Affinity Labeling and Cross-Linking of the Heavy and Light Chains of a Myeloma Protein with Anti-2,4-dinitrophenyl Activity*

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ABSTRACT: Eight bromoacetyl derivatives of 2,4-dinitrophenyl ligands, differing in length, charge, and optical configuration, were used for affinity labeling of a myeloma protein with anti-DNP activity. Fluorescence yields and absorption spectra showed that in affinity-labeled protein-315 the covalently attached ligands occupied the combining site in the same way as DNP ligands that are specifically bound noncovalently. The results showed that the anti-2,4-dinitrophenyl combining site imposed restriction on the chemical reaction between the reagent and amino acid residues of protein. Covalent labeling of amino acid side chains in the protein depends mainly on the length of the reagent and, in one case, on its optical configuration. The labeled residues were a unique tyrosine on the light chain and a unique lysine on the heavy chain. The use of a homologous series of reagents allows an estimate of the distance (less than 5 Å) between these two residues. On the basis of these results a bifunctional, bisbromoacetyl derivative of the 2,4-dinitrophenyl ligand was prepared and was found to react with both tyrosine and lysine. The covalent attachment of the bifunctional reagent to these two residues brought about the cross-linking of the heavy and light chains of the myeloma protein, to form a heavy-chain–light-chain complex linked at the combining site.

The affinity labeling of antibody molecules, originally performed with the diazonium salt derivatives of haptens, indicated the presence of tyrosine in combining sites of diverse specificities, and demonstrated that both light and heavy chains can contribute to the formation of this site (Wofsy *et al.*, 1962; Singer *et al.*, 1967). In order to develop affinity-labeling reagents with different chemical specificity, bromoacetyl derivatives of the DNP[1] hapten were prepared and tested for their specific covalent binding to anti-DNP antibodies (Weinstein *et al.*, 1969). These reagents have the advantage of reacting with a number of different amino acid residues (Korman and Clark, 1956) whose CM derivatives are well characterized and easily quantified following acid hydrolysis (Crestfield *et al.*, 1963).

Previous work (Haimovich *et al.*, 1970) has shown that the bromoacetyl derivatives of BADE and BADL combine covalently with a myeloma protein having anti-DNP activity (protein-315, produced by mouse plasmacytoma MOPC-315; Eisen *et al.*, 1968). Analysis of the modified protein revealed that BADE reacted exclusively with a tyrosyl residue on light chain and BADL with a lysyl residue on heavy chain. The present publication describes further analysis of the hapten-binding region of protein-315 through a study of the reactions of bromoacetyl derivatives of DNP ligands of various lengths. These studies provided information on the relative position of the labeled lysyl and tyrosyl residues in the binding site and led to the design of a bifunctional reagent which was able to react simultaneously with both residues. Since the labeled lysine and tyrosine are on different peptide chains, reaction with the bifunctional reagent brought about the covalent cross-linking of the heavy and light chains of the myeloma protein.

Materials and Methods

Protein-315 was isolated from sera of mice bearing tumor MOPC-315 as described (Haimovich *et al.*, 1970). Bromoacetyl derivatives of DNP-ethylenediamine and ε-*N*-DNP-L-lysine were prepared as described (Weinstein *et al.*, 1969). The [^{14}C]bromoacetyl derivatives BADB, BADO, D-BADL, BADGL, and BADH were prepared in a similar manner. Specific details concerning the synthesis of these compounds will be published elsewhere. All labeling reagents were purified by thin-layer chromatography on silica gel (Riedel-DE Haen AG, Sulze Hanover) developed with chloroform–*tert*-amyl alcohol–acetic acid (70:30:3, v/v) and eluted with dioxane. The bifunctional reagent DIBAB was prepared as follows.

γ-DNP-L-diaminobutyric Acid Hydrochloride (*I*). 1,3-Diaminobutyric acid (1.9 g) was dissolved in 15 ml of boiling water and 2 g of $CuCO_3$ was added with stirring over a period of 10 min. The excess $CuCO_3$ was removed by filtration and washed with 5 ml of hot water. $NaHCO_3$ (2 g) and fluorodini-

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[1] Abbreviations used are: DNP, 2,4-dinitrophenyl; BADE, *N*-bromoacetyl-*N*-'DNP-ethylenediamine; BADL, α-*N*-bromoacetyl-ε-*N*-DNP-lysine; BADB, α-*N*-bromoacetyl-γ-*N*-DNP-diamino-L-butyric acid; BADO, α-*N*-bromoacetyl-δ-*N*-DNP-L-ornithine; BADGL, α-*N*-bromoacetylglycyl-ε-*N*-DNP-L-lysine; DIBAB, α-*N*-bromoacetyl-γ-*N*-DNP-L-diaminobutyric acid *N'*-bromoacetylhydrazide; BADH, *N*-bromoacetyl-*N'*-DNP-hydrazide; H, heavy chain; L, light chain; PBS, 0.15 M NaCl–0.01 M potassium phosphate (pH 7.0).

