# STRUCTURAL PROPERTIES OF THE LIGAND BINDING SITES OF MURINE MYELOMA PROTEINS

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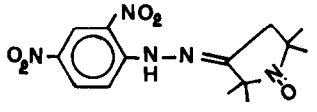
## 1. Introduction

The relationship of specific ligand binding to possible changes in antibody conformation remains a controversial issue. Many investigators have studied ligand induced apparent changes in antibody shape by a variety of generally indirect methods (reviewed in [4]) including: (i) the optical properties of antibodies or of bound ligands [1–3, 7], (ii) the susceptibility of antibody–ligand complexes to proteolytic digestion [5, 6], (iii) the rate of exchange of labile hydrogens of antibody molecules [7, 8] and (iv) sedimentation properties of antibodies [9, 10]. In the present experiments, we have used two immunospecifically purified mouse A-myeloma proteins to examine differences in the mobility of two spin labeled DNP ligands which are approximately the same size, but different in their configuration.



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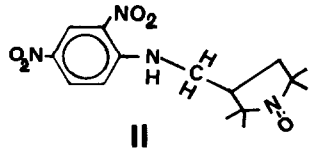

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Our working hypothesis is that the differences in configuration of the two spin labels may result in differences in folding of amino acid residues in the active sites around the DNP portion as well as the spin label moiety of these ligands. Use of the electron spin resonance (ESR) technique [11–13] enables us to infer differences in the extent folding of the antibody combining sites from a comparison of the immobilization of the spin label portion of the bound ligands.

## 2. Material and methods

Immunospecifically purified A-myeloma proteins produced by the mouse plasmacytomas MOPC-315 (Protein-315) and MOPC-460 (Protein-460) were obtained from the pooled serum of BALB/c mice according to the procedure of Eisen et al. [17]. The DNP hydrazone derivative of 1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidone (I) [13] was prepared according to the procedure Rozantzev and Neiman [14]. The *N*-[1-oxyl-2,2,5,5-tetramethyl-3-methyl aminopyrrolidinyl] 2,4-dinitrobenzene (II) (DNP-methylene-SL(5)) was prepared as described previously [12]. All ESR spectra were recorded on a Varian E-6 X-band ESR spectrometer at room temp. or at 4°. The immunoglobulin concentrations in buffered saline, pH 7.4, were approx. $5 \times 10^{-5}$ M and titrations were performed with spin la-



