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Affinity Labeling of a Phosphorylcholine Binding Mouse Myeloma Protein†

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ABSTRACT: The phosphorylcholine binding γA myeloma protein of the Balb/c plasmacytoma TEPC 15 was immunospecifically purified. A phosphorylcholine analog, *p*-diazonium phenylphosphorylcholine, was synthesized and used an an affinity-labeling reagent. More than 90% of the specific modification occurred on the light chains. The most substantially labeled residue was a light-chain tyrosine. This residue is in a homologous position to the tyrosine which became labeled in similar studies performed on a nitrophenyl binding mouse myeloma protein. This finding suggests: (1) that all immunoglobulin "antigen" binding sites are located in the same topological sector of the Fab region, (2) that the tyrosine which becomes labeled may not be chemically neutral, and (3) that when present, this residue plays a similar role in antibodies of diverse specificities.

A considerable number of human and mouse myeloma proteins have been discovered which have definable binding activities. Because such immunoglobulins are homogeneous and usually available in substantial amounts they are potentially useful for helping to define the structural basis of antibody specificity. One set of such proteins—those that bind nitrophenyl ligands—is being explored in several laboratories. Particularly by the technique of affinity labeling, it has already been possible to directly implicate certain sections of the light- and heavy-chain variable regions as playing a critical role in the structure of the combining sites.

It would be advantageous to explore in similar detail a set of immunoglobulins which bind very different ligands than those referred to above. Such studies would be helpful in delineating those features of immunoglobulin combining sites which may be common to many or all immunoglobulins and those which are unique to sites reacting with a particular determinant or set of determinants. The group of Balb/c mouse γA myelomas which share the property of binding phosphorylcholine should be useful in this regard. The studies of Potter and Leon (1968), Potter and Lieberman (1970), and particularly Leon and Young (1971) have shown that several of these proteins, while having related binding activities, are clearly distinguishable on the basis of their binding constants for phosphorylcholine and its analogs.

The study reported here is the first of a series in which we have used the technique of affinity labeling to explore the combining sites of these proteins. A phosphorylcholine analog, *p*-diazonium phenylphosphorylcholine (DPPC),[1] was used to specifically modify the combining sites of phosphorylcholine binding protein from the tumor, TEPC 15. A highly labeled peptide was isolated and sequenced, and this has permitted us to define its location in the primary structure of the protein.

Materials and Methods

Tumor Lines. The mouse plasmacytoma TEPC 15 was obtained from Dr. Michael M. Potter and was maintained by subcutaneous passage in Balb/c mice. Ascites was collected by paracentesis of Balb/c or (Balb/c × C57BL/) F_1 mice which had been inoculated intraperitoneally with 0.5 ml of a tumor cell suspension 1 month earlier. In general 3–6 ml of ascites could be obtained per mouse.

p-Diazonium Phenylphosphorylcholine (DPPC). Methyl iodide (2 mmoles) was added to 20 mmoles of dimethylaminoethanol in 10 ml ether at 4° with stirring. The mixture was then stirred at room temperature for 18 hr. The white precipitate of choline iodide was washed with ether and dried. The yield was approximately 100%. The choline iodide, *p*-nitrophenyl phosphorodichloridate (2 mmoles) (Aldrich), and dry quinoline (2 mmoles) were each dissolved in 0.5 ml of dry acetonitrile, mixed, and stirred at 0° in the dark for 4–8 hr (Bird, 1967). Then 1.0 ml of pyridine and 0.2 ml of H_2O were added, and the solution was incubated at room temperature for 30 min. The solvents were flash evaporated, the residue was dissolved in H_2O, and the solution passed through a 40-ml Amberlite MB-3 column equilibrated with H_2O. The effluent was lyophilized. The product was analyzed by thin-layer chromatography on cellulose and silica gel (Eastman Chromogram) using either isopropyl alcohol–NH_4OH–H_2O (7:2:1, v/v) or 1-butanol–acetic acid–H_2O (5:2:4, v/v). In either case only a single component was observed. It was positive for phosphate (Hanes and Isherwood, 1949) and absorbed ultraviolet light. *Anal.* Calcd for $C_{11}H_{17}N_2O_6P_1$: C, 43.4; H, 5.7; N, 9.2; P, 10.2. Found: C, 43.5; H, 5.9; N, 9.4; P, 10.0. It had a melting point (cor) of 243°. The uv spectrum had a minimum at 237 nm and a maximum at 287 nm (Figure 1). The extinction coefficient, $\epsilon_{287.1\text{ cm}}$, was 1.05×10^4 at pH 7. This compound, *p*-nitrophenylphosphorylcholine, was stored

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[1] Abbreviations used are: DPPC, *p*-diazonium phenylphosphorylcholine; BBS, 0.2 M sodium borate buffer with 0.16 M NaCl; SDS, sodium dodecyl sulfate.