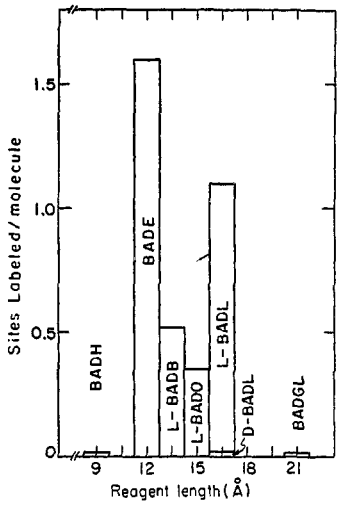

FIGURE 1: The extent of affinity labeling of protein-315 by different bromoacetyl reagents. Reaction was carried out with protein (1.0×10^{-6} M) and reagents (2.0×10^{-6} M) in 0.1 M $NaHCO_3$ (pH 9) for 24 hr at 37°.

trobenzene (3.6 g in 20 ml of ethanol) were added to the blue filtrate and the reaction mixture was stirred for 2 hr at room temperature. The green product was filtered, washed successively with water, ethanol, and ether, and then dissolved in 10 ml of 3 N HCl. After 10 min a yellow precipitate was formed which was collected and washed successively with 5 ml of cold 3 N HCl, 5 ml of cold water, ethanol, and ether. The yield was 2.1 g (65%), mp 254°. *Anal.* Calcd for $C_{10}H_{13}ClN_4O_6$: C, 37.50; H, 4.06; N, 17.50. Found: C, 37.28; H, 3.98; N, 17.64.

γ-DNP-L-diaminobutyric Acid Methyl Ester Hydrochloride (II). Compound I (1.6 g) was added to a chilled solution of thionyl chloride (1 ml) in methanol (10 ml) and the mixture was shaken for a few minutes until the solid had dissolved. The reaction mixture was left for 5 hr at room temperature after which 50 ml of dry ether were added. The precipitate was filtered and washed with ether. The yield was 1.69 g (95%), mp 125°. *Anal.* Calcd for $C_{11}H_{15}ClN_4O_6$: C, 39.52; H, 4.49; N, 16.73. Found: C, 39.81; H, 4.58; N, 16.52.

γ-DNP-α-Cbz-L-diaminobutyric Acid Methyl Ester (III). Compound II (1.69 g) was treated with 1 equiv of carbobenzoxy chloride (1.25 g) in a mixture of 5% $NaHCO_3$ solution and chloroform. After shaking for 20 min at 0° the reaction was allowed to proceed for an additional 20 min at room temperature. The chloroform layer was washed with 5% pyridine solution, dried over Na_2SO_4, and concentrated to dryness. This semicrystalline compound was used for the next step.

γ-DNP-α-Cbz-L-diaminobutyric Acid Hydrazide (IV). Compound III (1.1 g) was dissolved in 15 ml of anhydrous ethanol and hydrazine hydrate (0.5 ml) was added. The mixture was allowed to stand overnight at room temperature. The crystalline hydrazide was filtered and washed with cold ethanol and ether. The yield was 0.9 g (80%), mp 104–106°. *Anal.* Calcd for $C_{18}H_{20}N_6O_7$: C, 50.00; H, 4.81; N, 19.44. Found: C, 49.71; H, 4.68; N, 19.65.

γ-DNP-L-diaminobutyric Acid Hydrazide Dihydrobromide (V). Compound IV (0.86 g) was dissolved in glacial acetic acid (3 ml) and reacted with a 5-ml solution of HBr in acetic acid (45%). After 15 min at room temperature, 50 ml of dry ether was added. The precipitate was collected and washed with dry ether. The yield was 0.79 g (85%), mp 235°. *Anal.* Calcd for $C_{10}H_{16}Br_2N_6O_5$; Br, 34.78; N, 18.26. Found: Br 35.01; N, 18.04.

γ-DNP-α-bromoacetyl-L-diaminobutyric Acid Bromoacetyl Hydrazide (VI). Compound V (0.72 g) was dissolved in dimethylformamide (5 ml), to which was added triethylamine (0.56 ml), and treated with 5 equiv (1.3 g) of bromoacetic anhydride. After 1 hr at room temperature sodium bicarbonate was added to adjust the pH to 7. After another 20 min water was added and the precipitate formed was collected and washed with water. The yield was 75%, mp 135–138°. *Anal.* Calcd for $C_{14}H_{16}Br_2N_6O_7 \cdot 4H_2O$: C, 27.61; Br, 26.14; N, 13.72. Found: C, 27.19; Br, 25.3; N, 13.42.