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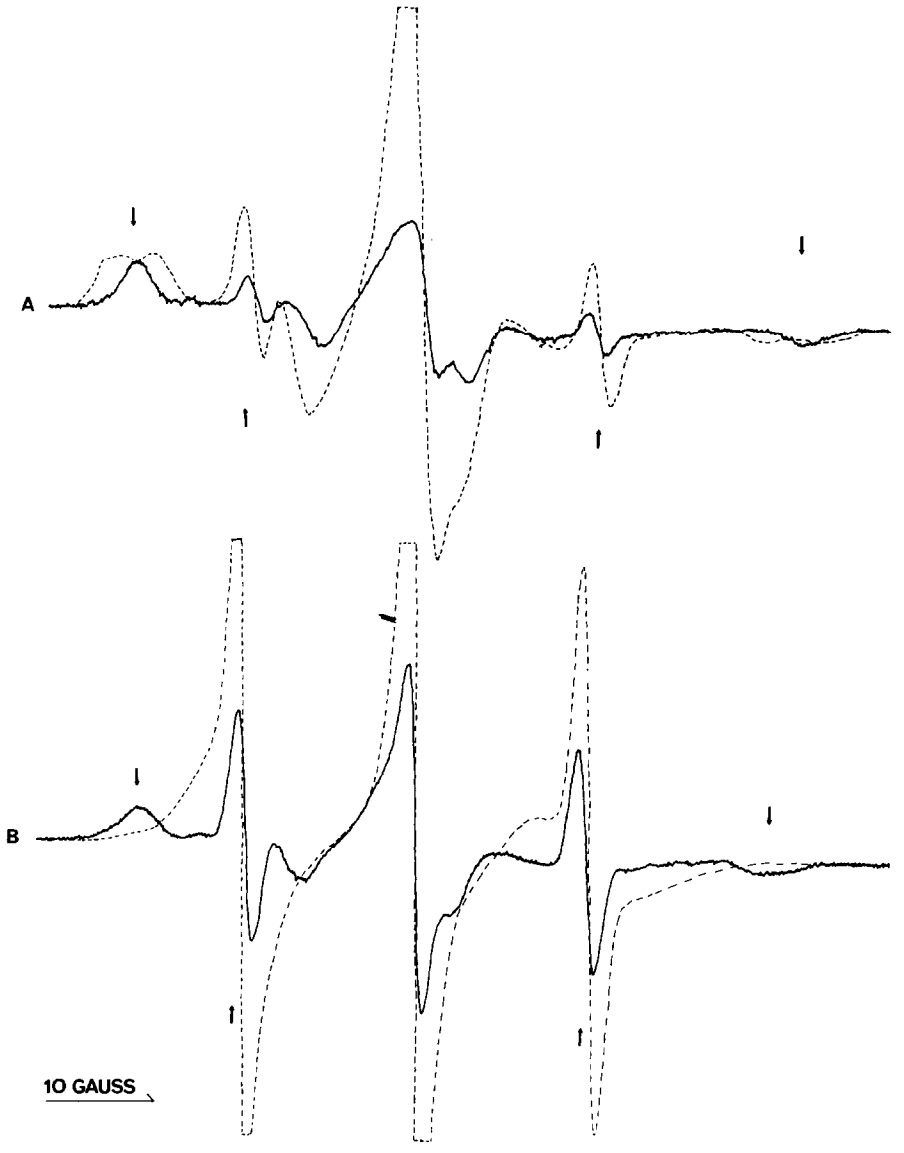


Fig.1. Electron spin resonance spectra of spin labeled DNP ligands in the presence of myeloma proteins: DNP-hydrazone-SL(5) binding (———) and DNP-methylene-SL(5) binding (------) by (A) protein-315, and (B) protein-460. The up arrow (↑) indicates free ligand spectrum and down arrow (↓) protein-bound ligand.

beled ligands such that final ligand concentrations were approx. $1 \times 10^{-5}$M. Antibody fluorescence quenching titrations were performed as described previously [13]. In the ESR and fluorescence quenching titrations stock solutions of the spin labeled ligands were prepared in methanol such that the final ligand addition to the solution of myeloma protein in buffered saline produced a total concentration of methanol of $< 2\%$ (v/v).

## 3. Results and discussion

The resonance spectra of antibody-bound and -free spin labeled ligands are shown in figs. 1 and 2. The high and low field peaks of the free ligand (↑) are well separated from the broad resonance peaks (↓) due to immobilization of the spin labeled ligand at the immunoglobulin active site. In a model system, the separation of the latter broad peaks, $A_{max}$, has been shown

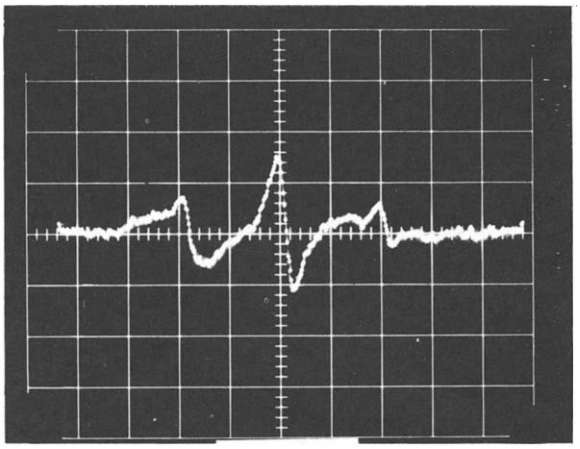

Fig.2. Electron spin resonance spectrum of low concentration ($1 \times 10^{-6}$M). DNP-methylene-SL(5) in the presence of excess protein-460 ($5 \times 10^{-5}$M) at 4°. The resonance spectrum was resolved by accumulating the weak signal eight times on a Fabri-Tek 1072 signal averager and the resulting trace displayed on the oscilloscope was photographed using a Polaroid camera. Each horizontal division is approx. eight gauss with field increasing to the right.

to be sensitive to changes of the viscosity of the medium, and therefore, the motional freedom of the spin label [12].

