid of mice carrying the MOPC 460 plasmacytoma using an ε-Dnp-Lys immunoabsorbent column (Goetzl and Metzger, 1970). The purified protein shows a single band in analytical acrylamide disc gel electrophoresis in Tris-glycine buffer at pH 8.6. Quantitative dansyl amino acid analysis (Varga and Richards, 1973) of the N terminal of protein 460 reveals the presence of only equimolar concentration of dansyl aspartic acid and dansyl glutamic acid which correspond to the N-terminal amino acid of light and heavy chains, respectively. The analytical method employed would have detected a 1% contamination of protein or peptides with free N-terminal groups.

Binding of [^{3}H]Dnp-N_3 to Protein 460. The dissociation constant, K_d, for 2,4-[^{3}H]dinitrophenyl-L-lysine (ε-Dnp-Lys) was 1.0×10^{-5} M at 20°; 2 mol of ε-Dnp-Lys was bound/mol of protein 460 present as the 7S monomer ($r = 2.0$).

The dissociation constant for [^{3}H]Dnp-N_3, $K_d = 3.5 \times 10^{-5}$ M, $r = 1.8$ (Figure 2a), was determined by the equilibrium dialysis method according to Eisen (1964) at 20° using 0.2-ml volume microdialysis cells (Gateway Immunoserum Co., Cahokia, Ill.). Additional lower binding sites were demonstrable at higher ligand concentrations. The Dnp-N_3 reagent competes effectively with ε-Dnp-Lys for the protein 460 binding site. A series of equilibrium dialysis cells were set up with increasing concentrations of cold Dnp-N_3; the cells were protected from light. Figure 2b shows that Dnp-N_3 displaces

[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; ε-Dnp-Lys, ε-2,4-dinitrophenyl-L-lysine; Dnp-AD, 2,4-dinitrophenylalanine diazoketone; Dnp-N_3, 2,4-dinitrophenyl 1-azide; N_2ph-F, 1-fluoro-2,4-dinitrobenzene; *r*, number of moles of reagent bound per 7S monomer of protein 460.

TABLE I: Labeling of Protein 460 by [^{3}H]Dnp-N_3.

Condition	Irradn Time (min)	mol of Affinity Labeling Reagent Covalently Attached/mol of 7S Monomer of Protein 460	mol of [^{14}C]Dnp-Ala Bound/mol of 7S Monomer of Protein 460 after Labeling	No. of Binding Sites Lost per 7S Monomer of Protein 460 after Labeling
Low-site occupancy[a]	30	0.75	1.6	0.4
High-site occupancy[b]	30	1.9	0.75	1.25
Control without irradiation[c]	30	0.06	2.00	0
Reagent and competing ligand[d] [competitor]		% of Incorp in Absence of Competitor		
10^{-4} M	30	76		
10^{-3} M	30	35		
10^{-2} M	30	7		
0	30	100		

[a] Monomeric protein 460 (3.35×10^{-5} M) in 0.2 M Tris-HCl–0.075 M NaCl (pH 8.0) buffer in the presence of [^{3}H]Dnp-N_3 (7.3×10^{-5} M) was irradiated with six 15-W General Electric F15T8-B1 lamps at a distance of 13 cm. [b] Same as footnote *a* except that the [^{3}H]Dnp-N_3 concentration was 2.90×10^{-4} M. [c] Same as footnote *a* but the protein–reagent mixture was maintained in the dark at 4°. [d] Same as footnote *a* except that ε-Dnp-Lys was also present.

[^{3}H]Dnp-Lys from the combining site of protein 460 in the dark.