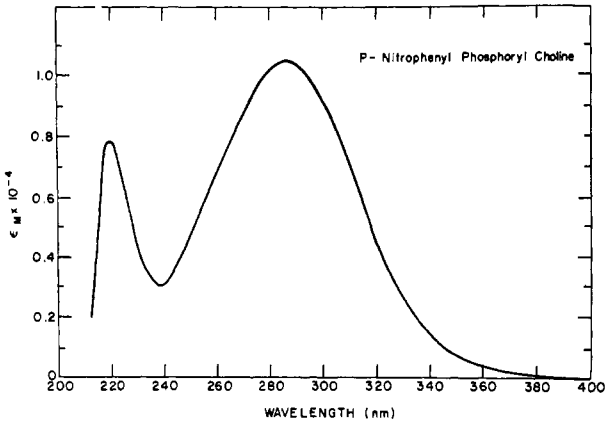


FIGURE 1: Spectrum of *p*-nitrophenylphosphorylcholine in 0.01 M sodium phosphate (pH 7.0).

dry at room temperature in the dark, and has been stable for over 9 months.[2]

p-Nitrophenylphosphorylcholine (100 mg, 0.3 mmole) was dissolved in methanol and quantitatively reduced with 1 atm of H_2 at room temperature for 1 hr in the presence of 10 mg of a 10% palladium on charcoal catalyst. The mixture was filtered on a sintered-glass funnel and flash evaporated. The amine oxidizes rapidly. It was therefore immediately dissolved in 2 ml of cold 0.1 M HCl and diazotized with $NaNO_2$ at 15–18°. The reaction was monitored with starch–KI paper to determine the exact amount of $NaNO_2$ necessary. The *p*-diazonium phenylphosphorylcholine (DPPC) solution was immediately frozen. At −80° it remained stable for 5 months.

The radioactive analog was made by employing [^{3}H]methyl iodide (50 mCi/mmole, New England Nuclear). The specific activity of *p*-nitrophenylphosphoryl[*methyl*-^{3}H]choline was determined by using the extinction coefficient determined at 287 nm and was 51.2 mCi/mmole. Radioactivity was determined in a Nuclear-Chicago liquid scintillation counter. The counting fluid was a mixture of toluene, Liquifluor (New England Nuclear), and NCS solubilizer (Amersham–Searle) (50:2:5, v/v). Quench corrections were made using the external standard source.

Mono-(p-azophenylphosphorylcholine)-N-acetyl-L-tyrosine. [^{3}H]DPPC (1 μmole) was added to 10 μmoles of *N*-acetyl-L-tyrosine in 1.0 ml of BBS and the solution left overnight at room temperature. The solution was chromatographed on thin-layer cellulose using the isopropyl alcohol–NH_4OH–H_2O solvent described above. A single major yellow component was observed and eluted with H_2O. It had a typical azotyrosine spectrum with a pH-dependent maximum at 475 nm (Figure 2). On the basis of the specific activity of the diazonium compound the azotyrosine analog had a molar absorbance at 475 nm of 1.26×10^4 in 0.1 M NaOH. The $\Delta\epsilon_{475nm}$ (base–acid) was 0.98×10^4. The pK_a of the phenolic hydroxyl, determined by spectrophotometric titration, was 9.3.

[2] An entirely different synthesis of this compound has been published by Kurioka (1968). The yield was very small and the melting point, 220° (uncor), was considerably lower than ours. Ultraviolet spectral parameters were not given. Still another type of synthesis has been employed by Ash Stevens Inc. (Detroit, Mich.) (personal communication). They prepared the chloride salt which is said to have a melting point of 167–168° (uncor). The wavelengths of maximum and minimum ultraviolet absorption are similar to ours though the molar absorptions are somewhat different ($\epsilon_{287\ nm}$ 9.9×10^3 and ϵ_{237} 2.0×10^3).

