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Localization of Affinity-Labeled Residues on the Heavy and Light Chain of Two Myeloma Proteins with Anti-Hapten Activity†

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ABSTRACT: Two mouse myeloma proteins with anti-2,4-dinitrophenyl (Dnp) activity (proteins-315 and -460) were affinity labeled with bromoacetyl derivatives of Dnp ligands. Bromoacetyl-N^{ϵ}-Dnp-L-lysine labeled a lysyl residue on the heavy chain of protein-315, and bromoacetyl-N^{1}-Dnp-ethylenediamine labeled a tyrosyl residue on the light chain of protein-315 and a lysyl residue on the light chain of protein-460. Partial sequencing of the isolated labeled tryptic peptides indicated that lysine-54 and tyrosine-34 were the labeled residues on heavy and light chains, respectively, of protein-315, whereas lysine-54 was the labeled residue on the light chain of protein-460. All of the labeled residues are located within restricted hypervariable segments of the variable regions of their respective chains, suggesting that the specific combining sites of the intact immunoglobulins are formed by amino acid residues of the hypervariable segments. A tryptophan residue was present in each of the affinity-labeled tryptic peptides and the Dnp moiety of the peptides from protein-315 showed a red shift in absorption spectrum, resembling that observed when Dnp ligands are specifically bound noncovalently in the combining sites of the intact protein or in those of conventionally raised anti-Dnp antibodies. The results suggest that in the isolated peptides (and perhaps in the intact protein as well) the Dnp group makes contact with Trp-49 or Trp-37 of heavy and light chain, respectively, of protein-315.

Previous work (Haimovich *et al.*, 1970; Givol *et al.*, 1971) has shown that the bromoacetyl derivatives of N^{ϵ}-Dnp-L-lysine (BADL)[1] and of N^{1}-Dnp-ethylenediamine (BADE) combine covalently with a myeloma protein having anti-Dnp activity (protein-315, produced by mouse plasmacytoma MOPC-315, Eisen *et al.*, 1968). It was demonstrated that BADL reacted with a lysyl residue on the heavy chain and that BADE reacted with a tyrosyl residue on the light chain of this protein. The labeled residues on the light and heavy chains are probably separated by a distance of 4–5 Å in the intact protein since the two chains could be cross-linked covalently by a bifunctional reagent that contained the bromoacetyl group at two positions, one resembling its position in BADE and the other its position in BADL (Givol *et al.*, 1971). These studies also provided evidence that the particular residues labeled by the bromoacetyl moieties of several different Dnp reagents are determined by the specific binding of the Dnp moiety, which continues to occupy the protein's combining site after the covalent bond is formed (Givol *et al.*, 1971; Haimovich *et al.*, 1972).

The present study was undertaken to determine the actual positions of the labeled lysyl and tyrosyl residues in the amino acid sequences of the heavy and light chains. We have also extended this analysis to a second Dnp-binding myeloma protein to determine if the differences in affinity and specificity of the two proteins are reflected by differences in their affinity labeling by these reagents. Protein-460, a mouse IgA immunoglobulin secreted by plasmacytoma MOPC-460, was selected for this purpose. Except for its unusually high affinity for 2,4-dinitronaphthol, this protein has lower affinity than protein-315 for a variety of Dnp ligands (Jaffe *et al.*, 1971). Nevertheless its reaction with Dnp ligands is accompanied by the general features of conventionally induced anti-Dnp antibodies, *i.e.*, quenching of the protein fluorescence and a red

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[1] Abbreviations used are: BADL, N^{α}-bromoacetyl-N^{ϵ}-Dnp-L-lysine; BADE, *N*-bromoacetyl-N^{1}-Dnp-ethylenediamine; Dnp, 2,4-dinitrophenyl; Gdn·HCl, guanidine hydrochloride.

shift in absorption spectrum of the bound ligand (*e.g.*, Little and Eisen, 1967). Studies similar to those conducted on protein-315 showed that BADL labeled only 0.3 site/molecule, or 15% of the combining sites of protein-460, whereas BADE labeled 1.2 sites/molecule of this protein or 60% of its combining sites; and 75% of the BADE-labeled sites was localized on the light chain.

The present publication describes the isolation and sequencing of the labeled peptides from the heavy and light chains of protein-315 and from the labeled light chain of protein-460. The heavy chain of protein-460 could not be similarly analyzed because, as noted above, it was insufficiently labeled with the reagents used (BADE and BADL). Tryptic peptides with the covalently bound Dnp moiety were isolated in high yields (65–90%) by affinity chromatography on anti-Dnp-Sepharose columns (Wilchek *et al.*, 1971). The positions of the labeled residues were localized largely on the basis of sequence homology with other immunoglobulins. They were found to be lysyl-54 and tyrosyl-34 on the heavy and light chains, respectively, of protein-315, and lysyl-54 on the light chain of protein-460. These results suggest that the combining sites of immunoglobulins are made up of amino acid residues from the hypervariable segments in the variable regions of immunoglobulin chains (Wu and Kabat, 1970; Kabat and Wu, 1972).

Materials and Methods

Affinity Labeling of Protein-315 and Protein-460. Protein-315 was purified from serum of tumor-bearing mice as described (Goetzl and Metzger, 1970). The labeling reagents [^{14}C]BADE and [^{14}C]BADL were prepared according to Strausbauch *et al.* (1971). The specific activity of BADL was 3.1×10^6 cpm/μmole and of BADE was 4.8×10^6 cpm/μmole. The conditions for affinity labeling of protein-315 with BADL were similar to those used by Haimovich *et al.* (1970), except that the protein concentration was 0.5 mg/ml and the molar ratio of reagent to protein as 1.3:1.0. The reaction was performed in 0.05 M $NaHCO_3$ (pH 9) at 37° for 20 hr, after which the reaction mixture was brought to pH 6.5 with acetic acid and concentrated 30-fold on a Diaflo membrane (PM10) at 4°. The concentrated solution was dialyzed against 0.1 M acetic acid and lyophilized. Protein-315 (200 mg) was also affinity labeled with BADE as described (Haimovich *et al.*, 1970). This labeled protein was precipitated with $(NH_4)_2SO_4$ at 40% saturation, redissolved in water, dialyzed against 1 M acetic acid, and lyophilized.

In preliminary experiments protein-460 was reacted with BADE and BADL as described (Haimovich *et al.*, 1970). After 20 hr, BADE labeled 1.2 sites and BADL labeled only 0.3 site per molecule of protein. In both cases the label was distributed between light and heavy chains in a ratio of 3:1. However, contrary to the findings with protein-315 (Haimovich *et al.*, 1970; Givol *et al.*, 1971) the BADE-labeled residues in protein-460 were predominantly lysine on the light chain and tyrosine on the heavy chain. In addition, 30 and 20% of the label on heavy and light chain, respectively, were found to be on cysteine, possibly as a consequence of incomplete alkylation and persistence of some SH groups in the purified protein. As heavy-chain labeling by BADE represented so small a proportion of combining sites we have confined our attention to the localization of the BADE-labeled lysine residue on the light chain of protein-460. The large-scale procedure used to label protein-460 (200 mg) with BADE was the same as for protein-315 (Haimovich *et al.*, 1970).