The ^{14}C-labeled compound was prepared as follows. Triethylamine (1 mmole) was added to a solution of V (0.5 mmole) in 3 ml of dimethylformamide. This was mixed with a solution of [^{14}C]bromoacetyl-*N*-hydroxysuccinimide ester (Cuatrecasas *et al.* (1969) (1.5 mmoles) in dioxane (3 ml). After 1 hr at room temperature 5 ml of 1 N $NaHCO_3$ was added and the precipitate formed was collected. This was purified on thin layer of silica gel developed with chloroform–benzyl alcohol–acetic acid (70:30:3, v/v; R_F 0.82).

Affinity labeling of protein-315 was performed by reaction of the protein (10^{-6} M) with the bromoacetyl, DNP derivative (2×10^{-6} M) in 0.10 M $NaHCO_3$ (pH 9.0) at 37° unless otherwise specified. Samples were assayed for covalent binding at various times by filtration of trichloroacetic acid precipitated protein on selectron filters BA 85/0 (Schleicher & Schull) as previously described (Weinstein *et al.*, 1969). Acid hydrolysates of ^{14}C-labeled protein were analyzed for CM-amino acid derivatives by high-voltage electrophoresis at pH 3.5 (Haimovich *et al.*, 1970). The electrophoresis runs included internal markers of CM-lysine and CM-tyrosine. Analytical separation of heavy and light chains by polyacrylamide gel electrophoresis, was carried out on 5% gels in 0.1% sodium dodecyl sulfate–0.14 M β-mercaptoethanol according to Shapiro *et al.* (1967). The 5% cross-linked gels were then stained with coomassie brilliant blue and treated by one of the following procedures: gels were analyzed for protein-containing bands by scanning at 570 nm in a spectrophotometric gel scanner (Gressel and Wolowelsky, 1968) kindly furnished by Dr. Jonathan Gressel. Radioactivity in the gel was analyzed by slicing the gel into 50 thin slices which were solubilized with Soluene (Packard Instrument Co.) and counted in toluene containing scintillant. Protein-containing bands were analyzed for CM-amino acid derivatives by 6 N HCl acid hydrolysis, performed directly upon thin slices of gel containing this protein; after 24 hr of acid hydrolysis, the hydrolysis tubes were opened, cooled, and then centrifuged to remove the residual polyacrylic acid. The hydrolysates were analyzed by high-voltage electrophoresis at pH 3.5, as mentioned above. Treatment by this technique of gels prepared with standard samples of labeled protein showed quantitative release of amino acids. Fluorescence quenching and difference spectral analyses were carried out as described (Eisen and Siskind, 1964; Little and Eisen, 1967).

Results

Labeling by Different Reagents. The extent of labeling obtained by reacting protein-315 with eight different reagents is given in Figure 1. The results indicate that the extent of labeling was dependent mainly on the length of the reagent and not on its charge since BADB, BADO, and BADL all have a carboxylate group near the bromoacetyl moiety, yet they labeled the protein to a different extent. Reagents which are

TABLE I: Characteristics of Reagent–Protein-315 Complexes.[a]

Reagent	K_a[b] (l./mole)	k_2 (min^{-1})	k_2 (Tyr) (min^{-1})	k_2 (Lys) (min^{-1})
BADE	3.57×10^5	100×10^{-4}	100.0×10^{-4}	10^{-4}
BADB	3.87×10^5	7×10^{-4}	5.9×10^{-4}	1.0×10^{-4}
BADO	21.00×10^5	4×10^{-4}	2.6×10^{-4}	1.4×10^{-4}
BADL	6.80×10^5	22×10^{-4}	1.3×10^{-4}	21.0×10^{-4}

[a] Reactions performed as described under Figure 2. The first-order limiting rate constant of labeling (*i.e.*, the rate of reaction at saturation of binding sites) is designated k_2 for convenience. k_2 at tyrosyl and lysyl residues were calculated by combining the k_2 values determined for the protein–reagent complex with the data for the relative rates of reaction with tyrosyl and lysyl residues (Figure 5). This is based on the assumption that the proportion of labeling at each residue is constant with time, as expected for reaction with a homogeneous protein. For conditions of labeling, see Figure 2. [b] The K_a values for BADE and BADL obtained by fluorescence quenching under conditions of labeling were 2.5×10^5 and 5.4×10^5 M^{-1}, respectively. Under these conditions (37°, pH 9.5) the K_a values are about one order of magnitude lower than at 4° and pH 7.4 (BADE, 5.3×10^6 M^{-1} and BADL, 1.9×10^7 M^{-1}). These differences are in accord with the pronounced influence of temperature on the affinity of anti-DNP antibodies and of protein-315 for DNP ligands.