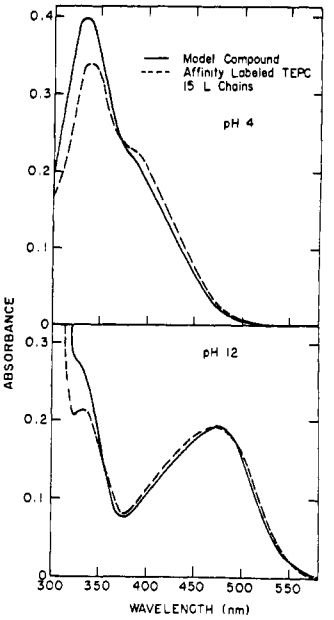


FIGURE 2: Spectra of mono-(*p*-azophenylphosphorylcholine)-*N*-acetyl-L-tyrosine (model compound) and purified light chains from affinity-labeled TEPC 15. The solvent was 0.1 M acetic acid–NaOH buffer (pH 4.0 and 12.0).

p-Diazonium Benzyldimethylhydroxyethylammonium Bromide. *p*-Nitrobenzyl bromide (3 mmoles) was dissolved in 15 ml of ether and dimethylaminoethanol (8 mmoles) in 2 ml of ether was added at 0° with stirring. The mixture was stirred overnight at room temperature. The *p*-nitrobenzyldimethylhydroxyethylammonium bromide was obtained as a white precipitate and was washed with ether and dried. The melting point (cor.) was 114°. *Anal.* Calcd for $C_{11}H_{17}BrN_2O_3$: C, 43.3; H, 5.6; Br, 26.2; N, 9.2. Found: C, 43.4; H, 5.6; Br, 24.7; N, 9.1.

This compound was reduced and diazotized according to the same procedures described above for *p*-nitrophenylphosphorylcholine. The *p*-diazonium compound was stored in solution in 0.1 M HCl at −80° and remained stable for over 6 months.

Immunoadsorbent. The dipeptide, glycyltyrosine (1.4 mmoles, Mann), was coupled to 400 ml of Sepharose 4B using the procedure of Cuatrecasas (1970). The gel was washed with pH 8.0 borate-buffered saline (BBS) and 0.4 mmole of DPPC was added. The mixture was stirred at room temperature overnight, washed with water, and stored in 1 M acetic acid at 4°. It has remained stable for more than 8 months.

Purification of TEPC 15 Protein. Ascites (100 ml) was brought to pH 8.6 with solid Tris and was reduced with 0.005 M dithiothreitol and alkylated with 0.011 M iodoacetamide (Goetzl and Metzger, 1970a). The solution was diluted 1:3 with BBS and applied to a 250-ml column of the immunoadsorbent (above). After washing the column with 2–3 l. of BBS until the effluent had an absorbance of less than 0.05 at 280 nm, the specifically bound protein was eluted with 250 ml of 10^{-3} M phosphorylcholine in BBS. The protein was concentrated to 20 mg/ml and dialyzed exhaustively against BBS. The yield from 100 ml of ascites was usually about 1.5 g.

Equilibrium Dialysis. Equilibrium dialyses were performed at room temperature in Lucite cells with 0.1-ml compartments (Eisen, 1971a) (Drummond) using phosphoryl[*methyl*-^{14}C]-choline (23 mCi/mmole, New England Nuclear).

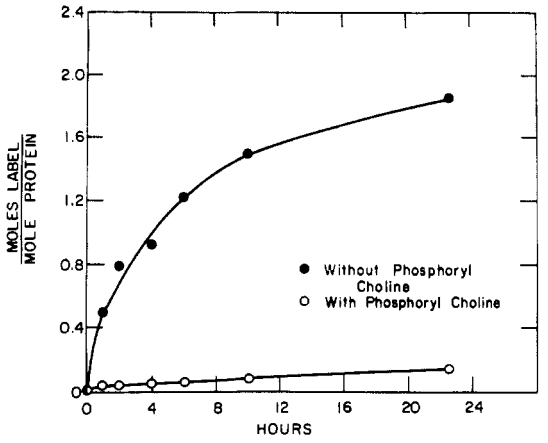


FIGURE 3: Kinetics of affinity labeling of TEPC 15 (5.0×10^{-6} M) with DPPC (3.0×10^{-5} M) in pH 8.3 BBS. Open circles indicate tubes containing disodium phosphorylcholine (5.0×10^{-3} M).