Labeling of Protein 460 with Dnp-N_3. To determine the extent and specificity of the reaction of Dnp-N_3 with protein 460, labeling was carried out at two reagent concentrations. [^{3}H]-Dnp-N_3 (sp act. 1×10^{13} cpm/mol) in ethanol was mixed with protein 460, 3.35×10^{-5} M in 0.2 M Tris-HCl–0.075 M NaCl (pH 8.0) buffer, to final concentrations of 7.3×10^{-5} and 2.90×10^{-4} M. Before adding the protein to the label, the ethanol was removed under a stream of nitrogen and the label was redissolved such that the ethanol concentration was less than 1% v/v. One-half of the combining sites is occupied by the Dnp-N_3 reagent at a concentration of 7.3×10^{-5} M and 86% of the sites is occupied at a reagent concentration of 2.90×10^{-4} M. The protein and reagent mixture was maintained at 4°, gently stirred, and irradiated at a distance of 13 cm from a bank of six, 15-W General Electric F158T-BL lamps which have a maximum energy output between 300 and 400 mμ (General Electric Co., 1965). After irradiating for 1 hr, the protein was freed of noncovalently bound reagent by dialysis against 0.2 M Tris-HCl–0.075 M NaCl (pH 8.0) buffer. Alternatively, 0.5-ml aliquots of labeled protein were freed of noncovalently bound reagent by passage through a 10-ml Sephadex G25 column in Tris buffer. The incorporation of reagent was determined by counting the radioactivity and measuring the optical density of the protein at 280 mμ, $E^{1\%}_{1\,cm}$ = 15.5 (Jaffe *et al.*, 1969). Irradiation of protein 460 and reagent in the presence of Dnp ligands and incubation of protein 460 and [^{3}H]Dnp-N_3 in the dark at 4° were included as controls. Table I shows the incorporation of reagent into protein 460. The ability of labeled protein 460 to bind Dnp ligands was demonstrated by equilibrium dialysis *vs.* [^{14}C]-Dnp-Ala ($K_d = 6.6 \times 10^{-6}$ M). After the incorporation of 2 mol of reagent into 1 mol of protein 460, 0.75 binding site/protein molecule, with a dissociation constant of 1.7×10^{-5} M, remained.

Location of Covalently Bound [^{3}H]Dnp-N_3 on Protein 460. Protein 460 labeled at 2.16×10^{-6} and 1.08×10^{-5} M reagent concentrations was completely reduced and alkylated with dithiothreitol and iodoacetic acid (Konigsberg, 1972). The labeled protein was dissolved in 0.5 M Tris-HCl–6.0 M guanidine-HCl–0.002 M EDTA (pH 9.0) buffer to a final concentration of 1%. Reduction, at 50° for 4 hr, was effected by making the solution 0.01 M in dithiothreitol; alkylation at room temperature was performed with neutralized iodoacetic acid at a final concentration of 0.022 M. Separation of heavy and light chains was by chromatography on Sephadex G100 in 6 M urea–1 M proprionic acid. Eighty-five per cent of the

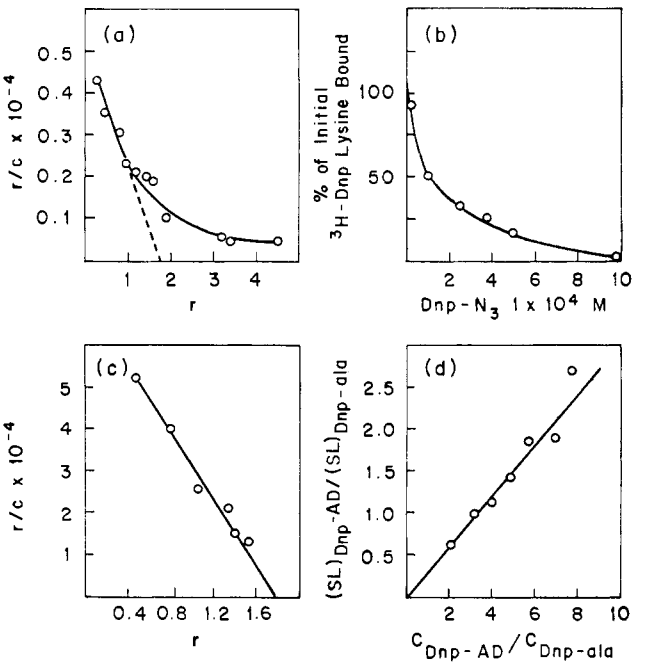

FIGURE 2: Binding studies of protein 460 with Dnp-N_3: (a) equilibrium dialysis of protein 460 with [^{3}H]Dnp-N_3 at 20°; (b) displacement of [^{3}H]Dnp-Lys from protein 460 by nonradioactive Dnp-N_3; (c) equilibrium dialysis of protein 460 with [^{3}H]Dnp-AD at 20°; (d) competition for protein 460 combining site between [^{14}C]Dnp-Ala and [^{3}H]Dnp-AD; slope = 0.31; binding constant of protein 460 for Dnp-Ala = 1.5×10^5 M^{-1}; binding constant of protein 460 for Dnp-AD = 0.45×10^5 M^{-1}; ratio = $(0.45 \times 10^5\ M^{-1})/(1.5 \times 10^5\ M^{-1}) = 0.30$; c = free concentration of competitor; (SL) = the concentration of binding sites occupied by a competitor.