Isolation of Heavy Chain from BADL-Labeled Protein-315. Preliminary experiments on reduction by dithiothreitol of the disulfide bonds of BADL-labeled protein-315 in 7 M Gdn·HCl or in 9 M urea, indicated that under these conditions there was modification of the Dnp group, leading to diminished binding by anti-Dnp antibodies. Only 20–50% of the label present in a tryptic digest of the reduced-alkylated protein was adsorbed by an anti-Dnp column (Givol *et al.*, 1970). On the other hand, when a tryptic digest was prepared from performic acid oxidized protein, 98% of the labeled peptides were specifically adsorbed by the anti-Dnp column. The effect of reduction on the Dnp group was partly overcome by adding N^{ϵ}-Dnp-aminocaproate during reduction. Reduction was used in preference to performic acid oxidation to avoid destruction of tryptophan and some other residues. Accordingly, for the isolation of BADL-labeled heavy chain, lyophilized protein-315 was dissolved (25 mg/ml) in 7 M Gdn·HCl–0.2 M Tris-HCl (pH 8.2) with N^{ϵ}-Dnp-aminocaproate at 0.5 mg/ml. To this solution dithiothreitol was added to give final concentration of 7 mM. After 30 min at room temperature the solution was alkylated with iodoacetamide (20 mM) for 20 min and dialyzed for 4 hr against 8 M urea–1 N propionic acid. The dialyzed solution was applied to a Sephadex G-100 column (3×140 cm) equilibrated and run with 8 M urea–1 N propionic acid, to separate heavy and light chains. The isolated heavy chain was dialyzed against 0.001 M HCl to remove urea and was digested with trypsin directly, or after lyophilization.

Isolation of Light Chains from BADE-Labeled Proteins-315 and -460. Labeled protein-315 and protein-460 were completely reduced and aminoethylated in 7 M Gdn·HCl according to Goetzl and Metzger (1970), and light and heavy chains were separated on Sephadex G-100 in 6 M urea–1 M acetic acid (Haimovich *et al.*, 1970). Aminoethylation was used, instead of alkylation with iodoacetamide (as in the heavy chain), because it aids fractionation of light-chain peptides (Schulenburg *et al.*, 1971). The labeled light chain was extensively dialyzed against 0.001 M HCl in preheated dialysis tubing (80° for 3 days) and then subjected to enzyme digestion.

Preparation and Isolation of Affinity-Labeled Peptides. The labeled heavy chain of protein-315 was digested with trypsin in 0.1 M NH_4HCO_3 (pH 8) at 37° with a 1:50 (w/w) ratio of enzyme to protein. Labeled light chains from proteins-315 and -460 were digested with trypsin for 2 hr in a pH-Stat at pH 8.6. Trypsin digestions were terminated by adding soybean trypsin inhibitor in equal weight to trypsin.

Labeled peptides were isolated in high yield (65–90%) from tryptic digests by affinity chromatography on Sepharose columns with covalently attached goat anti-Dnp antibodies (Givol *et al.*, 1970; Wilchek *et al.*, 1971). After collecting the unadsorbed peptides and washing the column, the Dnp-labeled peptides were eluted with 7 M Gdn·HCl or with 20% formic acid. They were then passed through Sephadex G-50 columns to remove Gdn·HCl or formic acid, and some eluted antibodies. Details are given in Figures 1 and 2. Further purification of the tryptic peptides, or of peptides derived from them, was accomplished, when necessary, by high-voltage paper electrophoresis at pH 3.5 or 6.5. Peptides were located on paper by staining with 0.25% ninhydrin and with specific stains for tyrosine (nitrosonaphthol), tryptophan (Ehrlich's reagent), and arginine (Ireverre, 1965). The Dnp-labeled peptide was located by its color (yellow), or by autoradiography. Peptides were eluted from paper with water or with 0.01 M NH_4OH.

Amino Acid Sequence of Purified Peptides. Sequence determinations were performed according to the Edman-dansyl

procedure (Edman, 1956; Gray, 1967). Dansylamino acids were identified by thin-layer chromatography on polyamide sheets (Cheng Ching Trading Co. Ltd., Taiwan) (Woods and Wang, 1967). In some instances the results were confirmed by analysis of the amino acid regenerated upon hydrolysis (6 N HCl, 20 hr, 150°) of the phenylthiohydantoin derivative obtained at each cycle of the Edman degradation (Van Orden and Carpenter, 1964). C-Terminal residues were determined by digestion with carboxypeptidases, followed by dansylation of the released amino acids, or by hydrazinolysis (Bradbury, 1956). Amino acid analyses were performed as described by Moore and Stein (1963). Peptides were hydrolyzed with 6 N HCl in sealed evacuated tubes at 110° for 24 hr. No corrections were made for destruction during hydrolysis. Tryptophan was determined by acid hydrolysis in the presence of thioglycolic acid (Matsubara and Sasaki, 1969) and also from the content of individual peptides as determined by staining with Ehrlich's reagent. The labeled lysine or tyrosine residues yielded upon acid hydrolysis carboxymethyl (CM)-lysine or CM-tyrosine, respectively, which emerged at known positions from the amino acid analyzer (Haimovich *et al.*, 1970).

Enzymes and Other Enzyme Digestions. Digestion of peptides with carboxypeptidases was performed in 0.2 M *N*-ethylmorpholine acetate (pH 8.5) for 2–3 hr at 37°, using 5 μg of enzyme/25 nmoles of peptide in 30 μl. Controls (enzyme alone and peptide alone) were run in parallel. The digests were subjected to dansylation, or directly applied to the amino acid analyzer.

Trypsin (TPCK treated), α-chymotrypsin (twice crystallized), carboxypeptidases A and B, and soybean trypsin inhibitor were purchased from Worthington. Thermolysin (three-times crystallized) was from Calbiochem.

Physical Measurements and Other Procedures. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer using dioxane-containing scintillant. Difference spectra were obtained on a Cary Model 14 spectrophotometer. Performic acid oxidation was carried out according to Hirs (1967). Blocking of amino groups by reaction with citraconic anhydride was performed according to Dixon and Perham (1968).

Reagents. Gdn·HCl, Ultra Pure, was purchased from Mann. Iodoacetamide (Fluka) was recrystallized prior to use. Dithiothreitol was obtained from Calbiochem. Hydrazine (>95%) was from Eastman Kodak and was distilled before use. Trifluoroacetic acid was obtained from Fluka. Phenyl isothiocyanate (British Drug Houses) was distilled before use. Sephadex and Sepharose were from Pharmacia, Uppsala.

