Photoaffinity Labeling of the Combining Region of Myeloma Protein 460. II. An Interpretation of the Labeling Patterns†

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ABSTRACT: The amino acid residues of the mouse γA immunoglobulin protein 460 modified with two photoaffinity reagents, 2,4-dinitrophenylalanyl diazoketone and 2,4-dinitro-1-azobenzene, are compared with the labeling pattern produced by bromoacetyl-based dinitrophenyl affinity labels. Both the diazoketone reagent and the bromoacetyl reagent *N*-bromoacetyl-*N'*-2,4-dinitrophenyl-L-ethylenediamine label lysine-54 on the light chain. The bromoacetyl and the diazoketone reagents do not modify the heavy chain, but the 2,4-dinitro-1-azobenzene reagent modifies tyrosine-33 and tyrosine-88 on the heavy chain. Protein NEW is a human γG immunoglobulin whose three-dimensional structure is known. Comparison with this protein shows that either there are substantial differences between the combining regions of proteins 460 and NEW, or that the labeling pattern is not compatible with reaction occurring only at a single binding site in each Fab fragment.

Affinity labeling reagents have been used to identify precisely the catalytic amino acid residues of certain enzymes such as the serine proteases (for review, see Knowles, 1972). It has been generally assumed that such reagents can be used with equal precision to identify amino acid residues in contact with the ligands binding to the combining region of immunoglobulins. It has indeed been shown that several classes of affinity reagents react to form covalent bonds with certain specific amino acid residues within the combining region. Whether such residues are in fact contact amino acid residues for the haptenic portion of the label remains unestablished. A number of investigators have studied both myeloma proteins and antibody populations directed against Dnp[1] with a number of different affinity reagents (Thorpe and Singer, 1969; Franek, 1971, 1973; Goetzl and Metzger, 1970a,b; Haimovich *et al.*, 1970, 1972; Hew *et al.*, 1973; Yoshioka *et al.*, 1973; Lifter *et al.*, 1974). An ideal affinity reagent should react with the contact amino acid residues binding the haptenic portion of the reagent molecule. Most affinity reagents that have been described have some limitations in this regard. One such limitation may be a narrow spectrum of reactivity with nucleophilic amino acid side chains. Another limitation is that in some affinity reagents, the distance between the haptenic and the reactive portion of the molecule is large (Eisen, 1971).[2]

Protein 460 is a mouse γA myeloma protein which binds ε-Dnp-Lys with a $K_0 = 1.0 \times 10^5$ l./mol at 20°. Two groups of investigators have reacted this protein with three different types of affinity reagent. Haimovich *et al.* (1972), using a radioactive Dnp-based affinity label (BADE) with a bromoacetyl reactive group situated in the extended form some 8.6 Å from the Dnp ring center, found no radioactivity attached by covalent bonds to the heavy chain, but did find that Lys-54 on the light chain had been modified by the affinity label. We have reacted two different Dnp-based photoaffinity reagents with protein 460. One reagent is a diazoketone (Dnp-AD), the other, an azide (Dnp-N_3), and in these compounds the reactive portions of the molecule in the photolyzed intermediates are separated from Dnp ring center by 5.6 and 3.6 Å, respectively. The diazoketone reagent reacts exclusively with Lys-54 of the L chain; the azide reagent reacts with Tyr-33 and Tyr-88 of the H chain. In addition, approximately 15% of the total radioactivity from the azide label is found widely spread over the light chain. The observed labeling pattern resembles those found by other workers who have used affinity reagents to detect the combining region (Goetzl and Metzger, 1970a,b; Haimovich *et al.*, 1972). We also find that reaction occurs at or close to the hypervariable regions of both H and L chains (Wu and Kabat, 1970). It has been suggested that such hypervariable regions may contain the contact amino acid residues which make up the combining site.

However, more recent evidence on the three-dimensional structure of the human γG myeloma immunoglobulin, "NEW," shows that the hypervariable regions are situated on polypeptide loops at the free end of the Fab fragment which extend into the solvent (Poljak *et al.*, 1973). There is evidence for the human γ-globulin NEW that a relatively large hapten, 3-(3-hydroxy-3,7,11,15-tetramethylhexadecyl)-2-methyl-1,4-naphthoquinone (a γ-hydroxyl derivative of Vitamin K_1), is bound in a shallow depression between the hypervariable loops, making contact with approximately 12 amino acid residues (Amzel *et al.*, 1974). Some of these residues are within 1-5 Å of the hypervariable regions; others are at a greater distance. Thus, it appears likely from the limited data available that the hypervariable regions are not really a three-dimensional array of contact amino acids shaped into a specific combining site complementary to the hapten. Instead, they form polypeptide loops accessible to the solvent, framing that region of the molecule to which haptens attach. Thus, individual hypervariable regions may be, but are not necessarily, involved directly in hapten binding.