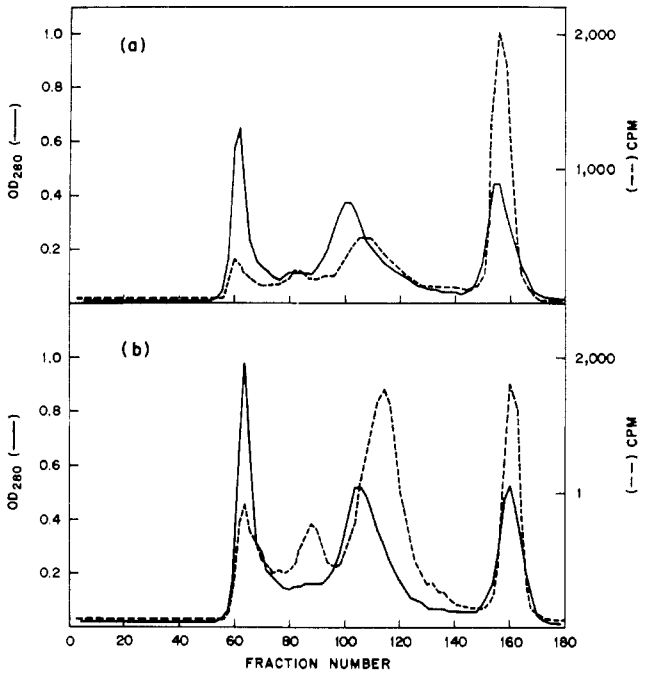

FIGURE 3: The distribution of [^{3}H]Dnp-N_3 in protein 460 heavy chain CNBr fragments under low- and high-site labeling conditions: (a) protein 460 was irradiated in the presence of 2.16×10^{-6} M [^{3}H]Dnp-N_3; the heavy chain was cleaved by CnBr, modified with maleic anhydride, desalted, and later, chromatographed in a Sephadex G75 column in 0.1 M NH_4HCO_3; (b) same as (a) except that the Dnp-N_3 concentration during irradiation was 1.08×10^{-5} M.

label was associated with the heavy chain and 15% of the label was associated with the light chain (Table II)

Papain digestion of labeled protein 460 revealed that approximately 80–85% of the radioactivity was located in the Fab fragments.

Heavy chains from protein 460 labeled at both reagent concentrations were cleaved with CNBr in 70% formic acid (Gross and Witkop, 1962) followed by reaction of the CNBr fragments with maleic anhydride (Butler *et al.*, 1969). The maleylated fragments were desalted on Sephadex G25. There was no loss of radioactivity during these procedures. The maleylated cyanogen bromide fragments were then chromatographed on Sephadex G75 in 0.1 M NH_4HCO_3. The elution profiles are shown in Figure 3. From the protein labeled using 2.16×10^{-6} M Dnp-N_3, most of the radioactivity was found in the smallest fragments, Figure 3a. In contrast, from the protein labeled using 1.08×10^{-5} M Dnp-N_3, the radioactivity was found in several cyanogen bromide fragments, Figure 3b, indicating that other less reactive residues are labeled as the extent of incorporation increases.

The location of the labeled peptides will be the subject of a subsequent paper (Hew *et al.*, 1973).

Binding of 2,4-Dinitrophenylalanyl Diazoketone (Dnp-AD) to Protein 460. The dissociation constant for the binding of Dnp-AD to protein 460 was 2.5×10^{-5} M as determined by equilibrium dialysis at 20°, Figure 2c. The competition between Dnp-AD and ε-Dnp-Lys for protein 460 was demonstrated by equilibrium dialysis, Figure 2d. A series of dialysis cells was set up which contained a constant amount of [^{14}C]Dnp-Ala and varying amounts of [^{3}H]Dnp-AD or ε-[^{3}H]Dnp-Lys. The presence of a second ligand should decrease the amount of the first ligand bound such that the ratio of the amount of each ligand bound should be proportional to the ratio of the association constants and free concentration of the two ligands (Markham and Benton, 1931). Competition for the same binding site is indicated if a plot of the ratio of the amount of each ligand bound *vs.* the ratio of the free concentrations of the two ligands yields a straight line with a slope equivalent to the ratio of the association constants. Dnp-Ala was shown to compete with both Dnp-AD and ε-Dnp-Lys.