too short (BADH) or too long (BADGL) failed to react with the protein. Of special importance is the difference in labeling between the bromoacetyl derivatives of the two optical isomers of ε-*N*-DNP-lysine (D-BADL and L-BADL). Since D-BADL did not react at all it appears that the bromoacetyl function in BADL is stereospecifically directed toward reaction with a protein residue. This is not due to differences in affinity of the protein for the ligands. Fluorescence-quenching titrations at 4°, pH 7.4 with the two reagents showed that both reagents were bound to protein-315 with equal affinity (1.9×10^7 and 2.0×10^7 M^{-1} for L-BADL and D-BADL, respectively).

In order to demonstrate further that the differences in covalent labeling of the protein were not due to differences in the affinity of the protein for the various reagents, the labeling reaction was examined with respect to its dependence on the concentration of reagent. The reaction of proteins with affinity-labeling reagents can be described by

$$A + H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AH \xrightarrow{k_2} L$$

where A is protein-315, H is the reagent, AH is the noncovalent complex, and L is the covalently labeled protein. A graphical treatment of the data according to the above equation was essentially as described by Kitz and Wilson (1962) and Shaw and Glover (1970), which yields the association constant (K_a) for a protein-labeling reagent complex and the first-order limiting rate of labeling (*i.e.*, k_2, the rate constant of covalent reaction at complete saturation of binding sites). An example is presented for the reaction with BADE. The labeling of protein-315 (pH 9.5, 37°) at several concentrations of BADE obeyed first-order kinetics over most of the labeling reaction (Figure 2). A double-reciprocal plot of the apparent first-order rate constants so obtained ($k_{app} = 0.69/t_{1/2}$) *vs.* labeling agent concentration (Figure 3) gave a positive intercept on the abscissa corresponding to a limiting rate constant (k_2) for covalent labeling by BADE of 0.01 min^{-1} at saturation of binding sites. This is equivalent to a half-lifetime of reaction of 69 min. From this value and the slope of the plot (K_a/k_2), an association constant (K_a) of 3.6×10^5 M was calculated for the noncovalent BADE-protein complex. Similar analyses were made for the reactions of BADB, BADO, and BADL with protein-315. These data, presented in Table I, show that there is no relationship between the affinity of the reagent for protein-315 and the rate of labeling, *i.e.*, in spite of similar association constants the rate of labeling by the different reagents varies substantially. It appears that the rate of reaction of the different reagents with a particular residue depends on its dimensions and the resultant positioning of the chemically reactive group of the bound reagent.

Fluorescence Quenching and Spectral Shift. The first-order

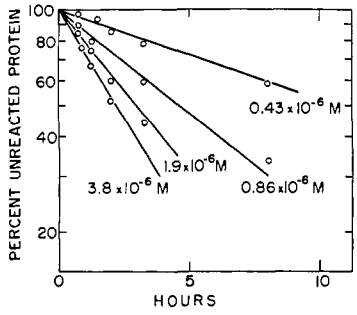

FIGURE 2: Labeling of protein-315 at various concentrations of BADE. Protein-315 was 1.5×10^{-7} M in 0.1 M $NaHCO_3$ (pH 9.5) at 37°. Rate of covalent reaction was determined as described in Materials and Methods.

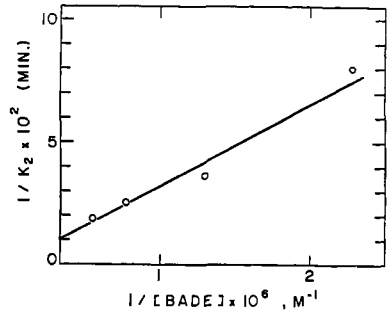

FIGURE 3: Saturation kinetics in the covalent labeling of protein-315 by BADE. The k_{app} of labeling at various BADE concentration (Figure 2) replotted in double-reciprocal fashion to obtain kinetic properties of the noncovalent protein–BADE complex.

