Contact Regions for Dinitrophenyl and Menadione Haptenst
in an Immunoglobulin Binding More Than One Antigen

(myseloma protein/combining site/sulfhydryl group/antibody heterogeneity)

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ABSTRACT Protein 460 is a mouse myeloma γA protein that competitively binds two small haptenst, 2,4-dinitrophenyl-L-Lysine (Dnp-Lys) and 2-methyl-1:4-naphthaquinone-S3[αH]thioglycollate (MenTG), to the antibody-combining region. The intact protein has a relatively inaccessible sulfhydryl group on each heavy chain. When it is substituted with a bulky reagent the binding affinity for MenTG decreases, while the binding of Dnp-Lys remains the same. Guanidine-HCl selectively reduces binding of Dnp-Lys; dimethylsulfoxide selectively reduces binding of MenTG. Papain digestion of protein 460 followed by column chromatography gave two fractions: one contained both binding activities and the other contained the sulfhydryl group. The affinity for Dnp-Lys of the first fraction is the same as that of the whole molecule, while affinity for MenTG is decreased. Since selective alteration of one or the other binding activity can occur in different ways, it seems likely that even though the hapten compete with each other, there is some spatial separation between the groups of contact amino-acid residues involved in the binding of these two haptenst. These findings do not support the hypothesis that an immunoglobulin molecule carries combining sites complementary only to a single hapten or to a structurally related series of haptenst, but rather suggests that the antibody-combining site may be a polyfunctional region capable of binding several structurally dissimilar haptenst. We discuss a mechanism whereby polyfunctional combining sites can give rise to an antibody population (immune serum) that has a high degree of specificity to a single hapten.

It has long been held that antibodies react most strongly with homologous eliciting antigen, but often also, with graded affinity, with structurally related substances (1). Many observations on the specificity of immune sera led to the idea that this specificity must, in turn, be reflected in a single binding specificity of the individual antibody combining site (2). However, recent reports indicate that single immunoglobulins may bind two or more determinants, some of which bear little or no structural relationship to each other (3, 4). It is not known whether a single, or closely spaced group of contact amino acids [such as those involved in binding the substrate in RNAse (5)] is responsible for antigen association, or whether the binding sites are diverse and spread over a relatively large area of the antibody-combining region.

Protein 460, a mouse γA myeloma immunoglobulin, binds the small determinant 2,4-dinitrophenyl-L-[3H]Lysine (Dnp-Lys) with an energy of interaction within the physiological range of elicited antibodies (6). It binds competitively 2-methyl-1:4-naphthaquinone (menadione) (3) and its thioglycollate derivative (Rosenstein, R. W. & Richards, F. F., in press) with interaction energies close to that of Dnp-Lys. Other haptenst also bind to the protein (3). In parallel with structural studies on the amino acid sequence of peptides involved in the binding region (7), we have attempted to find out whether one site or two spatially separated sites are involved in the binding of Dnp-Lys and 2-methyl-1:4-naphthaquinone-3-[3H]thioglycollate (MenTG). We have found that each combining region of protein 460 contains a single sulfhydryl group that may be used for the study of the geometry of the site. Preliminary evidence suggests that some other mouse γA myelomas with different binding specificities also contain sulfhydryl groups, but we know nothing so far of their location.

MATERIALS AND METHODS

MOPC 460 tumor cell line (isolated by Dr. Michael Potter) was given to us by Drs. Herman Eisen and Ernest Simms, and was maintained in BALB/c and (BALB/c x DBA/2)F1 hybrid mice. The dissociation constant (Ks) for Dnp-Lys was 0.97 × 10^6 liters/mol ± 0.15 SD (number of determinations, n = 28) at 20°; Ks for MenTG was 2.0 × 10^6 liters/mol ± 0.5 SD (n = 20) at 20°. Protein 460 was purified by methods (3, 8) that include partial reduction and alkylation to reduce the polymeric protein to 75 monomers. Where indicated, the polymeric (unreduced) protein was also used. MOPC 315 protein binds Dnp-Lys (9); HOPE 8 and TEP 15 proteins bind phosphoryl choline, but not Dnp-Lys (10); MOPC 173 protein does not bind Dnp-Lys or MenTG. Myeloma proteins were obtained through the courtesy of Drs. Eisen, Simms, and Potter.