TABLE II: Affinity of Protein 460 for Site-Specific Reagents.

Reagent	K_d (M)	Distribution of Label between Heavy and Light Chain (H/L)
Dnp-AD	2×10^{-5}	1/4
Dnp-N_3	5.0×10^{-5}	6/1
ε-Dnp-L-Lys	1×10^{-5}	

Labeling of Protein 460 with [^{3}H]Dnp-AD. Labeling was carried out at two reagent concentrations. [^{3}H]Dnp-AD (sp act. 3.8×10^3 cpm/mol) in dioxane was added to protein 460, 3.35×10^{-5} M in 0.2 M Tris-HCl–0.075 M NaCl (pH 8.0) buffer, to a final concentration of either 1.04×10^{-4} or 4.8×10^{-4} M. The dioxane concentration was less than 0.2% v/v. One-third of the combining sites is occupied by Dnp-AD at a reagent concentration of 1.04×10^{-4} M; 62% of the combining sites is occupied at a reagent concentration of 4.8×10^{-4} M. The mixture was maintained at 4° and irradiated at a distance of 3 cm from the lamps. The determination of the amount of label incorporated was as previously described. The following controls were included: (1) protein 460 irradiated without Dnp-AD; (2) protein 460 with Dnp-AD but without irradiation (dark reaction); (3) protein 460 irradiated in the presence of ε-Dnp-Lys; and (4) protein 460 irradiated in the presence of Dnp-AD and ε-Dnp-Lys. Table III shows the results of these experiments. The loss of ε-Dnp-Lys binding sites in the protein reacted with Dnp-AD was demonstrated by equilibrium dialysis with ε-[^{3}H]Dnp-Lys (specific radioactivity 8.3×10^{13} cpm/mol so that [^{3}H]Dnp-AD bound to the protein will be only a small amount of the total radioactivity on the dialysis cells).

Location of Covalently Bound [^{3}H]Dnp-AD on Protein 460. After labeling the immunoglobulin chains were separated and the ratio of radioactivity in the light and heavy chains was 3.5:1 (Table II).

Protein 460 was labeled at reagent concentrations of 1.04×10^{-4} or 4.8×10^{-4} M and the identity of the labeling patterns at both reagent concentrations will be shown in the accompanying paper (Hew *et al.*, 1973).

Discussion

Affinity labeling studies have been carried out with antibodies raised against haptens structurally related to the affinity labeling reagents (Wofsy *et al.*, 1962) and with homogeneous immunoglobulins having antibody-like activity (Haimovich *et al.*, 1970).

These affinity labeling reagents have had either a diazonium (Martin *et al.*, 1972) or a bromoacetyl moiety (Haimovich *et al.*, 1972), as the reactive group. Such reagents are restricted in their reactivity to amino acid residues having nucleophilic

TABLE III: Labeling of Protein 460 by [^{3}H]Dnp-AD.

Condition	Irradn Time (hr)	Mol of Affinity Reagent Attached by Covalent Bonds/mol of 7S Monomer of Protein 460	Mol of [^{3}H]Dnp-Lys Bound/mol of 7S Monomer of Protein 460 after Irradn	No. of Binding Sites Lost/7S Monomer of Protein 460 after Irradn
Low-site occupancy[a]	1	0.36	1.60	0.40
	2	0.47	1.38	0.62
	4	0.59	1.20	0.80
	6	0.58	0.92	1.08
	8	0.68	0.92	1.08
High-site occupancy[b]	1	0.56	1.50	0.5
	2	0.80	1.24	0.7
	4	1.08	0.84	1.12
	6	1.28	0.60	1.40
	8	1.36	0.48	1.52
Control with ligand[c]	1		1.9	0.1
	2		1.8	0.2
	4		1.6	0.4
	8		1.5	0.5
Control without ligand[d]	0		2.0	0
	8		2.0	0
Control without irradiation[e]	8	0.03	2.0	0
Label and competing ligand[f]	1	0.097		
	2	0.160		
	3	0.210		
	4$^5/_6$	0.260		
	7	0.290		
	8	0.310		