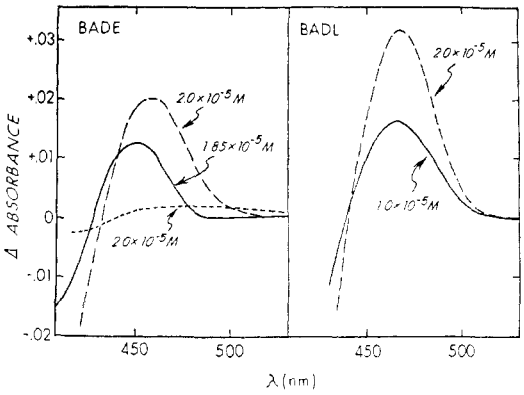

FIGURE 4: Difference spectra between free and protein-bound BADE and BADL. Spectra were obtained as the difference between a mixture of either BADL (2.9×10^{-5} M) or BADE (3.9×10^{-5} M) and protein-315 (1.0×10^{-5} M) or the affinity-labeled protein in the experimental cell *vs.* protein and ligands in separate tandem cells at the same final concentrations. (———) Affinity-labeled protein in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.0); (- - -) affinity-labeled protein in 7 M guanidine·HCl; (— — —) noncovalently bound ligands in PBS. Concentration of covalently bound ligands was determined by the amount of radioactivity and that of noncovalently bound ligand by assuming saturation of the protein ($n = 2$). Spectra determined at 4°. Concentrations shown in the figure are those of the bound ligands.

TABLE II: Fluorescence of Affinity-Labeled Protein-315.

Sample	Protein Fluorescence[a] (Arbitrary Units) PBS	7 M Gdn·HCl[b]	Q'[c] (%)
Protein-315[d]	68	106	53
BADE-labeled protein[e]	32	99	0 (± 2)
Control[f]	73		33
BADL-labeled protein[e]	39	86	0 (± 2)
Control[f]	71		25

[a] Protein concentration was 3.4×10^{-7} M. Fluorescence was measured at 4° at the same amplifier setting for the determination listed in each column (290-nm activation; 345-nm emission). [b] At the amplifier settings used for fluorescence determination in 7 M guanidine·HCl (Gdn·HCl) the values in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.0), for protein-315, BADE-labeled protein, and BADL-labeled protein were 43, 19, and 22, respectively. [c] 0.2 ml containing 1.9 nmoles of ε-DNP-lysine was added to 1.0 ml of the protein solution in PBS whose fluorescence is given in the first column, Q' is the decrease in fluorescence (quenching) as per cent of the fluorescence value in the absence of added ε-DNP-lysine (first column). [d] Protein-315 before incubation at 37° and pH 9.0. [e] 1.4 sites/molecule labeled, as described in Materials and Methods. [f] Protein treated under the conditions of labeling (0.1 M $NaHCO_3$, pH 9.0, and 37° for 20 hr in the control for BADE labeling and 46 hr in the control for BADL labeling) but without the reagent, and then extensively dialyzed against 0.15 M NaCl–0.01 M potassium phosphate (pH 7.0).

rate of reaction in the kinetic experiments indicate that the labeling reactions are site directed (Shaw and Glover, 1970). In order to evaluate if the DNP moiety in the covalent complex formed by affinity labeling occupies the same position as the DNP group in the noncovalent hapten–protein complex, the fluorescence yield of the modified protein and absorption spectrum of the bound DNP group were studied. Protein-315 was affinity labeled by BADE and BADL to the extent of 1.4 sites/molecule and was extensively dialyzed against 0.15 M NaCl–0.01 M potassium phosphate (pH 7.0) to remove the unreacted reagent. Further dialysis of a sample against 7 M guanidine·HCl did not reduce the amount of radioactive ligand bound to the protein. Fluorescence of the labeled protein and of control samples treated in the same way, but in the absence of affinity-labeling reagents, was also measured and the results are summarized in Table II. The fluorescence of the protein with covalently bound reagent was reduced by about 50% (actually 45% for BADL-labeled protein and 56% for BADE-labeled protein). This level of quenching is consistent with labeling of 1.4 out of 2.0 sites and a Q_{max} for noncovalently bound ligand of 69% (Eisen *et al.*, 1968). The absence of further quenching on addition of ε-DNP-lysine was probably due to inactivation during prolonged incubation of the protein at low concentration (0.15 mg/ml) under unfavorable conditions, which also caused some loss of activity of the control samples (Table II). In the presence of 7 M guanidine·HCl the affinity-labeled protein had almost the same fluorescence yield as the unlabeled protein. Hence, quenching by the covalently bound reagent (Table II), was dependent on native conformation of the immunoglobulin molecule.

Difference spectra between free and bound reagents (BADE and BADL) are shown in Figure 4, where the bound reagents were examined as covalent and noncovalent complexes with protein-315. The covalent complexes were affinity-labeled proteins prepared as described in Materials and Methods. The noncovalent complexes were prepared by simply mixing BADE or BADL with protein-315 in PBS at 4° (conditions at which no covalent reaction occurs). The difference spectra of both noncovalently and covalently bound BADL were identical, with a maximal ΔE_M at 468 nm of 1600 (ε-DNP-lysine gave similar results). Covalently bound BADE was somewhat different in its difference spectrum (maximal ΔE_M at 452 nm of 700) than the noncovalent bound BADE (maximal ΔE_M at 460 nm of 1000), but there was almost no difference in absorption spectra between free BADE and the covalently bound reagent when they were examined in 7 M guanidine·HCl. The affinity-labeled protein had the same sedimentation coefficient as unlabeled protein ($s_{20,w} = 5.95$ S at 0.5% concentration of protein), excluding the possibility that noncovalent complexes were formed between the DNP substituent on one protein molecule with unlabeled combining site of another.