Results

Labeled Lysine in the Heavy Chain of Protein-315

Isolation of the Labeled Tryptic Peptide. The extent of labeling of protein-315 with BADL and the presence of the labeled lysine residue on the heavy chain were described previously (Haimovich *et al.*, 1970; Givol *et al.*, 1971). In the experiment reported here, 390 mg of protein-315 (2.6 μmoles) was reacted with 3.4 μmoles of [^{14}C]BADL, and 0.9 mole of the labeling reagent became covalently bound per mole of protein. After separation of the fully reduced and alkylated chains 92% of the label was found with the heavy chain. After digestion with trypsin the labeled heavy chain (285 mg) was applied to a 10 × 2 cm column of anti-Dnp-Sepharose whose elution pattern is shown in Figure 1. The fraction that was unadsorbed by the column contained 88% of the absorbance at 280 nm and only 14% of the radioactivity. Upon elution with

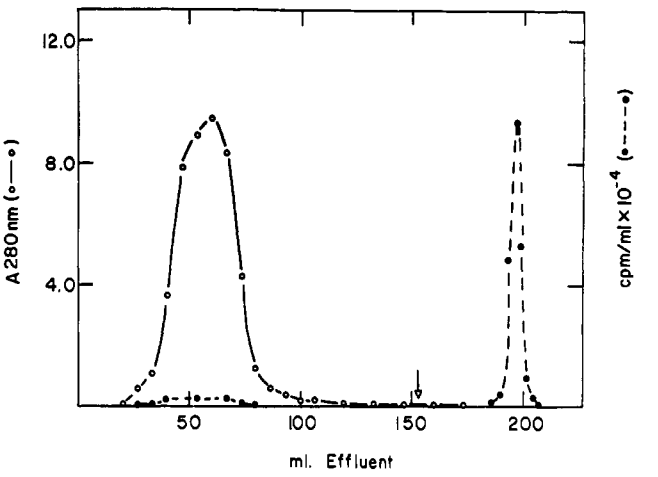


FIGURE 1: Isolation of the Dnp-labeled peptide from the BADL-labeled heavy chain of protein-315 using anti-Dnp-Sepharose column. A tryptic digest (39 ml) of 285 mg of heavy chain, containing 2.4 μmoles of Dnp label, was passed through a Sepharose column (2 × 10 cm) which contained 400 mg of goat anti-Dnp antibodies. The column was equilibrated and run with 0.1 M NH_4HCO_3. After the unadsorbed fraction had emerged the specifically adsorbed radioactive peptide was eluted with 7 M Gd·HCl (arrow).

7 M Gdn·HCl, 95% of the adsorbed radioactive material was desorbed from the antibody column and was applied to a Sephadex G-50 column equilibrated and run in 0.1 M NH_4HCO_3. The elution pattern of this column (Figure 2) demonstrates that about 7% of the radioactivity emerged at the front of the column together with some protein, which probably represents antibodies eluted by the Gdn·HCl from the antibody column (Wilchek *et al.*, 1971). Most of the radioactivity (90%) emerged from the column as a single fraction just before

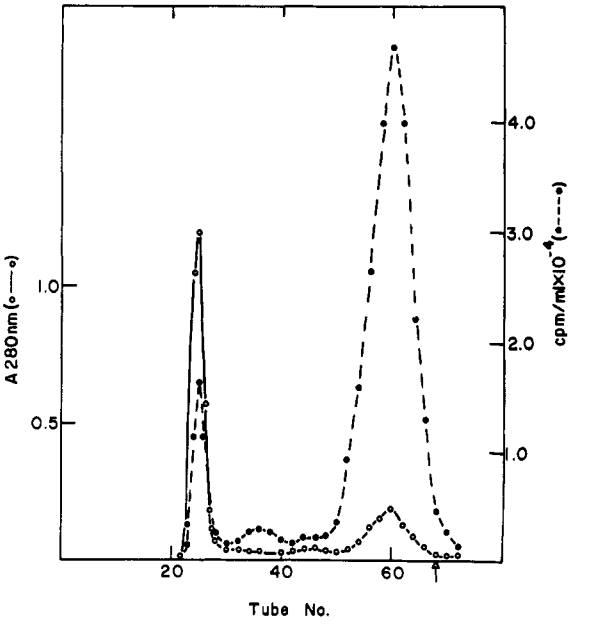


FIGURE 2: Further purification on the labeled peptide from BADL-labeled heavy chain of protein-315. The labeled material eluted with Gdn·HCl from the antibody column (Figure 1) was applied to a Sephadex G-50 column (2 × 120 cm) equilibrated and run in 0.1 M NH_4CHO_3. Fractions of 15 ml were collected. The material in tubes 53–65 was collected and used for further experiments. The arrow marks the appearance of Gdn·HCl.

TABLE I: Amino Acid Composition of Peptides from BADL-Labeled Heavy Chain of Protein-315.[b]

Residue	T	Th1	Th2	Th3	Th4
Lys	1.00	1.00			
Asp	3.06				3.20
Ser	1.75				1.96
Glu	1.20			1.10	
Pro	0.97				1.20
Gly	3.00		0.97		2.00
Ile	0.90				0.98
Leu	2.78	0.97	1.00	1.00	
Tyr	1.70				2.00
Phe	1.12		1.00		
Trp	1			1	
CMLys	0.92				1.00
Total	20	2	3	3	12
m, pH 3.5[a]		0.80	0.14	0.05	−0.16
m, pH 6.5[a]			0.00	−0.40	−0.38
Ehrlich's reagent	+	−	−	+	−
Nitrosonaphthol	+	−	−	−	+

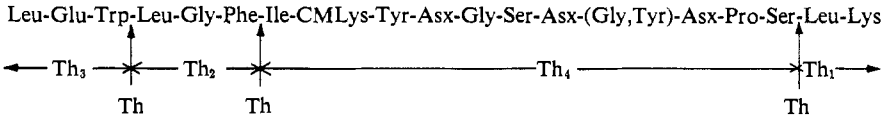
[a] Mobility (*m*) was calculated *vs.* the mobility of lysine (+1) at pH 3.5 and of aspartic acid (−1) at pH 6.5. [b] T is the affinity-labeled tryptic peptide and Th the thermolysin peptides derived from it.

the Gdn·HCl. This material was found to contain only one labeled peptide (1.6 μmoles, or 67.5% of the initial covalent label). This peptide was contaminated with some nonradioactive peptides which were removed by high-voltage electrophoresis at pH 3.5. The radioactive yellow material did not move from the origin and was eluted (70% yield) with 0.01 M NH_4OH and lyophilized.

Sequence of the Labeled Tryptic Peptide. Amino acid analysis of the radioactive peptide eluted from paper is given in Table I. It is a lysyl peptide of 20 residues, including 1 CM-lysine. The N-terminal residue was found to be Leu (by dansylation) and the C-terminal sequence was found to be Leu-Lys (by carboxypeptidases B and A). No other residues were released by longer incubation with carboxypeptidases. Six cycles of Edman degradation of the tryptic peptide indicated the following N-terminal sequence: Leu-Glu- ?-Leu-Gly-Phe-Ile. The residue at the third position was found to be Trp in a thermolysin derived peptide (see below).