[a] Monomeric protein 460 (3.35×10^{-5} M) in 0.2 M Tris-HCl (pH 8.0) buffer at 4° in the presence of Dnp-AD (1.05×10^{-4} M) was irradiated with six 15-W General Electric F15T8-B1 lamps at a distance of 3 cm. [b] Same as footnote *a* except that the Dnp-AD concentration was 4.8×10^{-4} M. [c] Protein 460 was irradiated in the presence of ε-Dnp-Lys (9.35×10^{-5} M). [d] Protein 460 was irradiated without any ligands. [e] Protein 460 was mixed with Dnp-AD (1.05×10^{-4} M) without any irradiation. [f] Same as footnote *a* except that the Dnp-AD concentration was 1.54×10^{-4} M and ε-Dnp-Lys was present at a concentration of 2.3×10^{-3} M.

side chains such as cystine, tyrosine, lysine, histidine, and methionine.

When these affinity labeling reagents reacted with antibodies, two types of results have been obtained. In one case nucleophilic amino acid residues may be present in the combining site, and reaction of the affinity labeling reagent with the residues may block the site and prevent the subsequent binding of additional ligands. In the other case, there may be no nucleophilic amino acid residues within the site, but the region around the site may contain one or more residues which can react with the affinity labeling reagent when it is not bound in the combining site. In this situation even though the modified protein still binds the specific ligand, the labeling required the prior binding of the reagent in the site.

As an example of the first case, TEPC 15, a mouse IgA myeloma protein which binds phosphorylcholine, was labeled with a diazonium affinity labeling reagent. Only a tyrosine at position 34 on the light chain reacted and binding of phosphorylcholine was abolished (Chesebro and Metzger, 1972). The second case is illustrated by protein 315, a Dnp binding mouse IgA myeloma protein. When this protein reacted with *m*-nitrobenzenediazonium fluoroborate, the same residue, Tyr-34, on the light chain, was modified but the number of combining sites for ε-Dnp-Lys was preserved, even though the binding energy was reduced (Goetzl and Metzger, 1970). One possible way of overcoming the limitations of these reagents with respect to reactivity is to use an affinity labeling reagent which has a broader spectrum of reactivity than those just described. Photoactivated affinity labeling reagents fulfill this requirement, since they generate very reactive intermediates upon irradiation.

The ability of an affinity labeling reagent to react *in situ* in the combining site depends on several factors: (1) the spectrum of reactivity of the reactive group; (2) the distance between the reactive group and the binding moiety on the affinity reagent; and (3) the half-life of the affinity reagent or its photoactivated intermediates relative to the length of time during which the reagent occupies the combining site, *i.e.*, equilibrium and rate considerations.

Affinity labeling reagents whose reactivity is restricted to amino acid residues having nucleophilic side chains may not react *in situ* for lack of a suitably positioned residue. The potential advantage of the photoaffinity labels is that the photogeneration of carbenes, ketenes, and nitrenes ensures that a relatively high concentration of reagent, capable of reacting with all heteroatomic side chains, is obtained in the combining site.

Dnp-AD and Dnp-N_3 are quite stable in the dark but give reactive intermediates upon irradiation. The covalent attachment of the reagents to protein 460 was minimal without

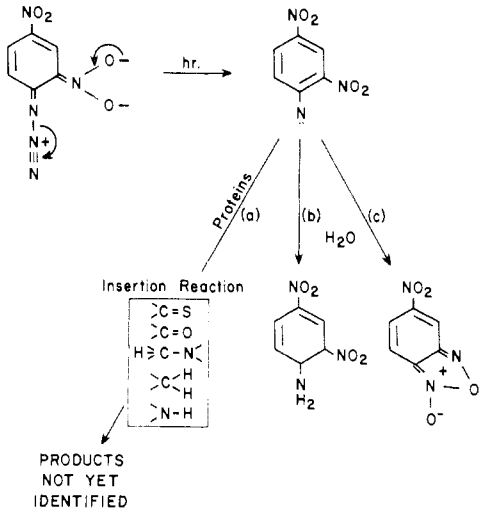

FIGURE 4: Generation of 2,4-dinitrophenylnitrene and its possible reaction products.