Rabbit anti-Dnp bovine gamma globulin serum (average intrinsic Ks for Dnp-Lys = 6.0 × 10^6 liters/mol at 20°) was given by Dr. Peter Jackson. Equilibrium dialysis and preparation of γA Fab-fragments was by the procedure of Eisen et al. (9). Light and heavy chains were separated in urea-propionic acid (11).
Synthesis of 5,5-dithiobis-(2,4-[H]dinitrophenyl) (Dnp-S-S-Dnp)

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\begin{array}{c}
\text{NO}_2 \quad \text{NO}_2 \\
\text{NO}_2 \quad \text{NO}_2 \\
\end{array}
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1 eq of [H]fluorodinitrobenzene in ethanol was reacted with 1.1 eq of sodium sulfide and the product was acidified and extracted three times with ether. The extract was dried under argon. It was then oxidized to minimal iodine excess in aqueous solution at pH 8.0 with 0.3 M KI. Yellow crystals precipitated (melting point, 290° with decomposition). The specific radioactivity was 5.9 Ci/mol. Nonradioactive Dnp-S-S-Dnp synthesized as above gave, on mass spectroscopy, a major fragment ion for the expected half molecule at m/e 199. Nonradioactive material cocrystallized to constant specific radioactivity with tritiated Dnp-S-S-Dnp.

Synthesis of 2-methyl-1,4-naphthaquinone-3-[H]thioglycollate (MenTG)

MenTG is an intermediate in the synthesis of the photoactivated affinity reagent 2-methyl-3-[H]-thioglycolyl-diazoketone-1:4-naphthaquinone. MenTG is the adduct of menadione with [H]thioglycolic acid. Details of this synthesis will be reported (Rosenstein, R. W. & Richards F. F., in press).

RESULTS AND DISCUSSION
Evidence for the presence of a heavy-chain cysteinyl sulphydryl group

We observed that addition of 0.01 M dithiothreitol (DTT) and \(2 \times 10^{-3} \) M EDTA increased the number of mol of hapten bound per mol of 7S monomer (r value) of some purified preparations of monomeric and polymeric MOPC 460 proteins from 1.0 to 1.75. This suggested to us the presence of a sulphydryl group that was relatively inaccessible to the alkylating reagents used during conversion of polymer to monomer. Spectrophotometric titration of both monomer and unreduced polymeric protein 460 with 5,5-dithiobis-(2-nitrobenzoate), Ellman's reagent (12), gave 1.35 \(\pm\) 0.05 SD mol of -SH group per 7S monomer (n = 3). Since this was less than the minimum symmetrical value of 2 expected, if one -SH group were associated with each combining region, another sulfhydryl reagent was designed on the Trojan Horse principle. It is known that the Dnp radical enters the site. The interaction energy of the Dnp moiety with the protein is not high, and on dissociation a relatively high local concentration of Dnp-S-S-Dnp is probably formed in the site, thus facilitating the formation of an S-S bond. [H]Dnp-S-S-Dnp (see Table 1) titrated 2.0 \(\pm\) 0.1 -SH groups per 7S monomer (n = 4). Equilibrium dialysis against 0.01 M DTT released all protein-bound tritium.

To confirm that a cysteine residue was reacting, we labeled unreduced polymeric protein 460 with \([1^4]C\)iodoacetamide (specific radioactivity 3.16 Ci/mol, New England Nuclear Corp.) in the presence of 4.3 M guanidine hydrochloride, until 1.5 mol of \([1^4]C\)iodoacetamide was bound. The labeled protein was hydrolyzed (at 110° for 24 hr in 5.7 N HCl under argon). A peak in the position of carboxymethylcysteine containing 98% of the applied radioactivity appeared when the hydrolysate was chromatographed (SAH Joelsen automatic amino-acid analyzer). The content of this peak behaved identically with authentic carboxymethyl cysteine on electrophoresis at pH 6.5 and on two-dimensional chromatography in butanol-acetic acid-water, 180:10:10, and also in ethanol-ammonia-water, 95:2.5:2.5. In addition it cocrystallized to constant specific radioactivity with authentic carboxymethyl cysteine.