Digestion of the tryptic peptide with thermolysin yielded four peptides which were isolated by high-voltage paper electrophoresis at pH 3.5. Their compositions are given in Table I. PEPTIDE Th1 (Leu,Lys) has N-terminal Leu and is the C-terminal peptide Leu-Lys. PEPTIDE Th2 (Gly,Leu,Phe) has the sequence Leu-Gly-Phe (by Edman-dansyl) and is compatible with residues 4–6 from the N terminus. PEPTIDE Th3 (Leu,-Glu,Trp) is the only tryptophan-containing peptide. Carboxypeptidase A released only Trp which was identified as DNS-Trp, and the N-terminal sequence was found to be Leu-Glu. From its mobility at pH 6.5 (Table I) it is clear that the second residue is glutamic acid and not glutamine. Hence the sequence Leu-Glu-Trp corresponds to the N-terminal sequence of the tryptic peptide. PEPTIDE Th4 (Asp_3,Ser_2,Pro_1,Gly_2,Ile_1,Tyr_2,-CM-Lys_1) was the only radioactive peptide in the thermolysin digest. Carboxypeptidase A digestion did not release any residue but hydrazinolysis identified Ser (60% yield) as the C-terminal residue. This suggests Pro-Ser as the C-terminal sequence, which is resistant to carboxypeptidase A. A portion of this peptide (0.15 μmole containing 4.65×10^5 cpm) was subjected to Edman degradation. Most of the yellow color, due to the Dnp group, was removed in the butyl acetate extract of the first cycle. This, however, was not accompanied by significant radioactivity in the extract. The amount of radioactivity present in the butyl acetate extract of each of the six Edman cycles was respectively 7800, 410,000, 14,000, 4500, 2700, and 2700 cpm. This indicates that the labeled CM-Lys residue is at the second position of peptide Th4.

The sequence of the first seven residues of this peptide was found to be Ile-CM Lys-Tyr-Asx-Gly-Ser-Asx. At the seventh cycle most of the peptide was extracted into the butyl acetate, preventing further analysis. The C-terminal sequence of Th4 was not determined. However, from the labeled tryptic peptide which was obtained from a performic acid oxidized heavy chain, a chymotryptic peptide with the following composition was isolated: $Lys_{1.00}$,$Asp_{0.98}$,$Ser_{1.12}$,$Pro_{1.20}$,$Leu_{0.92}$. The C-terminal sequence of this peptide was found by carboxypeptidase B and A to be Leu-Lys and the N-terminal sequence was found to be Asx-Pro-Ser. Hence the sequence of this peptide, Asx-Pro-Ser-Leu-Lys, is compatible with the C-terminal composition of peptide Th4 and with the sequence of peptide Th1. We deduce that the sequence of Th4 is Ile-CMLys-Tyr-Asx-Gly-Ser-Asx-(Gly,Tyr)-Asx-Pro-Ser, and that the sequence of the isolated tryptic peptide is



Sequence of the Citraconylated Peptide. In order to extend the sequence around the labeled lysine residue an attempt was made to obtain a larger labeled peptide. The labeled, reduced-alkylated heavy chain was treated with citraconic anhydride (3:1 weight ratio of anhydride to protein) in 0.1 M $NaHCO_3$ (pH 8.5). The pH was maintained with 5 N NaOH. After removal of excess reagent by dialysis the heavy chain was digested with trypsin and the labeled peptide was isolated on an anti-Dnp-Sepharose column and Sephadex G-50 as described for the tryptic peptide. Four cycles of Edman-dansyl degradation showed the N-terminal sequence Glx-Phe-Pro-Gly-Asx for this peptide. The blocking citraconyl groups were removed by incubating the peptide in 0.02 M HCl for 8 hr at room temperature. The peptide precipitated immediately after acidification but became completely soluble at the end of the incubation period. It was brought to pH 8, digested with trypsin, and the digest was fractionated on a Sephadex G-50 column equilibrated and run in 0.05 M NH_4OH (Figure 3). The compositions of the citraconylated peptide, as well as the tryptic peptides derived from it, are given in Table II.

PEPTIDE T_1 was identical with the tryptic peptide obtained previously. PEPTIDE T_2 (Asp_1,Glu_1,Pro_1,Phe_1,Lys_1) was found by dansyl-Edman to have the sequence Gln-Phe-Pro-Gly-Asn-

TABLE II: Amino Acid Composition of Peptides from BADL-Labeled Heavy Chain of Protein-315.[a]

Residue	Cit	T_1	T_2	T_3
Lys	1.97	1.17	1.07	
Arg	1.08	0.15		1.00
Asp	4.25	2.90	1.00	0.97
Ser	2.25	2.62		
Glu	3.12	2.00	1.04	
Pro	1.90	1.20	0.93	
Gly	4.27	3.15	1.04	
Ile	1.12	1.10		
Leu	3.12	3.25		
Tyr	2.00	1.80		
Phe	1.90	1.00	1.00	
Trp	1	1		
CMLys	0.97	0.95		
Total	*Ca.* 28	*Ca.* 21	6	2
m, pH 6.5			0.31, 0.00	0.49
Sakaguchi	+	−	−	+
Ehrlich	+	+	−	−

[a] Cit is the citraconylated affinity-labeled tryptic peptide and T_1, T_2, T_3, the tryptic peptides derived from it after removal of citraconyl groups. See Figure 3.

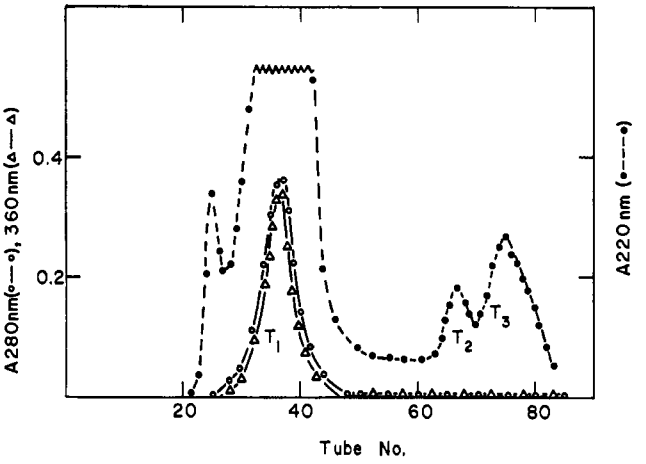
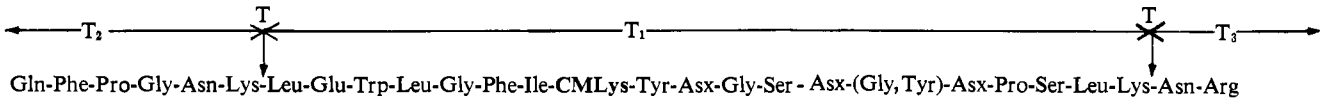


FIGURE 3: Separation of tryptic peptides from the labeled, citraconylated peptide obtained from BADL-labeled heavy chain of protein-315. After removing citraconyl groups the peptide (see text) was digested with trypsin and run on a Sephadex G-50 column in 0.05 M NH_4OH. Fractions of 10 ml were collected.