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[1] Abbreviations used are: BADE, *N*-bromoacetyl-*N'*-2,4-dinitrophenyl-L-ethylenediamine; BADL, α-N-bromoacetyl-ε-2,4-dinitrophenyl-L-lysine; Dnp-AD, 2,4-dinitrophenylalanyl diazoketone; Dnp-N_3, 2,4 dinitrophenyl 1-azide.

[2] However, the hope has been expressed that despite the limitations of individual affinity reagents, the labeling pattern of a number of different affinity reagents might yield information on the locus of hapten binding.

TABLE I: A Matrix Showing Approximate Distances between Certain L and H Chain Residues in Protein NEW.[a]

	H 34	H 54	H 88	L 31	L 54
H 34	0				
H 54	11 Å	0			
H 88	23 Å	15 Å	0		
L 31	17 Å	13 Å	30 Å	0	
L 54	D	D	D	D	0

[a] The numbered residues are *homologous* to residues labeled in other myeloma proteins by affinity reagents. The L chain hypervariable region corresponding to the one containing L 54 is deleted in protein NEW. "D" = deleted. Homologous residues H 34, H 88, and L 54 were labeled by Dnp-N_3 and Dnp-AD on protein 460. L 31 was labeled both in protein 315 and TEPC 15 by a Dnp-based bromoacetyl label and phosphorylcholine-based diazonium affinity reagent. Amino acid residue H 34 of NEW H chain is analogous to residue 33 of the H chain of protein 460. H 88 has the same number both in NEW and protein 460.

We do not know the distance between the affinity labeled residues in protein 460. There is evidence that there is a close similarity between the structure of MOPC 603, a mouse γA_2 myeloma immunoglobulin protein binding phosphorylcholine (Padlan *et al.*, 1973), and protein NEW (Poljak *et al.*, 1973). Since MOPC 603 may be expected to resemble protein 460, it is useful to consider the actual distances between those amino acid residues of protein NEW which are likely to be homologous to the labeled residues of protein 460. Table I gives the distance between the residues of protein NEW which are homologous in primary amino acid sequence to the affinity labeled residues in other immunoglobulins. It is likely that these correspond in position to the affinity labeled residues, though this has not been established with certainty. Table I indicates that some of the distances between individual labeled residues are large. Reagents such as Dnp-N_3 and Dnp-AD which measure respectively 3.6 and 5.6 Å from the ring center to the reactive atom of the photolyzed intermediates react with residues homologous to tyrosine-34 and tyrosine-88 which are 23 Å apart in the heavy chain of protein NEW. Unless these distances are much closer together in protein 460 than in NEW, this finding suggests that the affinity reagents may react with immunoglobulins in situations other than when the haptenic portion of the label is bound to contact residues.

Table II reviews the amino acid residues labeled by various affinity reagents both in myeloma proteins with ligand binding activity and in natural antihapten immunoglobulin populations. This table shows that a homologous residue, tyrosine-34, is labeled in two different immunoglobulins which bind structurally dissimilar ligands. In one instance, reaction with tyrosine-34 reduces the K_0 of the immunoglobulin for the hapten, but does not destroy binding. In the other instance, reaction with the affinity reagent completely destroys the binding capacity. Tyr-34 is in fact a "constant" residue close to a region of high variability.

The affinity labeling pattern of protein 460 may be summarized: (a) most of the incorporated radioactivity is localized at three residues, lysine-54 in the light chain and tyrosines-33 and -88 in the heavy chain; (b) the bromoacetyl Dnp-based affinity label (BADE) used by Haimovich *et al.* (1972) also reacts with lysine-54 in the light chain; (c) there is reason to believe that the overall geometry of the protein 460 combining region resembles that of protein NEW. The residues on immunoglobulin NEW analogous to the labeled H chain residues tyrosine-33 and tyrosine-88 are separated by a distance of 23 Å. Therefore, the possibility exists either that in protein 460 the overall geometry of the site is different and residues 33 and 88 in the heavy chain are much closer together in protein 460 than in NEW; or, alternatively, that there are additional preferred sites on protein 460 where covalent interaction with the affinity reagent occurs. One of several possibilities is that there might be low affinity hapten binding sites containing highly reactive amino acid residues. If the affinity constants of such sites are low enough, these might be missed by conventional binding methods such as equilibrium dialysis or fluorescence quenching. Recently, three groups of workers have found evidence for the existence of not only a single high affinity hapten binding site, but also the existence of one or more low affinity binding sites for the same hapten in the Fab fragment (Padlan *et al.*, 1973; Amzel *et al.*, 1974). Since the concentration of hapten in the immediate vicinity of a high affinity site could well be greater than the "free" concentration, binding at neighboring low affinity sites could well be significant.[3]