Protein 460, labeled as above, was dialyzed free of guanidine-HCl, and, after partial reduction and alklylation, light and heavy chains were separated in urea-propanolic acid on Sephadex G100 (11). The A<sub>280</sub> ratio of the heavy- and light-chain peaks was 2.9:1. Of the total A<sub>280</sub> units applied, 92% were recovered, and 92% of the applied \(1^4\)C was recovered in the heavy-chain peak.

When the native protein 460 molecule is labeled with \([H]\)-iodoacetamide for 48 hr, 0.6 mol of \([H]\)iodoacetamide are bound per 7S monomer. Digestion of this material by papain, followed by chromatography (Table 1) gave two fractions. The first bound Dnp-Lys and MenTG, but contained no radioactivity derived from \([H]\)iodoacetamide. The second fraction contained all the radioactivity and bound neither hapten. Sodium dodecyl sulfate-polyacrylamide gel analysis (13) of the first fraction gave a single predominant band of molecular weight 24,000 and a less intense band of molecular weight 16,000. Both bands had aspartic and glutamic acids as the N-terminal residues; a pattern similar to that of the whole molecule. This fraction thus contains the N-terminus of the heavy chain (glutamic acid) and of the light chain (aspartic acid).

The second fraction gave a complex pattern, suggesting that the pattern of papain digestion of alkylated MOPC 460 protein differed from that of IgG molecules. Although it is tempting to speculate that the -SH group is present in a part of the Fc-fragment close to the Fab-fragment, the atypical complexity of the papain digestion pattern counsels caution. Heavy-chain structural studies with both affinity-labeled and -SH-labeled heavy chains of MOPC 460 protein are in progress and should give a more accurate localization of the -SH group.

Alkylation of the sulfhydryl group with \([H]\)iodoacetamide (New England Nuclear Corp., 3.18 Ci/mol) was extremely slow when the native polymeric protein was used. A maximum of 0.6 mol radioactivity per mol 7S monomer was incorporated after exposure for 48 hr at 4° to a 20-times molar excess of iodoacetamide. Reaction with \([1^4]C\)iodoacetide (New England Nuclear Corp., 3.4 \(\times\) 10<sup>4</sup> cpm/mol) proceeded at about half the rate, and labeled 0.3 mol -SH group per mol 7S monomer. Both reaction rates increased rapidly in the presence of 4.3 M guanidine-HCl. 1.85 mol of \([H]\)iodoacetamide and 1.5 mol of \([1^4]C\)iodoacetide were incorporated per mol 7S monomer in the presence of 20-fold molar excess reagent in 12 hr at 4°. These results suggest to us that the sulfhydryl group is relatively inaccessible to these reagents and may be buried in a hydrophobic region of the molecule.

Selective alteration of hapten binding affinities

It has been previously reported that menadione inhibits binding of Dnp-Lys to protein 460 (10), and we have con-
Table 1. Selective alteration of protein 460 hapten binding

<table>
<thead>
<tr>
<th>Modifying agent*</th>
<th>[\textsuperscript{[H]}]Dnp-Lys</th>
<th>[\textsuperscript{[H]}]MenTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_a \times 10^8$</td>
<td>$r$ Value after</td>
</tr>
<tr>
<td></td>
<td>liters/mol</td>
<td>modification(\dagger)</td>
</tr>
<tr>
<td>(A) 4.3 M Guanidine-HCl</td>
<td>$&lt;0.05$</td>
<td>$&gt;2.0$</td>
</tr>
<tr>
<td>(B) Papain digestion</td>
<td>$0.95$</td>
<td>$1.24$</td>
</tr>
<tr>
<td>(C) 2% MesSO</td>
<td>$0.86$</td>
<td>$1.98$</td>
</tr>
<tr>
<td>(D) (-\text{SH} ) alkylation by</td>
<td>$1.0$</td>
<td>$1.40$</td>
</tr>
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\(\dagger\) Before modification: $0.97 \pm 0.15$ SD, $n = 28$.  
\(\ddagger\) Before modification: $1.5$.  