Labeled Residues and Chains. Previous results showed that BADE and BADL differ in the specificity of their labeling. BADL labeled lysine in the heavy chain, whereas BADE-labeled tyrosine in the light chain (Haimovich *et al.*, 1970). It was of interest to determine whether BADB or BADO exhibit similar specificity. Figure 5 gives the results of labeling of protein-315 by these four reagents. It is shown that labeling by BADE and BADL, and to some extent BADB, are specific for residue and chain, while BADO is the only reagent which appreciably labels both chains. However, the extent of labeling by BADO (Figure 1) is smaller than that obtained with the other reagents. When radioactive label was incorporated into both chains, the label on H chain was lysine and on L chain it was tyrosine. These results, taken together with those of Figure 1 again illustrate the restriction imposed by the

Reagent		Labeled residue and chain	
		%Tyr (L)	%Lys (H)
BADE	DNP-NH-CH_2-CH_2-NH-X	96	4
BADB	DNP-NH-CH_2-CH_2-CH(COOH)-NH-X	87	13
BADO	DNP-NH-CH_2-CH_2-CH_2-CH(COOH)-NH-X	66	34
BADL	DNP-NH-CH_2-CH_2-CH_2-CH_2-CH(COOH)-NH-X	5	95

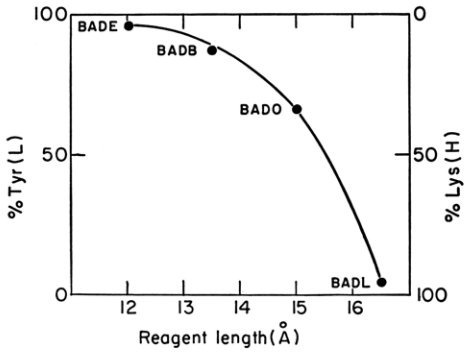

FIGURE 5: The specificity of affinity labeling of protein-315 by different reagents. The upper part of the figure gives the proportion of peptide chain and amino acid residue labeled by the different reagents. This was determined from heavy and light chains isolated on sodium dodecyl sulfate polyacrylamide gels (see Materials and Methods). The lower part gives a schematic representation of the relation between the above specificity and the size of reagents. X = $COCH_2Br$.

anti-DNP combining site on the reaction of the protein residues with the bromoacetyl group. A difference of 3 Å in length (between BADB and BADL) results in a marked change in specificity, in both residue modified and chain labeled.

The kinetics of reaction with either tyrosine (light chain) or lysine (heavy chain) by each of the reagents can be estimated from the values for k_2 (Table I) and the amount of label introduced into each of the residues (Figure 5). The data (Table I) clearly show that with increasing length of reagent the rate of reaction with tyrosine decreases and the rate of reaction with lysine increases.

If indeed the specificity of the chemical reaction is a function of the positioning of the bromoacetyl group, the foregoing results indicate that the distance between the labeled lysine (heavy chain) and the labeled tyrosine (light chain) is approximately equal to the difference in length (about 5 Å) between BADE and BADL (Figure 5).

Cross-Linking of the Heavy and Light Peptide Chains. Consideration of the above results led to the prediction that a bifunctional reagent with two bromoacetyl groups separated by a distance equal to the difference in length between BADE and BADL, would react simultaneously with both lysine and tyrosine and thus would cross-link the heavy and light chains. The bifunctional reagent DIBAB, $DNPNH(CH_2)_2CH(—NHCOCH_2Br)CO(NH)_2COCH_2Br$, almost satisfies this requirement.