Extinction Coefficient. The extinction coefficient of purified TEPC 15 protein was determined by measuring the absorbance at 280 nm and determining the protein concentration by refractometry on a Brice-Phoenix refractometer assuming a refractive index increment of 0.00188 ml/10 mg (Kabat and Mayer, 1961). The $\epsilon^{0.1\%}_{280nm,\ 1\ cm}$ was 1.34.

Affinity Labeling. The purified protein of TEPC 15 at (0.5–5) $\times 10^{-5}$ M was reacted with [^{3}H]DPPC using 1–3 moles of reagent/mole of combining sites. The reaction was run at 0° in pH 8.3 BBS, and was stopped by addition of a 500-fold molar excess of resorcinol. In some cases, in order to attempt to protect the antibody sites from labeling and thus test the specificity of the labeling reaction, a 10- to 100-fold molar excess of disodium phosphorylcholine[3] was included in the reaction mixture. For analytical experiments the protein was precipitated with 10% trichloroacetic acid in the presence of a 100-fold molar excess of disodium phosphorylcholine, washed three times with 10% trichloroacetic acid, and then redissolved in 1% sodium dodecyl sulfate for determination of radioactivity and OD_{280}.

Peptide Analysis. Amino acid analysis on the Beckman 120C AutoAnalyzer and by paper electrophoresis, carboxypeptidase B digestion, hydrazinolysis, fingerprinting, and dansyl-Edman analysis were all performed as previously described (Goetzl and Metzger, 1970b). Thin-layer chromatography for identification of dansylamino acids was performed on a small scale using 5 × 5 cm polyamide sheets according to Konigsberg (personal communication). α-Dansylhistidine was identified by dissolving the lyophilized, hydrolyzed, dansylated peptide directly in absolute ethanol prior to analysis by thin-layer chromatography.

N-Bromosuccinimide Cleavage. N-Bromosuccinimide (100 nmoles) was added to 50 nmoles of peptide in 0.4 ml of 0.1 M acetic acid. The mixture was incubated at 25° for 3 hr, and then passed over a Sephadex G-10 column in 0.1 M acetic acid. The peptides obtained were analyzed for N-terminal amino acids by the dansyl chloride method in the usual manner.

Materials. Soybean trypsin inhibitor and trypsin which had been treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone were purchased from Worthington Biochemical Corp. and each was stored as a 1% solution in 0.001 N HCl at −20°. Thermolysin was purchased from California Biochemical Corp. and was dissolved in the appropriate buffer just prior to use. Carboxypeptidase B treated with diisopropyl fluorophosphate (Worthington) was stored at −20° as a stock solution (6.1 mg/ml) and diluted prior to use.

Dansyl-Cl and dansylamino acids were purchased from Pierce Chemical Co. Polyamide chromatography sheets were obtained from Cheng Chin Trading Co., Ltd. (Gallard-Schlesinger Chemical Manufacturing Co.). Other reagents and resins were the same as those described previously (Goetzl and Metzger, 1970b).

Results

Purification of TEPC 15 Protein. Immunospecifically purified protein gave a single line when assayed by immunoelectrophoresis *vs.* a potent anti-ascites antiserum. Using the extinction coefficient determined by refractometry (Methods) and assuming a molecular weight of 1.5×10^5 (Jaffe *et al.*, 1971) the protein had a valence of 1.9 and an association constant of 2.2×10^5 when assayed by equilibrium dialysis using phosphoryl[*methyl*-^{14}C]choline. The binding data when plotted by the method of Scatchard were linear.

Modification of TEPC 15 Protein with DPPC. The kinetics of the reaction of DPPC with TEPC 15 protein are shown in Figure 3. The presence of site-saturating concentrations of phosphorylcholine markedly reduced the rate of labeling. The reaction of DPPC with a nonphosphorylcholine binding protein was similarly slow. No reaction of DPPC with phosphorylcholine itself was observed.