Whether formation of covalent bonds between affinity reagents and amino acid residues occurs while the haptenic portion of the reagent is bound by the protein probably depends on the proximity of a reactive amino acid residue and also on the reaction rate of the reagent with that residue. The chemical half-life of the reagent in water may be a useful guide (Knowles, 1972), but is unlikely to give precise information on reaction rates with amino acid residue side chains. It is not possible to forecast whether covalent bond formation occurs while the affinity reagent (or photoaffinity reagent) is bound by the protein or whether reaction occurs at a highly reactive residue near the site. Ruoho *et al.* (1973) expected that covalent bond formation between an azide affinity reagent and acetylcholinesterase would occur during the lifetime of the reagent–protein complex but demonstrated with the use of a scavenger molecule (*p*-aminobenzoic acid) that this as probably not the case. They also used 4-azido-2-nitrophenyl-L-lysine as a photoaffinity reagent for anti-Dnp antibodies and concluded that in this instance reaction had occurred prior to hapten-antibody complex dissociation. This illustrates that no predicition about rates or mechanism with either conventional or photoaffinity reagents can be made without detailed knowledge of the geometry of the interaction and lifetime of the intermediates. So caution should be exercised if scavenger molecules such as *p*-aminobenzoic acid are used which are expected to react with "free" active photolytic intermediates. Low affinity interactions with small molecules are common in immunoglobulins (Varga and Richards, 1974) and have to be rigidly excluded before conclusions can be drawn with the use of scavengers. In

[3] Very recently, evidence has been presented (Haselkorn *et al.*, 1974) that kinetic measurements of the association between Dnp-based ligands and the γA mouse myeloma immunoglobulin protein 315 support the view that there are multiple binding sites in an immunoglobulin combining region. These authors propose the existence of an initial "encounter complex" followed by formation of a second "final" complex in which a distinct subsite for the Dnp ring and three other ligand binding subsites may be identified. These proposals are relevant to the theme of this paper. For instance, tyrosine 34 of the L chain, the "common" residue labeled in antibodies of different specificities, is a hydrophobic residue located (by analogy with protein NEW) on a solvent exposed hypervariable loop. Such a residue might well be involved in an "encounter complex." Also, the existence of several subsites binding a single hapten might further complicate interpretation of labeling patterns.

TABLE II: Summary of Affinity Labeling Experimental Results on Some Immunoglobulins Related to the Present Study.

Affinity Reagent	Protein	Ligand Binding Set of Proteins	H Chain Residue(s) Modified		L Chain Residue Modified	Effect of Reaction on Ligand Binding	Ref
BADL	Protein 315 IgA mouse myeloma	Dnp, menadione	Lys-54				*a, b*
BADE	Protein 315 IgA mouse myeloma	Dnp, menadione			Tyr-34		*a, b*
MNBDF[n]	Protein 315 IgA mouse myeloma	Dnp, menadione			Tyr-34	Reduces association constant from 7.5×10^5 to 1.5×10^4; no effect on valence	*c*
BADE	Protein 460 IgA mouse myeloma	Dnp, $K_0 = 1 \times 10^5$ l./mol., menadione, $K_0 = 2 \times 10^4$ l./mol.	Tyr; Cys		Lys-54; Cys	Not reported	*b*
Dnp-AD	Protein 460 IgA mouse myeloma	Dnp, $K_0 = 1 \times 10^5$ l./mol., menadione, $K_0 = 2 \times 10^4$ l./mol.	*l*		Lys-54	Stoichiometric loss of binding with incorporation	*d, e*
Dnp-N_3	Protein 460 IgA mouse myeloma	Dnp, $K_0 = 1 \times 10^5$ l./mol.; menadione, $K_0 = 2 \times 10^4$ l./mol.	Tyr-33 Tyr-88		*l*	Loss of binding activity; remaining binding at reduced association constant	*d–f*
MNBDF[n]	Guinea pig IgG	Dnp	Tyr			Not reported	*g*
MNBDF[n]	HPC-3 IgG mouse myeloma	Dnp, $K_0 = 8.4 \times 10^4$ l./mol.	Tyr				*h*
DPPC[m]	TEPC-15 IgA mouse myeloma	Phosphorylcholine, $K_0 = 2.2 \times 10^5$ l./mol.		Tyr-34	Loss of binding with incorporation		*i*
MNBDF[n]	Porcine IgG	Dnp	Tyr 1st hypervariable	Tyr-33 (λ) 83 chain	Not reported		*j*
MNBDF[n]	Mouse and rabbit IgG	Dnp	Tyr	Tyr	Not reported		*k*

[a] Haimovich *et al.* (1970). [b] Haimovich *et al.* (1972). [c] Goetzl and Metzger (1970a, b). [d] Yoshioka *et al.* (1973). [e] Hew *et al.* (1973). [f] Lifter *et al.* (1974). [g] Ray and Cebra (1972). [h] Martin *et al.* (1972). [i] Chesebro and Metzger (1972). [j] Franek (1971, 1973). [k] Thorpe and Singer (1969). [l] Labeling reported, modified residue unidentified. [m] DPPC, *p*-diazoniumphenylphosphorylcholine. [n] MNBDF, *m*-nitrobenzenediazonium fluoroborate.

addition, Haimovich *et al.* (1970) have presented evidence that bound ligand and ligand attached by covalent bonds cause similar spectral changes in the ligand-protein complex. However, these data cannot by themselves be taken as evidence that in both complexes hapten is bound in exactly the same configuration.