\(* (A) 1 \times 10^{-4}$ mol/ml purified polymeric protein 460 in 0.2 M Tris·HCl buffer (pH 8.0) containing 0.075 M NaCl (Buffer 1) plus solid guanidine·HCl (Mann ultra-pure) to final concentration 4.3 M in 5.0 ml final volume. The mixture was left at 4°C for 12 hr, then dialyzed against three changes of Buffer 1 at 4°C for 12 hr each. Equilibrium dialysis was done in micro-equilibrium cells (Gateway Immuno-serum Co., Cahokia, Ill.) at 20°C with Dnp-Lys, $(2.57 \times 10^{11}$ cpm/mol) or MenTG $(4.63 \times 10^{11}$ cpm/mol). Final volume of each side was 50 ml. The equilibrium dialysis conditions were followed throughout. Conclusion: Dnp-Lys binding affinity reduced at hapten 0.73 mM (See Fig. 1). MenTG binding unaffected. (B) The procedure was that of Eisen et al. (9) modified by the use of 2.4 × 25-cm DES5 (Whatman) column and a linear gradient elution system consisting of 500 ml 0.01 M phosphate buffer (pH 8) (Buffer 2) plus 0.05 M NaCl for column equilibrium and as initial buffer and 500 ml Buffer 2 plus 0.5 M NaCl as final buffer. Conclusion: MenTG binding affinity reduced; $r$ value increased (See Fig. 1). Dnp-Lys binding unaffected. (C) $1 \times 10^{-4}$ mol/ml purified polymeric protein 460 in 0.01 M phosphate buffer (pH 7.5) 0.15 N in NaCl (Buffer 2) plus 2% v/v redistilled MesSO; final volume, 1.0 ml. The mixture was kept at 20°C for 30 min, then filtered through a 2.0 × 12.0-cm Sephadex G25 (medium) column equilibrated in Buffer 3. Conclusion: MenTG binding affinity reduced; $r$ value increased (See Fig. 1). Dnp-Lys binding unaffected. (D) $1 \times 10^{-4}$ mol/ml purified polymeric protein 460 in Buffer 3 plus $1 \times 10^{-7}$ mol/ml Dnp-S-S-Dnp $(6.4 \times 10^{13}$ cpm/mol) in acetone (1% v/v of final volume of 1.0 ml). Conclusion: MenTG binding affinity reduced; $r$ value increased. Dnp-Lys binding unaffected.

\(\dagger\) Before modification: $0.97 \pm 0.15$ SD, $n = 28$.  
\(\ddagger\) Before modification: $1.5$.  

\(\ddagger\) Before modification: $2.0 \pm 0.55$ SD, $n = 20$.  

\(\ddagger\) Before modification: $2.0$.

Aren't the conditions (Table 1) and the Jenard test (Fig. 1) sufficient to prove that the hapten displaces the other from the combining site? Four different experiments are presented below in outline, and in Table 1 in detail. Each experiment modifies protein 460 in a different way. With each modification, only one of the two hapten binding activities is modified while the other is left intact.

(A) Denaturation with Guanidine·HCl. Polymeric protein 460 was treated with 4.3 M guanidine·HCl and then dialyzed free of guanidine·HCl. The $K_a$ for Dnp-Lys decreased by about 1.5 orders of magnitude, while the $K_a$ and $r$ value for MenTG was unchanged (Table 1).

It is known that antibodies treated with denaturing agents and subsequently renatured will regain only partial binding activity (14, 15). It is likely, therefore, that our dialyzed preparation contained both denatured and undenatured molecules of protein 460. A Scatchard plot of Dnp-Lys binding to renatured protein 460 showed a sharp break at $3 \times 10^{-2}$ M ligand. The break separates two slopes: one with a $K_a$ of $1.0 \times 10^4$ liters/mol (the $K_a$ of unmodified protein) and the other with a $K_a$ of $5 \times 10^3$ liters/mol (Fig. 1, left). This indicated the presence of two populations of protein 460 molecules with respect to Dnp-Lys binding; however, with respect to MenTG binding, there was only one population. The $K_a$ and $r$ value of this population was unchanged by the treatment. It seems likely, therefore, that the denaturation conditions were either insufficient to disturb the MenTG binding site or that this site was restored to complete activity by the renaturation conditions (Table 1). Whatever the cause, it does not seem likely that a single local site could be involved in the binding of both hapten.