For comparison, reactions of TEPC 15 with DPPC and two other diazonium reagents, *p*-diazonium benzoic acid and *p*-diazonium benzyldimethylhydroxyethylammonium bromide (*p*-diazonium phenyl analog of choline), were carried out in the presence and absence of phosphorylcholine. The reactions were stopped at 5 hr with resorcinol, and the proteins were trichloroacetic acid precipitated, washed, redissolved in 1% SDS, and analyzed for radioactivity (DPPC) or spectrally for azotyrosine groups by absorbancy at pH 5 and 12 at 475 nm using $\Delta\epsilon_{475}$ (base–acid) 0.78×10^4 for tyrosine azobenzoic acid and 1.0×10^4, assumed for the tyrosine azobenzyldimethylhydroxyethylammonium group. The results shown in Table I indicate only DPPC gives a significant amount of specific labeling. In both the other cases less than 0.08 mole of specific labeling was observed.

Properties of Labeled TEPC 15 Protein. The specifically labeled protein, with 2 moles of DPPC/mole of 7S TEPC 15 protein, when studied by equilibrium dialysis using phosphoryl[*methyl*-^{14}C]choline, showed total absence of measurable binding activity. On the other hand, a preparation reacted with DPPC in the presence of phosphorylcholine had binding properties indistinguishable from those of the native protein.

The distribution of the label bound to specifically modified TEPC 15 protein, was investigated on an analytical scale by disc electrophoresis in 7.5% polyacrylamide with 0.1% sodium dodecyl sulfate, and on a preparative scale by gel filtration using Sephadex G-100 equilibrated with 5 M guanidine hydrochloride. The results were the same by both methods and at all labeling reaction times studied (1–24 hr). Ninety per cent of the label was on the light chains, and ten per cent on the heavy chains. By contrast, a preparation reacted with DPPC in the presence of phosphorylcholine had 60% of the small amount of label associated with the heavy chains and 40% with the light chains.

[3] Disodium phosphorylcholine was prepared by ion exchange of calcium phosphorylcholine (Calbiochem) with Dowex 50 (Na^+) in 1% NaCl. The calcium was removed because it precipitated with borate ions at certain pHs and interfered with subsequent analysis.

TABLE I: Labeling of TEPC 15 by Various Diazonium Reagents.[a]

Labeling Reagent	Moles of Azotyrosine/Mole of Protein	
	Without Phosphorylcholine	With Phosphorylcholine
p-Diazonium phenylphosphorylcholine	1.24	0.20
p-Diazonium benzyldimethylhydroxyethylammonium bromide	0.39	0.32
p-Diazonium benzoic acid	0.91	0.84

[a] TEPC 15, 3.5×10^{-5} M, in pH 8.3 BBS. A 3- to 5-fold molar excess of labeling reagent over sites was used in each case. The reaction time was 5 hr at 0°. Samples to be protected from labeling contained 2.5×10^{-3} M phosphorylcholine.

Isolation of Labeled Peptide. Specifically labeled TEPC 15 protein (10^{-4} M) was reduced with 5×10^{-3} M dithiothreitol in 5 M guanidine hydrochloride, adjusted to pH 8.6 with Tris-HCl, for 40 min at room temperature. The protein was reacted with a 100-fold molar excess (over SH groups) of ethylenimine and then applied to a Sephadex G-100 column equilibrated with 5 M guanidine hydrochloride. The labeled light chains were isolated and the spectrum was examined at pH 4 and 12 (Figure 2). The spectra were virtually identical with those of the model compound, mono(*p*-azophenylphosphorylcholine)-*N*-acetyl-L-tyrosine. The light chains were then dialyzed against 0.1 M acetic acid and lyophilized.