The possibility that the hapten binding to the combining region of an immunoglobulin will give rise to conformational changes should also be borne in mind. Such changes might produce proximity relationships between amino acid residues which are not there in the absence of hapten. Although it seems likely that small conformational changes will occur in proteins whenever a ligand is bound, the best evidence available at present suggests that large conformational changes which might materially alter distance relationships do not occur in two hapten binding immunoglobulins which have been studied by X-ray crystallography. Fourier difference maps of 4.5-Å resolution of MOPC 603, a mouse γA_2 myeloma immunoglobulin, show no evidence of conformational change in the presence and absence of the hapten phosphorylcholine (Padlan *et al.*, 1973). Fourier difference maps of NEW, a human γG myeloma immunoglobulin, in the presence and absence of the hapten 3-(3-hydroxy-3,7,11,15-tetramethylhexadecyl)-2-methyl-1,4-naphthoquinone, also show no conformational changes visible at 3.5-Å resolution (Amzel *et al.*, 1974). While these results do not exclude small conformational changes below the limits of resolution of these studies, they suggest at least that large conformational changes are not an obligatory concomitant of hapten binding.

In conclusion, the results both of our studies and those of others cast some doubt that affinity reagents react exclusively with the contact residues of a single hapten binding site. We feel that affinity labeling procedures by themselves are insufficient for delineating combining sites and that other indepen-

dent methods such as X-ray crystallography are needed for site identification. Since there is some doubt that affinity reagents react exclusively at or relatively near the binding site, their most useful function may be to identify proteins binding ligands in complex systems such as membranes.

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Antibodies to the Codons ApApA, ApApC, and ApUpG†

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ABSTRACT: Antibodies to three triplet codons (ApApA, ApApC, and ApUpG) were elicited in rabbits by immunization with trinucleoside diphosphate-bovine serum albumin conjugates. Both cold and tritiated ApUpG used in these studies were synthesized by a modification of the method of P. Leder *et al.* ((1965), *Biochemistry 4,* 1561). The specificities of the antibodies were determined by gel diffusion, microquantitative precipitation, and radioimmunossay. Precipitation analysis using rabbit serum albumin conjugates showed that antibodies to ApApC and ApUpG recognized the entire trinucleotide sequence but also cross-reacted with the components of the hapten closest to the carrier protein. However, radioimmunossay using radiolabeled ApUpG and unabsorbed anti-ApUpG globulin showed that there was a population of antibody highly specific for the ApUpG sequence. Precipitation analysis showed that the anti-ApApA immunoglobulin reacted only with its parent antigen and not at all with conjugates of A or ApA, even without prior absorption. This unusually high specificity may reflect the unique three-dimensional conformation of the ApApA molecule. Many immunizations were required to obtain antibodies specific for ApUpG and, even then, the antibody was of low titer. The possibility that synthesis of antibody specific for ApUpG may inhibit further protein synthesis by reaction with the initiator codon is discussed.

In 1964, this laboratory reported a new method for the preparation of immunogenic protein conjugates of ribonucleosides and ribonucleotides which were used to elicit nucleic acid reactive antibodies highly specific for the purine or pyrimidine determinant groups (Erlanger and Beiser, 1964). Subsequently, this method was used, in our laboratory as well as in others, to prepare antibodies to inosine (Bonavida *et al.,* 1971; Inouye *et al.,* 1971), to methylated bases (Levine *et al.,* 1971; Sawicki *et al.,* 1971), and to various dinucleotides (E. Nahon in Beiser and Erlanger, 1966; Wallace *et al.,* 1971; Erlanger *et al.,* 1972). The unusually high specificity of the antibodies and their ability to bind to single-stranded regions of nucleic acids made them useful tools for probing biological and biochemical systems (Klein *et al.,* 1967; Seegal *et al.,* 1969; Wallace *et al.,* 1969; Freeman *et al.,* 1971; Liebeskind *et al.,* 1971, 1974; Erlanger *et al.,* 1972) and for the examination of the architecture of metaphase chromosomes (Dev *et al.,* 1972; Schreck *et al.,* 1973).

The purpose of this paper is to report the elicitation and properties of antibodies specific for the codons ApApA,

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