**Fig. 1.** (Left) Scatchard plot of [\textsuperscript{[H]}]Dnp-Lys binding to protein 460 denatured with 4.3 M guanidine·HCl and subsequently partially renatured (for conditions, see Table 1). Anundenatured control preparation of MOPC 460 protein gave an $r$ of 1.5. Slope (--) corresponds to $K_a = 1.25 \times 10^4$ liters/mol; $r = 0.37$; Slope (---) corresponds to $K_a = 0.27 \times 10^4$ liters/mol. (Right) Scatchard plot of [\textsuperscript{[H]}]MenTG binding to protein 460 treated with 2% MesSO (for conditions, see Table 1). An undenatured control preparation of protein 460 gave an $r$ of 2.0. Slope (--) corresponds to $K_a = 2.2 \times 10^4$ liters/mol; $r = 1.75$. Slope (---) corresponds to $K_a = 0.06 \times 10^4$ liters/mol.

$r$, mol hapten bound/mol 7S monomer; $c$, free hapten concentration.
(B) Papain Digestion and Chromatography. Protein 460 was digested with papain, and the digestion products were separated on DE52 cellulose (Table 1). Two fractions were eluted from the column: the first contained both the Dnp-Lys and the MenTG binding activities. $K_a$ for MenTG was reduced by nearly 78%; the $r$ value per Fab-fragment was 0.95. $K_a$ and $r$ values for Dnp-Lys binding were not affected to a significant degree. This treatment is yet another way of showing that the binding sites for MenTG and Dnp-Lys can be differentially affected.

(C) Denaturation with Dimethylsulfoxide (MeSO). Polymeric protein 460 was treated with 2% (v/v) MeSO and subsequently dialyzed free of the MeSO. The renatured protein showed a reduction of $K_a$ for MenTG from $2.0 \times 10^4$ to less than $5 \times 10^{-3}$ liter/mol; Dnp-Lys binding remained unaffected. A Scatchard plot of the MenTG binding to protein 460 again showed the presence of two populations of protein that differed in their binding to MenTG. One population retained the original $K_a$; the second population had a $K_a$ reduced by about half an order of magnitude. Again an increase in $r$ value for MenTG binding was noted (Table 1). Both of these phenomena have been discussed previously.

(D) Substitution of -SH Group with Iodoacetamide or Dnp-S-S-Dnp. Alkylation of the -SH group with iodoacetamide (about 0.6 mol -SH group alkylated per 7S monomer) produced no effect either on $K_a$ or the $r$ value of the protein 460 population or on any fraction of this population.

The sulfhydryl group on the heavy chain of the polymeric undenatured protein 460 molecule alkylates rapidly and completely with Dnp-S-S-Dnp. As a consequence, $K_a$ for MenTG drops from $2.0 \times 10^4$ to less than $1.5 \times 10^4$ liters/mol. $K_a$ for Dnp-Lys is unchanged (Table 1).

This fact implies that the nature of the contact between MenTG and protein 460 has been altered by the substitution of the -SH group by Dnp-S-S-Dnp. The mechanism might be either through a steric effect or a conformational alteration of the protein. It seems unlikely to us that access of MenTG to the binding cavity has been reduced without a direct effect on the binding site. If this were so, there would be no effect on the equilibrium point of the reaction, although the reaction rates might be slowed. The effect is, however, confined to MenTG binding, and Dnp-Lys binding is not affected.