This material was then redissolved in 1 ml of 10 M urea, diluted 1:7 with 0.2 M Tris-HCl (pH 8.0) containing 0.01 M $CaCl_2$ and digested with trypsin (1% w/w ratio) at 37° for 3 hr. The flocculated material was pelleted by centrifugation for 10 min at 1500 rpm, and soybean trypsin inhibitor (equal weight to the amount of trypsin used) was added to the separated supernatant. The precipitate was then redissolved in 10 M urea and rediluted and redigested with trypsin as before. Three such cycles were usually sufficient so that no flocculated material remained after the last digestion. The supernatant fractions were then pooled, lyophilized, redissolved in 1 M acetic acid, and applied to a Sephadex G-50 column equilibrated with 1 M acetic acid. A typical elution pattern is shown in Figure 4. The major peak representing about 47% of the applied and recovered radioactivity, was lyophilized and applied to a 10-ml CM-Sephadex (C-50) column equilibrated with 0.01 M pyridine acetate (pH 3.3). After thorough washing of the column, a gradient with 1.0 M pyridine acetate (pH 4.5) was begun. A single major radioactive component was eluted which represented 72% of the radioactivity applied. The eluate when examined by ninhydrin analysis showed no major peptide peaks on either side of the peak coeluting with the radioactivity. The amino acid composition of the radioactive peptide is shown in Table II.

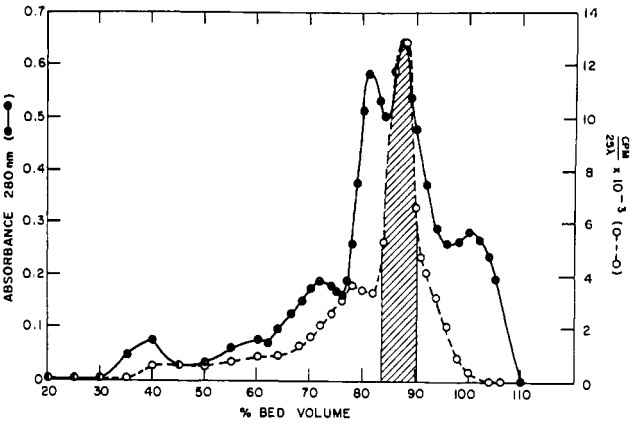


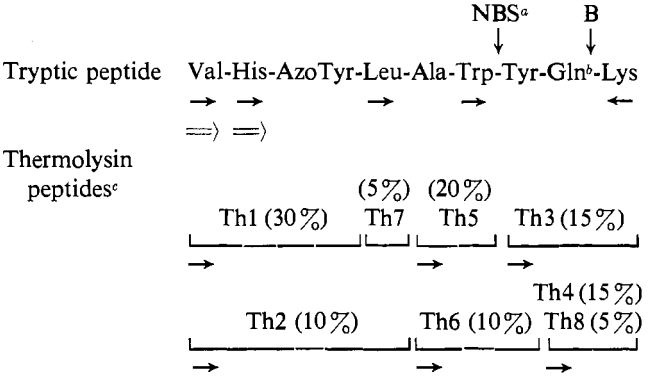
FIGURE 4: Sephadex G-50 fractionation of tryptic digest of affinity labeled TEPC 15 light chains in 1.0 M acetic acid. Shaded area indicates material used for further purification on CM-Sephadex (C-50).

TABLE II: Composition of Labeled Tryptic Peptide.

	Moles
AzoTyr	1.00[a]
Lys	1.12 ± 0.06
His	0.81 ± 0.15
Trp	0.82 ± 0.29
Glu	1.19 ± 0.15
Ala	1.08 ± 0.08
Val	0.86 ± 0.08
Leu	1.01 ± 0.06
Tyr	0.98 ± 0.07

[a] Azotyrosine was determined by radioactivity and the other amino acids were quantitated relative to it.

TABLE III: Sequence of Labeled Tryptic Peptide and Resulting Thermolysin Peptides.



Legend symbols: →, dansyl-Edman; ⟹, subtractive Edman; ← hydrazinolysis; B, carboxypeptidase B digestion.

[a] *N*-Bromosuccinimide cleavage. [b] Determination of Gln *vs.* Glu at this position was done by high-voltage paper electrophoresis at pH 6.5 in pyridine acetate buffer using thermolysin peptides Th3 and Th4. [c] Numbers in parentheses indicate approximate yield of each peptide.

TABLE IV: Comparison of Labeled Peptide Sequences from MOPC 315 and TEPC 15 to Mouse and Human κ-Chain Sequence Data for This Region.