Lys, which is compatible with the N-terminal sequence of the citraconyl peptide. Upon electrophoresis at pH 6.5 this peptide gave two ninhydrin-positive spots, which on elution were found to have the same composition (Table II). One of the peptides was neutral and had a blocked N-terminal residue (no dansyl residue detected), presumably due to cyclization of Gln duration the incubation at acid pH. The other peptide, with N-terminal glutamine, was basic, indicating that both dicarboxylic acids were in amide form.

PEPTIDE T_3 (Asp_1,Arg_1) had N-terminal Asp. From its mobility (Table II) it must be Asn-Arg. It represents the C-terminal sequence of the citraconylated peptide.

The above analyses demonstrate that the sequence around the labeled lysine residue in the heavy chain of protein-315 is as follows



This sequence has considerable homology (35–50% identical residues) with the region 41–69 in the heavy chains of human myeloma proteins of subgroup II, indicating that the labeled lysine is at position 54 from the N terminus (Figure 4). This is supported by the finding that CNBr cleavage of unlabeled and [^{14}C]BADL-labeled heavy chain yielded a purified radiolabeled fragment that corresponded to the N terminal *ca.* 160 residues of this chain, including its entire V_H segment (Francis *et al.*, 1972).

Labeled Tyrosine in the Light Chain of Protein-315

Protein-315 labeled with BADE contained 1.4 moles of label/mole of protein and 95% of this label was present on the light chain. Hydrolysis and paper electrophoresis (Haimovich *et al.*, 1970) confirmed the earlier finding that the labeled residue was Tyr (see CM-Tyr, Table III). The reduced and aminoethylated light chain was digested with trypsin and the labeled peptide was isolated on an anti-Dnp-Sepharose column. The isolated tryptic peptide thus obtained contained 76% of the radioactivity initially present in the labeled protein. Its amino acid analysis was identical with that of the labeled tryptic peptide obtained by Goetzl and Metzger (1970) from the light chain of protein-315 affinity labeled with *m*-nitrobenzenediazonium fluoroborate (Table III).

By the dansyl method the N terminal of the BADE-labeled peptide was found to be serine and carboxypeptidase B digestion showed that the C-terminal residue was arginine, in agreement with similar data of Goetzl and Metzger (1970) on the labeled peptide they isolated. The light chain of protein-315 contains only two tyrosine residues, one at position 34 and the other at 88 (Schulenburg *et al.*, 1971). From the composition of the BADE-labeled peptide it was clear that the labeled tyrosine could only be Tyr-34, the same residue that was labeled by *m*-nitrobenzenediazonium (Goetzl and Metzger, 1970).

Spectrum of the Labeled Tryptic Peptides Isolated from Protein-315. Since the binding of Dnp-haptens by anti-Dnp antibodies is accompanied by a red shift in absorption spectrum of the Dnp group (Little and Eisen, 1967), the spectrum of Dnp attached to the isolated peptide was examined. Figure 5A demonstrates that the Dnp group, covalently attached to the BADL-labeled tryptic peptide, showed a red shift (ΔE_M at 455 nm = 760 and ΔE_M at 386 nm = 1200), which was similar to that observed with conventional anti-Dnp antibodies and with intact protein-315 (Givol *et al.*, 1971; Eisen *et al.*, 1968). If the shift is due to an intramolecular, low-affinity interaction between the peptide's tryptophan (Trp 49, Figure 4) and Dnp residue (bound to Lys-54, Figure 4) the difference spectrum should disappear after splitting the peptide with thermolysin (see sequence of BADL-labeled peptide). As is shown in Figure 5B this is indeed the case. The BADE-labeled peptide isolated from the light chain of protein-315 also showed a spec-

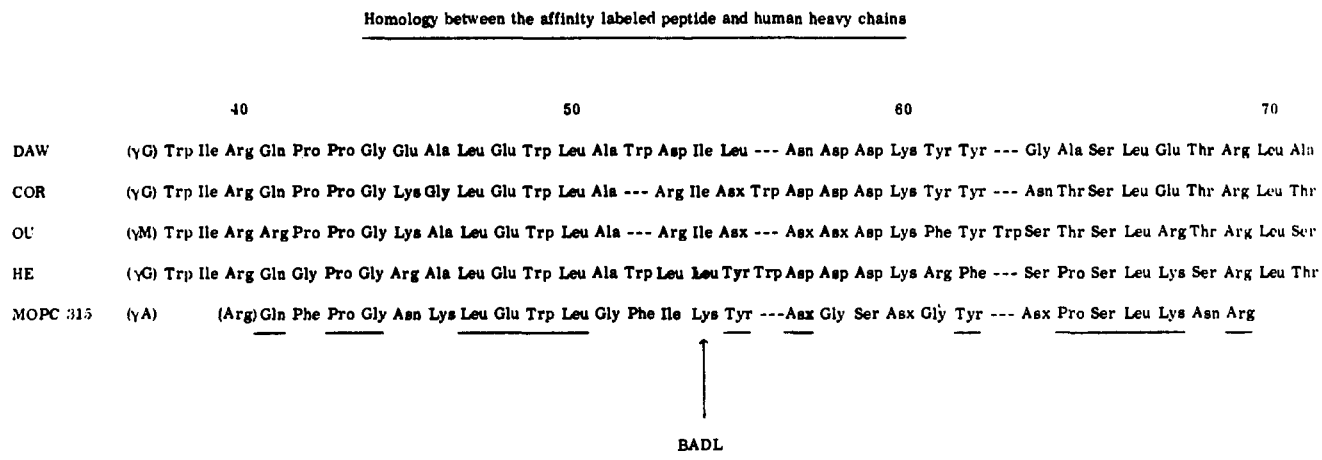


FIGURE 4: Sequence homology between the BADL-labeled peptide from the heavy chain of protein-315 and heavy chains of four human immunoglobulins. The sequences for the human chains are from Press and Hogg, 1970 (DAW and COR), Putnam *et al.*, 1971 (OU), and Cunningham *et al.*, 1969 (HE). Underlined residues are identical with protein HE or DAW. Residues are numbered according to Cunningham *et al.* (1969).

tral red shift with maxima at 450 nm (ΔE_M = 530) and at 384 nm, probably due to interaction between Trp at position 37 and the Dnp attached to Tyr-34 (Figure 6; see Goetzl and Metzger, 1970; Schulenburg *et al.*, 1971).

Labeled Lysine in the Light Chain of Protein-460

Isolation of Labeled Tryptic Peptide. Protein-460, affinity labeled with BADE, contained 1.2 moles of label/mole of protein; 75% of this label was present on the light chain and after hydrolysis with 6 N HCl the labeled residues on the light chain were identified as CM-lysine (80%) and CM-cysteine (20%).