Recently we have shown that the differences in $A_{max}$ can be used to detect alterations of antibody active sites during the immune response [13]. Generally, a decrease of $A_{max}$ from 63 gauss indicates increasing mobility of the spin label. The $A_{max}$ value of I [DNP-hydrazone-SL(5)] when bound in the active sites of the protein-315 was 62.4 ± 0.5 gauss, however, two peaks were observed when II [DNP-methylene-SL(5)] was specifically bound by a separate aliquot of the same myeloma protein. The separation of the outer peaks was 66.3 ± 0.5 gauss and that of the inner peaks was 57.3 ± 0.5 gauss. A detailed account of the separation of the resonance peaks will be reported in a later communication. Nevertheless, these $A_{max}$ values indicate strong interactions of the spin label moiety with the immunoglobulin active sites. DNP-hydrazone-SL (5) was also found to be strongly immobilized when bound in the active sites of the protein-460 (fig.1B). The protein-460 gave an $A_{max}$ value of 62.4 ± 0.5 gauss with the binding of DNP-hydrazone-SL(5), but no broad resonance peaks could be detected with the binding of DNP-methylene-SL(5), although the low field peak intensity was higher than the high field peak intensity indicating the presence of an asymmetric spectrum superimposed on an isotropic free spin label spectrum. At lower temperature (4°) when $1 \times 10^{-6}$M of II was added to the same concentration of protein-460, an intermediately immobilized resonance spectrum was resolved (fig.2), indicating very little interaction of the spin label moiety with the immunoglobulin active site. The asymmetric resonance spectrum observed at room temp. (fig.1B dotted line spectrum) is due mainly to the binding of the DNP portion of the ligand.

Fig.3 shows the results of the fluorescence quenching titrations of both myeloma proteins with spin labeled ligands I and II in comparison to fluorescence quenching of protein-315 by ε-DNP-L-lysine and protein-460 by 2,4-dinitronaphthol. As was expected, the titrations indicated uniform intrinsic binding with the spin labeled ligands consistent with the chemical uniformity of these immunoglobulin preparations [12, 15, 16]. The most important result of these experiments is that the two myeloma proteins each bind I and II with nearly identical affinities. There are significant differences in the extent of immobilization of the two spin labels. Although, there are differences in $A_{max}$ values of the resonance spectra of I and II (fig. 1A) nevertheless, the two ligands are strongly immobilized at the protein-315 binding site. However, the resonance spectra in fig.1B indicate that the nitroxide moieties of these two ligands have very different mobility at the protein-460 binding site. Namely, protein-460 can immobilize label I but fail to make significant contact with the pyrrolidine portion of label II (fig.2). These results indicate that the active site of the higher affinity protein-315 is more flexible or adaptable than that of the protein-460 as indicated by their respective ability to accommodate the bulky pyrrolidine nitroxide labeled ligands. These observations are in accord with our previous studies on the binding properties of primary and secondary anti-TNP antibodies [13].

Although the two spin labeled ligands used in this study have approximately the same size, they differ in that the DNP-hydrazone-SL(5) may be a slightly more rigid, linear molecule than DNP-methylene-SL(5) due to the nature of their respective covalent linkage. One would expect that a linear ligand would have less steric hindrance and, therefore, greater mobility than a bent ligand, if the protein active site interacts only with the DNP portion of the ligand. The $A_{max}$ value of DNP-