Figure 6 shows the kinetics of labeling of the myeloma protein by DIBAB. After treatment of the protein (1 mg/ml) with DIBAB (4 moles/mole of protein) in 0.1 M $NaHCO_3$ (pH 9.0), 37° for 24 hr, sodium dodecyl sulfate and β-mercaptoethanol

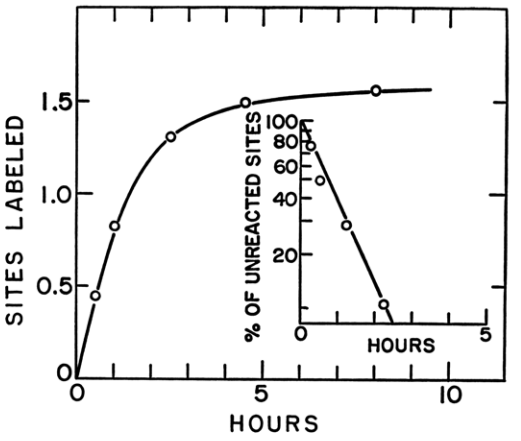

FIGURE 6: Kinetics of labeling of protein-315 by the bifunctional reagent DIBAB. Conditions are as in Figure 1. Insert: replotting the data to indicate the apparent first-order rate constant of reaction as in Figure 2.

were added to a final concentration of 1% and 0.14 M, respectively. After 3 hr of further incubation at 37° the solutions were dialyzed against the buffer used for the sodium dodecyl sulfate acrylamide electrophoresis supplemented with glycerol (30%), β-mercaptoethanol (0.14 M), and bromophenol blue (0.002%) and analyzed. The electrophoretic pattern of samples treated with either DIBAB or monofunctional reagents (Figure 7) clearly shows the appearance of a new band in the DIBAB-treated protein. The position of this band corresponds to cross-linked light chain–heavy chain with a molecular weight of 72,000. Figure 7B shows the scanning pattern of the stained acrylamide gel together with the distribution of radioactivity in the gel. About 50% of the protein chains became cross-linked as an H–L covalent complex and this band also contained about 50% of the bound reagent as measured by radioactivity. Most of the additional radioactivity was present in the heavy-chain fraction. Analysis of the [^{14}C]CM-amino acids in the acid hydrolysate of the DIBAB-labeled protein revealed only CM-tyrosine and CM-lysine. The H–L band

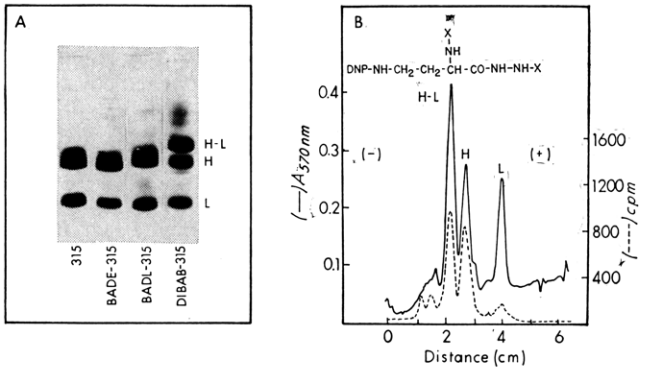

FIGURE 7: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of DIBAB-, BADL-, and BADE-labeled protein-315. The conditions for labeling and sodium dodecyl sulfate acrylamide electrophoresis are given in the text. Protein (100 μg) in 100 μl was applied to the gel. After the run, the gel was stained, and either scanned at 570 nm or sliced to 50 slices (1.3 mm) which were dissolved in Soluene and counted in toluene scintillant. (A) Electrophoretic pattern of affinity-labeled protein-315, protein migration from top to bottom. (B) Distribution of protein and radioactivity in the gel of DIBAB-labeled protein. H, heavy chain; L, light chain. X = $COCH_2Br$.

contained both CM-lysine and CM-tyrosine (51 and 49%, respectively), whereas the H band contained predominantly CM-lysine (89%).

Discussion

Some of the advantages of the bromoacetyl group as the reactive portion of affinity-labeling reagents are evident in this study. This group may be incorporated into a variety of haptens, beginning with commercially available radioactively labeled bromoacetic acid. The affinity-labeling reagents thus made can introduce the label into a number of different amino acid side chains (tyrosine, lysine, histidine, methionine, cysteine), and most of the CM derivatives formed in this manner are stable to acid hydrolysis. The CM derivatives are easily and rapidly separated and quantified. Finally, bromoacetyl derivatives of haptens of various length and configuration make it possible to map the relative position of labeled residues in the region of the specifically bound haptenic group.

Evidence that these labeling reagents react specifically at or near the hapten combining site is provided by a number of observations. The reagents do not react with normal immunoglobulins, and the reaction with protein-315 or anti-DNP antibodies is strongly inhibited by DNP-lysine. The reaction is almost stoichiometric, yielding close to one labeled group per combining site. The chemical reaction of the bound reagent apparently depends on whether or not its exact positioning permits it to approach the reactive amino acid residue.