The labeled, reduced and aminoethylated, light chain was digested with trypsin. A precipitate formed during the digestion contained 80% of the radioactivity present in the light chain, and all of the precipitated radioactivity was associated with lysine ([^{14}C]CM-lysine after hydrolysis). The precipitate was washed with 0.14 M NaCl–0.01 M potassium phosphate (pH 7.0) and then dissolved in 0.1 M acetic acid. The pH was

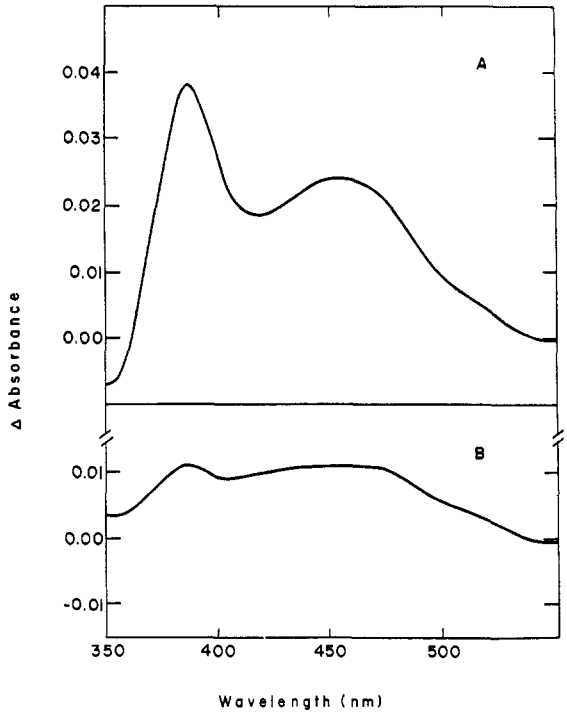


FIGURE 5: Difference absorption spectrum of the labeled peptide obtained from BADL-labeled heavy chain of protein-315. The labeled peptide and free BADL were at 3.15 × 10^{-5} M in 0.1 M NH_4HCO_3. The concentration of the peptide was based on the specific radioactivity of BADL. The difference spectrum was measured in a Cary Model 14 spectrophotometer. (A) Before digestion with thermolysin; ΔE_M at 455 nm was approximately 760; ΔE_M at 386 nm was approximately 1200. (B) After digestion with thermolysin.

TABLE III: Amino Acid Compositions of the Labeled Tryptic Peptides Isolated from Affinity-Labeled Light Chain of Protein-315.

Residue	From Protein Labeled with BADE[a]	From Protein Labeled with *m*-Nitrobenzenediazonium Fluoroborate[b]
Lys	1.14	1.00
His	1.00	0.98
Arg	0.95	1.02
Asp	3.70	3.82
Thr	4.63	4.60
Ser	3.51	3.27
Glu	2.07	2.12
Pro	1.20	1.12
Gly	4.23	4.06
Ala	2.15	2.09
Val	1.15	1.00
Ile	1.93	1.84
Leu	2.10	1.91
Tyr	0.93[c]	1.00
Phe	1.03	0.95
Trp	1.20[d]	0.73
Total	33	33

[a] From this work. [b] From Goetzl and Metzger (1970). [c] Determined as CM-Tyr. [d] Determined by staining and spectrophotometrically; not corrected for light scattering.

TABLE IV: Amino Acid Composition of the BADE-Labeled Tryptic Peptide (T) from the Light Chain of Protein-460, and the Chymotryptic (C) and Thermolysin (Th) Peptides Derived from It.[a]

Residue	T	C1	C2	C3	C4	C5	CLP[b]	ThLP[b]
Lys	1.13				0.95			
His	1.90		0.91	1.02				
Arg	1.00						0.92	
Asp	2.92		2.00				1.02	
Thr	0.93		0.85					
Ser	4.72	2.03	0.95		0.91		0.97	
Glu	4.00	1.00			3.00			
Pro	2.20				2.08			
Gly	2.25		1.17		1.03			
Val	1.87		0.95				1.03	
Ile	1.02					1.00		0.73
Leu	4.93	0.96		1.00	2.94			
Tyr	2.64		0.70			0.76		0.79
CM Lys	0.93						1.00	1.00
Trp	+[d]			+[d]				
Total	34	4	8	2	11	2	5	3
Yield[c] (%)		17	25	69	24	41	95	40

[a] The composition of the tryptic peptide is the average for four different preparations; that of chymotryptic peptides is the average of two different preparations. Free tyrosine was also obtained on gel filtration and high-voltage paper electrophoresis of the chymotryptic digests. [b] CLP and ThLP are chymotryptic and thermolysin BADE-labeled peptides. [c] The amount of tryptic peptide digested was taken as 100%. Peptides C3 and C5 were purified by gel filtration or high-voltage electrophoresis only. Peptides C1, C2, and C4 were further purified by paper chromatography. [d] Identified by staining with Ehrlich's reagent and absorbance at 278 nm. Trp was obtained also by CpA digestion of C3.

raised to pH 5.0 with 1 M NaOH and the solution was applied to an anti-Dnp-Sepharose column. The adsorbed Dnp-labeled peptide was eluted with 20% formic acid and resolved from impurities on a Sephadex G-50 column equilibrated and run in 20% formic acid. The isolated peptide contained 92% of the radioactive label present in the precipitate of the light-chain digest.

Sequencing of the BADE-Labeled Tryptic Peptide from the Light Chain of Protein-460. Because the whole tryptic peptide was insoluble in $NaHCO_3$, dansylation was performed in presence of 4 M urea (Gray, 1967). The insoluble dansylated peptide was washed with 0.2 M $NaHCO_3$ and hydrolyzed for 18 hr in 6 N HCl. The N-terminal amino acid was serine. Digestion of the tryptic peptide by carboxypeptidase B liberated arginine.

The isolated labeled tryptic peptide was lyophilized and dissolved in 0.2 M acetic acid. Ammonia was added to adjust the pH to 8.5 (at this pH the peptide was completely insoluble) and the peptide was digested with either chymotrypsin or thermolysin (1:50 weight ratio) for 4 hr at 37°. (In one preparation chymotrypsin digestion was carried out for 19 hr.) After adding diisopropyl fluorophosphate to a final concentration of 0.001 M (in order to inactivate the enzymes and to avoid digestion of antibody molecules on the adsorbent), the chymotrypsin or thermolysin digests were applied to an anti-Dnp-Sepharose adsorbent and the effluent, containing the unlabeled peptides, was saved for subsequent studies (below). The retained, labeled peptides were eluted with 20% formic acid and further purified on Sephadex G-50 column equilibrated and developed with 20% formic acid. One labeled chymotryptic peptide was obtained; however, the thermolysin digest yielded several labeled peptides, which were resolved by high-voltage paper electrophoresis at pH 3.5. The amino acid compositions of the labeled tryptic peptide, the chymotryptic peptides derived from it, and the principal labeled thermolysin peptide are given in Table IV.