	[a]30	31	32	33	34	35	36	37	38	39
MOPC 315 (λ)	-Ser-	Asp-	Tyr*-	Ala-	Ser-	Trp-	Ile-	Glu-	Glu-	Pro-
TEPC 15 (κ)	Val-	His-	Tyr*-	Leu-	Ala-	Trp-	Tyr-	Gln-	Lys	
Mouse and human κ[b]	Asn_6	Ser_8	Phe_3	*Leu_{12}*	*Ala_5*	*Trp_{16}*	*Tyr_{14}*	*Gln_8*	Gln_9	Lys_{12}
	Gly_4	Asn_4	*Tyr_7*	Met_4	Asn_4		Phe_1	Glx_5	Glx_6	Arg_1
	Asx_3	Asx_2	Asn_1	Ser_1	Asx_3		Leu_1	Leu_2		Gly_1
	Ile_2	Thr_2	Leu_1		Asp_1					Asx_1
	Ser_2	Lys_1	Trp_1		Gln_3					
	Lys_1	*His_1*			His_1					

[a] The numbering system used is from Wu and Kabat (1970). [b] Cumulated mouse and human κ data are from Wu and Kabat (1970), Melchers (1969), and D. J. McKean, M. Potter, and L. Hood (manuscript in preparation). Data are expressed as the total number of times each amino acid was observed at each position noted. The residues set in italic type indicate that this amino acid was also found at the corresponding position in TEPC 15.

Sequence Analysis of Labeled Peptide. The results of the sequence analysis are given in Table III. The carboxy-terminal residue, determined by both hydrazinolysis and carboxypeptidase B digestion, was lysine. Dansyl-Edman analysis of the amino-terminal end gave unequivocal results for positions 1, 2, and 4: Val-His-(?)-Leu. Position 3 gave no identifiable dansylamino acid. After *N*-bromosuccinimide cleavage two amino-terminal amino acids were found by dansylation: Val and Tyr.

The labeled peptide (150 nmoles) was digested with thermolysin (2% w/w) for 6 hr at 37° in a pH 8.2 0.1 M ammonium acetate buffer containing 0.01 M $CaCl_2$. The solution was successively chromatographed (butanol–acetic acid–H_2O, pH 3.6) and electrophoresed (pyridine acetate, pH 3.6) on Whatman No. 3MM paper. The ninhydrin-positive spots were cut out, eluted, and analyzed. The results are shown in Table III. These data were adequate to give an unambiguous sequence for the labeled tryptic peptide. All the predicted thermolysin cleavages were observed (Matsubara, 1970). This sequence is very similar to a section of the variable region of human and mouse myeloma protein light chains (Table IV) but to no other region of other light-chain sequences. Its approximate position in the TEPC 15 protein light chain can therefore be unambiguously defined.[4]

Discussion

The residue which was most substantially affinity labeled in the TEPC 15 protein is exactly homologous to the light-chain tyrosines, which were affinity labeled in similar studies on the nitrophenyl binding myeloma protein of plasmacytoma MOPC 315 and on pig anti-DNP antibodies. Not only do these proteins interact with vastly different ligands but also the labeling reagents used in the pertinent studies were substantially different: *m*-nitrophenyldiazonium (Goetzl and Metzger, 1970b; Franek, 1971), *N*-(2,4-dinitrophenyl)-*N'*-(bromoacetyl)ethylenediamine (Haimovich *et al.*, 1970; Eisen, 1971b), and *p*-diazonium phenylphosphorylcholine in the present work.