Not only do these reagents exhibit selective reactivity in regard to the particular amino acids they modify, but the kinetics of the reaction also suggest the necessity of an exact positioning of the reactive bromoacetyl group in the vicinity of the residue to be labeled. BADE and BADL react quite well with protein-315, but the reagents of intermediate length, BADB and BADO, react poorly even though they bind with approximately the same affinity to the protein (Table I). On the other hand, BADO and BADB can effectively carry out affinity labeling of rabbit and goat anti-DNP antibodies (P. H. Strausbauch, unpublished results). In protein-315, it is clear that the rate of reaction with a particular amino acid residue depends on the length of the labeling reagent. Thus, BADE reacted 80 times faster with the tyrosyl residue on the light-chain as compared to the heavy-chain lysyl residue, and BADL reacted at least 21 times faster with the lysyl residue than with tyrosyl residue (Table I). Reagents which are too short (BADH) or too long (BADGL) failed to react covalently. These results strongly support the view that the labeling reactions are sterically directed in the reversible complexes of protein and reagent.

Further support is provided by the results with the bromoacetyl derivatives of DNP-D- and L-lysine (Figure 1). L-BADL labeled the heavy-chain lysine, but D-BADL did not react chemically at all, though protein-315 has the same intrinsic affinity for both reagents.

The reduced fluorescence yield of the affinity-labeled proteins (Table II) and the spectral shift of the covalently bound ligand (Figure 4) indicate that the DNP group can occupy that part of the combining site which is specific for DNP while the bromoacetyl function reacts with tyrosine or lysine. Evidence to be presented elsewhere shows that the labeled tyrosyl residue is at position 34 of the light-chain (Tyr_{34}) while the labeled heavy-chain lysyl residue has been tentatively placed at position 54 (Lys_{54}).

The series of labeling reagents examined in this study suggests that the distance between the side chains of labeled lysine and tyrosine residues is approximately 5 Å (the difference in length between BADL and BADE). Since these two residues are on different peptide chains the bifunctional reagent DIBAB, designed on the basis of these data, proved capable of reacting with both residues, thus cross-linking the heavy and light chains. It should be noted that this cross-linking is intramolecular and only between heavy and light chains. The scanning pattern of the gels (Figure 7) showed that about 50% of the protein was cross-linked. This is less than expected if both functional groups of all the covalently bound reagent (Figure 6) had reacted. Most of the remaining covalently bound reagent, which did not cross-link chains, was found on the heavy chains. This may be due to the fact that the terminal bromoacetyl function corresponds to that of BADL, in respect to distance from the DNP group, whereas the bromoacetyl at the middle of DIBAB resembles that of BADB, which labels less well than BADE (Figure 1). It is of interest that, whereas the labeling by either BADL or BADE is specific for residue and chain (Haimovich *et al.*, 1970), the labeling by DIBAB yielded both CM-tyrosine and CM-lysine in the cross-linked H–L component. This is the first report of cross-linking of protein peptide chains which is directed by a site-specific reagent.

Because the distances between the bromine substituents and the DNP group in the affinity-labeling reagents are fairly great (9 Å for BADE and 13 Å for BADL) and the reagent is flexible, it is not possible to state with precision how far Tyr_{34} is from the specifically bound DNP moiety. However, the previous result of Goetzl and Metzger (1970) that Tyr_{34} is labeled by a *m*-nitrobenzenediazonium salt strongly suggests that Tyr_{34} must be within a few Å of the DNP combining site. Since our findings with DIBAB place Tyr_{34} about 5 Å from the labeled lysine of the heavy chain (Lys_{54}), the results provide a preliminary view of the topology of the combining region of protein-315, with apparent intimate participation of the heavy and light chains.

References

Crestfield, A. M., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 2413.

Cautrecasas, P., Wilchek, M., and Anfinsen, C. B. (1969), *J. Biol. Chem. 244*, 4316.

Eisen, H. N., Simms, E. S., and Potter, M. (1968), *Biochemistry 7*, 4126.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Goetzl, E. J., and Metzger, H. (1970), *Biochemistry 9*, 3862.

Gressel, J. B., and Wolowelsky, J. (1968), *Anal. Biochem. 24*, 157.

Haimovich, J., Givol., D., and Eisen, H. N. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1656.

Kitz, R., and Wilson, I. B. (1962), *J. Biol. Chem. 237*, 3245.

Korman, S., and Clark, T. H. (1956), *J. Biol. Chem. 221*, 133.

Little, J. R., and Eisen, H. N. (1967), *Biochemistry 6*, 3119.

Shapiro, A. L., Vinuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.

Shaw, E., and Glover, G. (1970), *Arch. Biochem. Biophys. 139*, 298.

Singer, S. J., Slobin, L. I., Thorpe, N. O., and Fenton, J. W., II (1967), *Cold Spring Harbor Symp. XXXII*, 99.

Weinstein, Y., Wilchek, M., and Givol, D. (1969), *Biochem. Biophys. Res. Commun. 35*, 694.

Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.