The unlabeled peptides from the chymotryptic digestion,

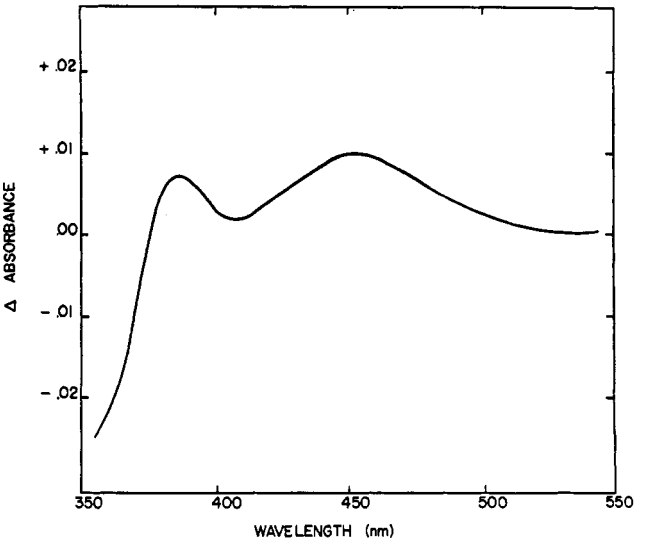


FIGURE 6: Difference spectrum of labeled peptide from BADE-labeled light chain. The peptide and BADE were at a concentration of 1.7×10^{-5} M in 0.14 M NaCl–0.01 M potassium phosphate (pH 7.0). ΔE_M at 450 nm was appryximately 530.

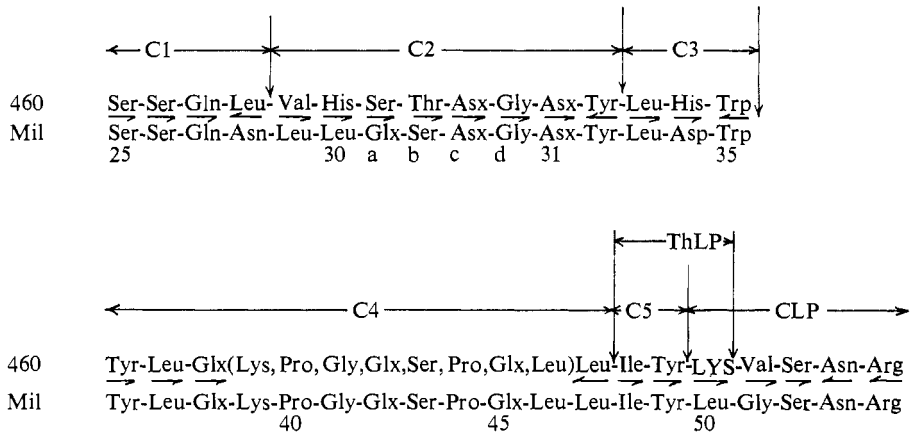


FIGURE 7: Partial sequence of the BADE-labeled tryptic peptide from the (kappa) light chain of protein-460, and its homology with human (kappa) Bence-Jones protein Mil. The chymotryptic (C) peptides (Table IV) derived from the BADE-labeled tryptic peptide are arranged to maximize homology with protein Mil (Dreyer *et al.*, 1967). The labeled lysine is in capitals. → Edman degradation; ← carboxypeptidase digestion. a, b, c, and d designate inserted residues in some light chains at these position (Milstein and Pink, 1970). Though it is labeled 50, the labeled lysine is the 54th residue from the N terminus. In the text this lysine is referred to as Lys-54. ThlP and CLP are labeled peptides derived by thermolysin and chymotrypsin, respectively, from the tryptic peptide.

which had passed unretarded through the anti-Dnp-Sepharose adsorbent (600 nmoles), were lyophilized, redissolved in 1 ml of 0.1 M HN_4HCO_3, and separated by either gel filtration on a Sephadex G-25 Superfine column (1 × 135 cm), equilibrated and developed with 0.1 M NH_4HCO_3, or by high-voltage paper electrophoresis at pH 3.5. Further purification of some of these peptides was achieved by ascending paper chromatography in a butanol–acetic acid–water system. The amino acid compositions of the peptides thus purified are also given in Table IV.

Sequencing of the Chymotryptic Peptides. The amino acid sequences of the purified chymotryptic peptides were determined by Edman-subtractives and Edman-dansyl methods and by carboxypeptidase A and B digestion. In Figure 7 the peptides are aligned for maximal homology with protein Mil, a human κ chain (Dreyer *et al.*, 1967) whose sequence is strikingly similar to that of the tryptic peptide.[2] Further support for this alignment was obtained from the composition of the thermolysin peptide (Table IV) and from sequencing of the tryptic peptide on an automatic sequencer.[2] The results indicate that the labeled lysyl residue in the light chain of protein-460 is at position 54 from its N terminus, which corresponds to residue 50 in some other light chains with deletions of four residues after position 30 (a, b, c, and d in Figure 7) (Milstein and Pink, 1970).

Discussion

The results demonstrate that bromoacetyl derivatives of Dnp ligands label unique residues in the variable regions of proteins-315 and -460. Only a single labeled peptide was isolated from either the heavy chain of BADL-labeled protein-315 or from the light chain of this protein after labeling with BADE. The light chain of BADE-labeled protein-460 contained some labeled cysteine (20%), but most of its label was also localized in a single peptide.

The positions of the labeled residues are summarized in Table V, along with results from other similar studies. It is notable that Tyr-34 in the light chain of protein-315 was labeled by both *m*-nitrobenzenediazonium (Goetzl and Metzger, 1970) and by BADE, which in its extended configuration is about 6 Å longer than the diazonium reagent. It seems likely therefore that BADE assumes a coiled configuration when it is bound in the protein's combining site, allowing the bromoacetyl moiety to approach the same residue as the reactive group of the diazonium reagent. This suggests that BADL might also be found in the coiled configuration, allowing it, in spite of its considerable extended length (*ca.* 17 Å), to label residues that are close to the site at which the Dnp moiety is bound.

In each of the four affinity-labeling experiments with the two myeloma proteins, and in practically every study with conventionally raised anti-Dnp antibodies (Table V), the labeled residues fall within one of the restricted hypervariable segments of the variable domains of light or heavy chain (Franek, 1971; Milstein, 1967; Wu and Kabat, 1970; Kabat and Wu, 1972; Kehoe and Capra, 1971).

The hypervariable segments occupy positions 24–34, 50–56, and 89–97 of light chains, and positions 31–35, 50–65, 81–87, and 95–102 of heavy chains (Kabat and Wu, 1972); altogether they add up to about 30% of the residues in variable segments. If affinity-labeling reagents were to combine with residues anywhere in the variable segments (V_L or V_H), the probability would be less than 0.01 that in four out of four experiments (with two myeloma proteins and three different reagents) all labeled residues would fall in just the hypervariable segments. It would seem, rather, that residues in these restricted segments of light and heavy chains actually constitute the specific combining sites, and it is for this reason that they are labeled so consistently. Other indications that the hypervariable segments form the specific combining sites are provided by the remarkable similarity of sequences in these sections in diverse immunoglobulins with similar binding specificities (Capra and Kunkel, 1970).