Summary. Alkylation with a bulky reagent, i.e., Dnp-S-S-Dnp (10 Å, maximum dimension) affects MenTG binding, while iodoacetamide (3.5 Å, maximum dimension of the carbboxymethyl group) does not. Since Dnp-Lys binding is unaffected, it suggests that the distance between the -SH group and the MenTG site is greater than 3.5 Å, but less than 10 Å. It is also necessary to account for the competitive binding of the two haptons. The immunoglobulin combining site is believed to measure $25-44 Å \times 10-17 Å$ (16). In a cavity of this size, Dnp-Lys (18.2 $\times 7.9 \times 3.9$ Å) could, for instance, adhere to one wall blocking the entry of MenTG (12.5 $\times 6.9 \times 2.4$ Å), which might adhere to the opposite wall of the cavity. The attachment of MenTG could block binding of Dnp-Lys in the same way. Attachment of Dnp-S-S-Dnp (9.5 $\times 10.1 \times 7.6$ Å) within 9.5 Å of the MenTG site could displace or perturb attachment of MenTG without affecting Dnp-Lys binding. Such models are only speculative; we have not excluded conformational changes secondary to hapten binding. Fortunately, the sulfhydryl group in close conjunction to the active site has proved a valuable tool for studying spatial relationships in the site. Studies will be reported elsewhere (Rosenstein, R. W. Konigsberg, W. H., & Richards, F. F., manuscript in preparation) in which fluorescence, NMR, and electron spin probes have been attached to the -SH group for measurement of the probe–hapten and interhapten distances. These -SH derivatives should also prove useful if the protein can be crystallized. Preliminary studies with MOPC 315, HOPC 8, TEPC 15, and MOPC 173 proteins indicate that all these γA proteins derived from mouse myeloma have reactive -SH groups, although their location and effect on binding is not yet known. The heavy-chain amino-acid sequence of protein 173 from residues 1–104 is published (17). There are only two cysteine residues, and these are in the correct position for the first intrachain sulfhydryl loop.

It seems possible that low degrees of alkylation of this "hidden cysteine" with iodoacetamide, as well as possible heavy metal complex formation, may have been responsible for the initial difficulties in finding the correct hapten valency for γA myelomas with antibody activity (9). We do not know how widespread the phenomenon of spatially separated hapten sites is in immunoglobulins. Certainly, the findings of Parker and Osterland (18) suggest that it is perhaps relatively common.

We have considered the measurable binding phenomena for Dnp-Lys and MenTG without attempting to distinguish between binding in the physiological range of elicited antibodies and binding of lesser energies of interaction. The $K_a$ of protein 460 for Dnp-Lys corresponds to the average intrinsic $K_a$ for early anti-Dnp immunoglobulin preparations in the rabbit. This point is reviewed in (3). The $K_a$ of protein 460 for MenTG is lower than this, but we have no information on the average intrinsic $K_a$ in natural antibody populations for this hapten. Protein 460 does not exhibit general binding for hydrophobic compounds (3). This is important because it has been pointed out that low-energy interactions between small hydrophobic molecules and myeloma proteins are more common than might be expected on the basis of random immunogenetic selection, and this argument has been used to characterize such binding as "nonspecific" (18). We would add that there would seem to be no contradiction in finding high frequency of interaction between single determinants and individual myeloma proteins unless it is assumed that each myeloma protein has no more than a single "true" specificity.

However, these findings (with the considerations mentioned above) again raise the possibility that a single immunoglobulin may bind a number of chemically dissimilar antigens in different parts of the combining region. This poses a second question. If this is true, how can such polyfunctional immunoglobulins give rise to antibody populations (immune sera) having a high degree of specificity towards a single hapten? We believe that polyfunctional combining regions could be compatible with high specificity in immune sera for the following reasons. Antigen selection of antibody-producing
unrelated inhibitors at or around their binding cavities or clefts (22). Polyfunctional combining regions eliminate the necessity for postulating a single antibody for each binding specificity, thus reducing the need for undue complexity in some theories of the origin of antibody diversity (23). There are also some epidemiological implications in polyfunctional combining regions. If two structurally linked specificities, A and B, are present in a single combining region, and antigen B is lost from the environment, the presence of antigen A would continue to assure the survival of specificity B during the natural selection process. Linked specificities of this type could also account for the occasional anomalous cross-reactions found in immune sera.

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