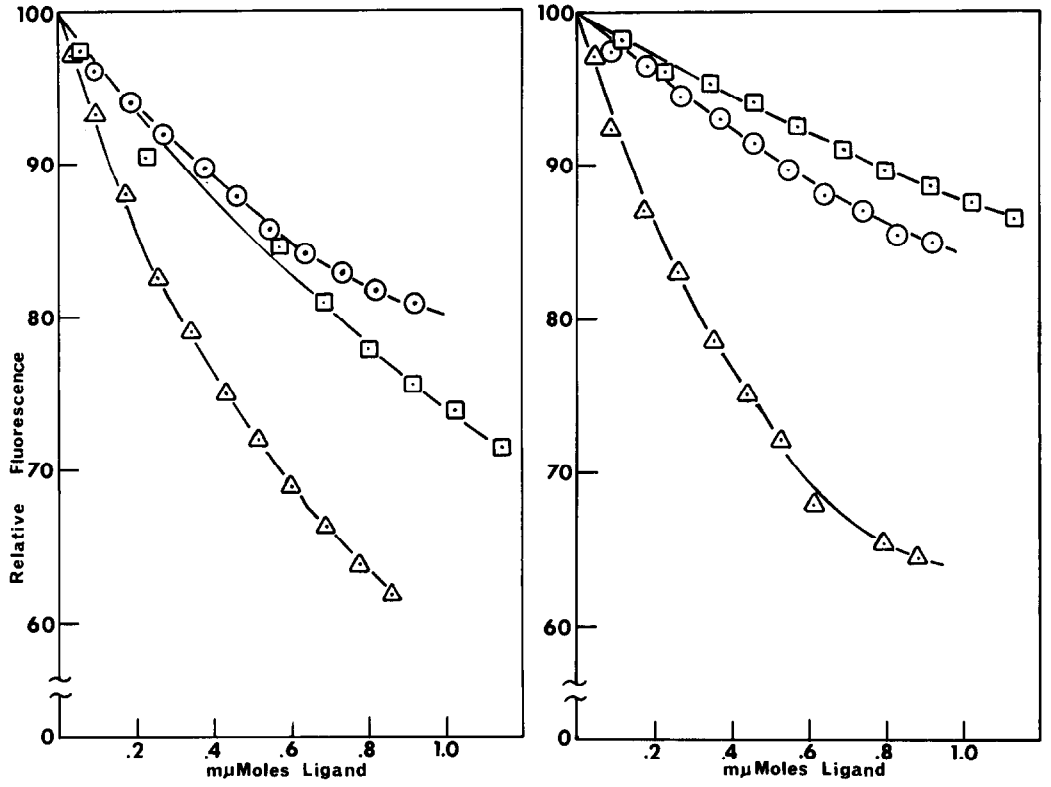


Fig.3. Fluorescence quenching titrations of 315 protein (left panel) at 25° by spin labels II (□), $K = 8.6 \times 10^5 M^{-1}$ and I (○), $K = 4.9 \times 10^5 M^{-1}$ and ε-DNP-L-lysine (△), $K = 4.5 \times 10^6 M^{-1}$. Fluorescence quenching titrations of 460 protein (right panel) at 3° by spin labels I, (○) $K = 7.5 \times 10^5 M^{-1}$ and II (□), $K = 5.6 \times 10^5 M^{-1}$ and 2,4-dinitronaphthol (△), $K = 2 \times 10^6 M^{-1}$.

hydrazone-SL(5) complex with the protein-460 indicates that the immunoglobulin active site is large enough to accommodate all or part of the spin label resulting in strong immobilization (see fig.1B). However, only very weak immobilization of the spin label moiety resulted from the binding of the DNP portion of DNP-methylene-SL(5) by the active site of protein-460. Fluorescence quenching data indicates that DNP-hydrazone-SL(5) has slightly greater affinity to protein-460 than DNP-methylene-SL(5). Although it is not clear whether the DNP portion of each ligand has a similar or identical steric relationship with the active site of the protein-460 we have detected that the spin label portion located at a fixed distance from the DNP group is making extensive contact (DNP-hydrazone-SL(5)) or very little contact (DNP-methylene-SL(5)) with the active site. The large difference in the extent of immobilization of these two structurally similar ligands at the same antibody active site could result from either of two causes: (i) the two ligands are binding to different regions of a conformationally rigid active site, or (ii) alternative conformations of the active site are selectively stabilized by structurally different ligand molecules due to the binding of a particular ligand.

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