The lysyl residues that were labeled in the heavy chain of protein-315 and in the light chain of protein-460 are replaced by other residues in the sequences of many other light and heavy chains (Dayhoff, 1969, also Figures 4 and 7). Hence

[2] We thank Dr. L. Hood for pointing out the similarity between the labeled peptide from protein-460 and a segment of the Mil κ chain. The automatic sequencer results establishing the sequence of the light chain of protein-460 from the N terminus to position 35 will be given in a separate communication with Dr. L. Hood.

TABLE V: Affinity-Labeled Residues in Various Anti-Dnp Immunoglobulins.

Protein	Reagent	Labeled Residue	Reference
315	BADL	Lys-54 on H chain	This work
315	BADE	Tyr-34 on L chain	This work
460	BADE	Lys-54 on L chain	This work
315	MNDB[a]	Tyr-34 on L chain	Goetzl and Metzger (1970)
Pig anti-Dnp	MNDB	Tyr-33 and Tyr-93 on L chain	Franek (1971)
Rabbit anti-Dnp	MNDB	Tyr-96 on H chain	Singer and Thorpe (1968)
Mouse anti-Dnp	MNDB	Tyr-86 on L chain	Thorpe and Singer (1969)
Guinea pig anti-Dnp	MNDB	Tyr-33 and positions between 99 and 119 on H chain	Cebra *et al.* (1972)

[a] MNDB = *m*-nitrobenzenediazonium fluoroborate.

these residues seem not only to be present in the combining site but they probably help determine its specificity. The same argument applies less forcibly to Tyr-34, labeled in the light chain of protein-315, because this amino acid is present at this position (designated 32 in the numbering used by Wu and Kabat, 1970) in about 50% of all light chains. While selective labeling at variable and especially at highly variable positions is understandable, affinity labeling at an invariant position has also been reported: an early study by Singer and Doolittle (1966) suggested that Tyr-86 of light chain was specifically labeled (by nitrobenzenediazonium) in a preparation of mouse anti-Dnp antibodies; and Tyr occurs at position 86 in all (20/20) light chains (Wu and Kabat, 1970). While it may appear paradoxical that an invariant residue should be affinity labeled, it is apparent that invariance in linear sequence need not correspond to invariance in three-dimensional position. With different neighbors and in different V_L segments, Tyr 86 could occupy quite different three-dimensional positions and contribute in different ways to different sites, or not contribute at all. Another possibility is that light-chain Tyr-86 is part of a limited, conservative region in the combining sites of all antibodies, regardless of their specificity, as suggested by Singer and Doolittle (1966).

It is of interest that while BADL labeled Lys-54 in the heavy chain of protein-315, it did not label the neighboring Tyr-55 (Figure 4). Similarly, BADE labeled Lys-54 in the light chain of protein-460, but not the neighboring Tyr-53. It seems likely, therefore, that in each of these chains the tyrosine hydroxyl group is not vicinal to the neighboring ϵ-amino of Lys-54 in the combining site of the intact protein. Besides emphasizing the extraordinary dependency of the covalent labeling reaction on steric factors, these findings imply that the Dnp reagent is highly immobilized when bound specifically (noncovalently) in the active site, and that the amino acid residues that make up the site are also fixed in a relatively rigid configuration. If the polypeptide chains were flexible in this region, or if the bound ligand were mobile, some labeling of Tyr-55 and Tyr-53 (on the heavy and light chains of proteins-315 and -460, respectively) should have been found.

The absorption spectra of the labeled peptides isolated from protein-315 provide additional information on the protein's combining site. The red shift in the absorbance of the Dnp group implies interaction with the indole ring of a tryptophanyl residue (Little and Eisen, 1967) in each of these peptides. Though not proved, it is likely that Trp-49 in the peptide isolated from the heavy chain (Figure 4) forms an intramolecular charge-transfer complex with the Dnp moiety covalently bound to lysine-54. Trp-37 in the light chain of protein-315 probably forms a similar complex with the Dnp group attached to Tyr-34 of this chain, since the corresponding peptide also exhibits the red shift (Figure 6). It is possible, but by no means certain, that in the combining site of the intact immunoglobulin Trp-34 of the light chain or Trp-49 of the heavy chain, or both of these Trp residues, interact with noncovalently bound Dnp ligand and account for its red shift (Eisen *et al.*, 1968; Givol *et al.*, 1971). In complexes of the charge-transfer-type donor and acceptor groups approach to approximately within the sum of their van der Waals radii. Hence if both of these tryptophan residues interacted with the same molecule of specifically bound Dnp it would mean that in the intact combining site these heavy- and light-chain Trp residues would lie within about 3–5 Å of each other. That other residues from the two chains can be in such close proximity was previously suggested by affinity-labeling studies with a bifunctional reagent that covalently linked the chains of protein-315 through attachment to a tyrosine residue on the light (presumably Tyr-34) and a lysine residue on the heavy chain (presumably Lys-54) (Givol *et al.*, 1971).

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Peptide Comparison of Two Histocompatibility-2 (H-2b and H-2d) Alloantigens†

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ABSTRACT: The peptide composition of two H-2 alloantigen glycoproteins (class I) of different antigenic profile was compared by several methods. In one set of studies, nonradioactively labeled class I alloantigen fragments from spleens of *H-2*^{b} and *H-2*^{d} strain animals were cleaved by CNBr and the resultant peptides compared by ion-exchange chromatography. Approximately 90% of the peptides were similar, and 10% were different. In another set of studies, peptides radiolabeled with either [^{3}H]- or [^{14}C]amino acids were prepared by CNBr cleavage and trypsin digestion of H-2b and H-2d alloantigens which had been purified by a combination of conventional methods as well as immunological techniques. Comparison of the double-label peptide patterns showed a sharing of about 80–90% of the peaks and differences in about 10–20%. Such findings confirm and extend previous data using different techniques and sources of antigen, and support the hypothesis that the H-2 antigenic sites are determined by primary amino acid structure. The great similarity of most of the peptides stresses the anticipated similarity to be expected from products of allelic forms of the same genetic region.

The murine H-2 histocompatibility transplantation alloantigens comprise a complex system of immunologically identifiable glycoproteins which are integrated into the cellular membrane. These antigens are controlled by a genetic region on the IXth mouse linkage group—a region which is exemplified by its extreme degree of polymorphism (Klein and Shreffler, 1971; Snell and Stimpfling, 1966).

Considerable information about the antigens has been obtained through the study of immunologically active glycoprotein fragments released from their membrane location by papain proteolysis. Two classes (class I and class II) of glycoprotein fragments (85% protein and 70% carbohydrate) carrying H-2 antigenic sites were isolated from each of two mouse strains (*H-2*^{b} and *H-2*^{d}), differing in their known H-2 specificity profile (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970a,b). The glycoprotein fragments of each class appeared to be the gene product of one of the two postu-

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