photolysis (the dark reaction). Both Dnp-AD and Dnp-N_3 compete with ε-Dnp-Lys for the combining site of protein 460. They can be displaced by ε-[^{3}H]Dnp-Lys or [^{14}C]Dnp-Ala in the dark (Figure 2b,d). Thus, prior to photolysis both reagents behave as nonreactive, competitive ligands with ε-Dnp-Lys. The site directed labeling of protein 460 by Dnp-AD and Dnp-N_3 was shown by the following experiments: (1) the avidity of protein 460 for the reagents and the competition between reagent and ε-Dnp-Lys for the combining site; (2) inhibition of labeling by the presence of ε-Dnp-Lys; (3) after the covalent incorporation of reagent, the loss of binding activity which could be correlated with the number of moles of reagent bound; (4) demonstration that the radioactivity incorporated into the labeled protein was located predominantly in the Fab fragment; and (5) the amino acid sequence analysis which also confirms that the photoaffinity reagents are attached by covalent bonds to the variable portions of both the heavy and light chains. Details of these experiments will be reported in subsequent papers. Although Dnp-AD and Dnp-N_3 can generate reactive intermediates upon irradiation, the rate of reaction, the reaction mechanism, and the pattern of labeling by these two reagents differ significantly. The reaction of Dnp-N_3 with protein 460 is much faster but less specific compared to Dnp-AD. The incorporation of approximately 2 mol of Dnp-N_3/mol of protein is completed within 1 hr of irradiation while Dnp-AD requires 8–10 hr for the same extent of incorporation.

Dnp-N_3 generates a nitrene upon photolysis. Figure 4 gives a series of possible products which could result from the nitrene intermediate. The principle reactions of the nitrene are probably (a) reaction with amino acid side chains in the protein, (b) hydrolysis to 2,4-dinitroaniline, and (c) cyclization to form 4-nitrobenzene-1,2-furazan *n*-oxide. If a reaction takes place resulting in the formation of covalent bonds while the Dnp group is in the combining site, suitable residues within a sphere of 3.1 Å from the center of the Dnp ring should react.

Dnp-N_3 labels the heavy chain predominantly and is located in several different regions when a high reagent concentration is employed. When lower reagent concentrations are used, labeling was restricted to two variable region peptides (C.-L Hew *et al.*, manuscript in preparation). The existence of more than two combining sites on protein 460 for Dnp-N_3 (Figure 2a) may account for the wider distribution of radioactivity as the concentration of reagent is increased and the inability to exactly correlate the incorporation of reagent with loss of Dnp binding activity.

Dnp-AD produces a carbene on photolysis which may react directly or form a ketene *via* the Wolff rearrangement (Wolff, 1902). The carbene has the potential of inserting into C—H, C—N, and C=O bonds while the ketene is attacked by nucleophiles. Irradiation of protein 460 in the presence of a nonreactive ligand such as ε-Dnp-Lys, or the continued irradiation of protein 460 in the presence of the photolysis product of Dnp-AD, results in the loss of binding sites without incorporation of ligand (Table II). No binding sites are lost if the protein is irradiated in the absence of ligand. This photoinactivation, which may be due to the destruction of sensitive residues in the binding region, is much slower than the covalent incorporation of reagent and accompanying site loss and does not suggest the independence of site loss and affinity labeling reagent incorporation.

In subsequent papers we will present evidence for the location of the attachment of Dnp photoactivated reagents on both the light and heavy chains of protein 460.

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Affinity-Labeled Peptides Obtained from the Combining Region of Protein 460. Light Chain Labeling Patterns Using Dinitrophenyl Based Photoaffinity Labels†

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ABSTRACT: Two radioactive photoaffinity reagents, based on the 2,4-dinitrophenyl group, have been used to label a homogeneous mouse myeloma protein (protein 460). After separation of the heavy and light chains, a portion of the light chains was allowed to react with maleic anhydride and then digested with trypsin while another portion was digested directly with trypsin. With one of the reagents, 2,4-[^{3}H]-dinitrophenylalanyl diazoketone, which labels the light chain predominantly, nearly all of the reagent was found attached to an ϵ-amino group of lysine residue 54 *via* an amide linkage. In the case of the other reagent, 2,4-[^{3}H]dinitrophenyl azide, which labels chiefly the heavy chain, only a small fraction (15%) of the reagent which had reacted with the protein was found on the light chain. In contrast to the labeling with the diazoketone reagent, where the reagent attacked a single residue, radioactivity from azide label was found mainly in three light chain peptides which spanned residues 29–58, 62–77, and 78–108.