These results suggest to us the following. (1) The binding sites on the proteins from tumors MOPC 315 and TEPC 15 are located in approximately the same regions in the three dimensional structure of these proteins. Furthermore, we have previously cited the evidence (Goetzl and Metzger, 1970a) that the MOPC 315 protein site is located in the same region as the combining site on conventionally induced anti-dinitrophenyl antibodies. Together, these results suggest that all immunoglobulin combining sites are in the same location in the Fab region, and argue against the notion (Eisen *et al.*, 1967) that the combining sites on different immunoglobulins are located in different regions. (2) The residue in question may have some special reactivity not shared by other residues which participate in the combining site. It seems more than fortuitous that the same residue should become labeled despite the major stereochemical differences in the labeling reagents used. If steric factors were solely responsible for determining which residues became labeled one would have expected quite different results with the disparate reagents employed. Recent studies (Fuchs and Givol, 1968; Szpirer and Jeener, 1970; Otchin and Metzger, 1971) with the nitrating reagent, tetranitromethane, have shown that at a level of 1 nitrotyrosine/mole of Fab essentially all combining activity is lost. Although there is no evidence that the critical tyrosine in the nitration studies is the same as the light-chain tyrosine so far implicated in the affinity-labeling studies,[5] these findings do suggest that such special residues exist in antibody combining sites. This does not imply that the mechanism by which the pertinent residues became labeled was not basically of the affinity type. In all these studies, either by the use of a weakly cross-reacting but chemically similar reactive reagent, or by studying the reaction kinetics, or both, it could be shown that the labeling reagent was not simply reacting as a group specific reagent. The data do raise the possibility that the relevant tyrosine was labeled not only because it was near the reactive end of the reagent, but also because it was chemically more reactive compared to adjoining residues. (3) The relationship of the labeled tyrosine to the combining site is uncertain. As discussed elsewhere (Singer, 1967) the fact that the labeling reaction fulfills all the criteria of affinity labeling means that the labeled resi-

[4] The data shown in Table IV suggest to us that position 38 may actually be deleted in the TEPC 15 light chain, so that the COOH-terminal lysine would become homologous to the lysine usually seen at position 39. Sequence data on the residues immediately adjacent should resolve this ambiguity.

[5] In the study by Otchin and Metzger (1971) the relevant tyrosine appears primarily in the F_d' region. The locations of the tyrosines nitrated in the other studies have not been described.

due is in the combining site as *operationally* defined but does not necessarily mean it is in the site as defined *topologically*. In the absence of X-ray diffraction data the best that one can do is to employ an affinity-labeling reagent in which the reactive group is (a) as close to the determinant as possible and (b) in a stereochemical relationship to the determinant similar to that known to be important from binding studies with ligand analogs. With these comments in mind it is useful to review those factors which speak for and against the labeled tyrosine being an intimate part of the combining site in the studies with *m*-nitrophenyldiazonium on MOPC 315 and with *p*-diazonium phenylphosphorylcholine on TEPC 15.

Studies with m-Nitrophenyldiazonium and MOPC 315. The evidence that the residue is in the site is as follows. (i) Substituents on the meta and para positions on the benzene ring relative to the nitro group(s) are known to affect binding (Jaffe *et al.*, 1971). (ii) The diazonium group is immediately adjacent to the nitrophenyl group and in view of (i) can almost be considered a part of the determinant. (iii) The tyrosine which becomes labeled is in a sequence position immediately adjacent to one of the "hypervariable" regions (Wu and Kabat, 1970; Franek, 1970) which because of this hypervariability are implicated as directly participating in the combining sites of immunoglobulins.

The evidence against this tyrosine being directly in the combining site is as follows. (i) The binding activity while markedly diminished is not entirely lost after labeling. (ii) Affinity-labeled MOPC 315 when reduced with dithionite (which puts an NH_2 group at the 3 position of the relevant tyrosine) is as active as native protein (Hadler and Metzger, 1971).

Studies with DPPC and TEPC 15. The evidence that the residue is topologically in the site is as follows. (i) The binding activity is entirely lost after labeling. (ii) Phosphorylcholine analogs with consitutents at the distal end of phosphorylcholine, *e.g.*, *p*-nitrophenylphosphorylcholine, glycerolphosphorylcholine are less strongly bound than phosphorylcholine itself (B. Chesebro, unpublished observations; Leon and Young, 1971). This suggests that such constituents are sterically interacting with the combining site (on the other hand, it is possible that the weaker binding could simply be due to a reduction of the negative charge on the phosphate). (iii) The tyrosine which becomes labeled is the "same" one labeled by *m*-nitrophenyldiazonium with MOPC 315 and in pig anti-DNP. The evidence that that tyrosine is in the site of these immunoglobulins is quite suggestive (see above).

Since the reactive diazonium group could be quite distal to the phosphorylcholine determinant, 5–6 Å as estimated from molecular models, it is conceptually possible that the labeled tyrosine is not directly in the site; however, whatever the role of the tyrosine in question, it is likely that it is similar in immunoglobulins of diverse binding properties.

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