In a previous paper (Yoshioka *et al.*, 1973), we have reported the labeling of the combining region of protein 460 with two radioactive photoaffinity reagents based on dinitrophenyl (Dnp).[1] This is one of several haptens known to bind to the combining region of this protein (Jaffe *et al.*, 1971; Rosenstein *et al.*, 1972). One Dnp reagent, 2,4-[^{3}H]-dinitrophenylalanyl diazoketone ([^{3}H]Dnp-AD), reacts predominantly with the light chain. The other photoaffinity reagent, 2,4-[^{3}H]dinitrophenyl azide ([^{3}H]Dnp-N_3), is attached predominantly to the heavy chain. This paper deals with the isolation of light chain peptides and the location of the photoaffinity-labeled aminoacid residues in the light chain of protein 460 labeled with [^{3}H]Dnp-AD and [^{3}H]Dnp-N_3.

Materials and Methods

Preparation of Protein 460 and the Separation of Light and Heavy Chains. The purification and the labeling of protein 460 with [^{3}H]Dnp-AD and [^{3}H]Dnp-N_3 have been described in the accompanying paper (Yoshioka *et al.*, 1973). The light and heavy chains were reduced with a 50-fold molar excess of dithiothreitol compared to disulfides in 0.5 M Tris-HCl–6 M guanidine-HCl (pH 8.0) buffer for 4 hr at 50°. Alkylation was effected with a 2.5 molar excess of iodoacetic acid over reducing agent; the pH was maintained by the addition of NaOH. They were then separated on a Sephadex G100 column in 6 M urea–1 M propionic acid. The labeled light and heavy chains were dialyzed exhaustively against 0.2 N acetic acid and lyophilized.

Maleylation Procedure. In a typical experiment 1×10^{-6} mol of labeled light chain was suspended with stirring in 10 ml of 0.2 M phosphate buffer (pH 8.8) and approximately 1×10^{-4} mol of solid maleic anhydride (Butler *et al.*, 1968) was added in five equal portions over a 30-min period. The pH was adjusted with 2.0 N NaOH so that it remained between 8.5 and 9.5. Two milliliters of a 1 N pH 9.0 hydroxylamine solution was added at 20° (Freedman *et al.*, 1968). The mixture was stirred for 2 hr, dialyzed against 0.05 M (pH 8.0) NH_4HCO_3 buffer, and then lyophilized. All the radioactivity was recovered in the maleylated L chain.

Tryptic Digestion of the Dnp-AD-Labeled Maleylated 460 Light Chain. The Dnp-AD-labeled maleylated L chain (0.45 μmol) in 0.2 M NH_4HCO_3 (pH 8.0) buffer was digested with Tos-PheCH_2Cl-treated trypsin (1:200) for 1 hr at 37° and then inactivated with a 2 molar excess of soybean trypsin inhibitor (Schwarz/Mann, Orangeburg, N. Y.). The digest was chromatographed on a 2 × 90 cm Sephadex G50 column in 0.1 M NH_4HCO_3. A peak which accounted for 84% of the total radioactivity was pooled and the material purified by DEAE-cellulose ion exchange chromatography using a 1 ×

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[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; ϵ-Dnp-Lys, ϵ-2,4-dinitrophenyl-L-lysine; Dnp-AD, 2,4-dinitrophenylalanine diazoketone; Dnp-N_3, 2,4-dinitrophenyl 1-azide; Men, menadione (2-methyl-1,4-naphthoquinone); N_2ph-F, 1-fluoro-2,4-dinitrobenzene; *r*, number of moles of reagent bound per 7S monomer of protein 460; Tos-Phe-CH_2Cl, α-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone. BADL, α-*N*-bromoacetyl-ϵ-dinitrophenyl-L-lysine; BADE, *N*-bromoacetyl-*N'*-2,4-dinitrophenyl-L-ethylenediamine.