Biochem. J. (1977) **165**, 199–206
Printed in Great Britain

Comparison of the Dimensions of the Combining Sites of the Dinitrophenyl-Binding Immunoglobulin A Myeloma Proteins MOPC 315, MOPC 460 and XRPC 25 by Spin-Label Mapping

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(*Received* 26 *October* 1976)

The mouse immunoglobulin A myeloma proteins MOPC 315, MOPC 460 and XRPC 25 all possess dinitrophenyl (Dnp)-binding activity. Differences in specificities were shown by measuring the affinities of a variety of haptens. By using a series of Dnp-spin-labelled haptens, the dimensions of the binding sites of the three myeloma proteins were compared by the method described for protein MOPC 315 [Sutton, Gettins, Givol, Marsh, Wain-Hobson, Willan & Dwek (1977) *Biochem. J.* **165**, 177–197]. The dinitrophenyl ring is rigidly held in all three sites. The depths of the sites are all 1.1–1.2nm, but there are differences in the lateral dimensions at the entrance to the sites. For protein XRPC 25 these dimensions are 0.75nm×0.8nm, which may be compared with 0.85nm×1.1nm for protein MOPC 315 and ≥1.0nm×1.1nm for protein MOPC 460. The site in protein MOPC 460 is more symmetrical with respect to the plane of the dinitrophenyl ring than in either of the other two myeloma proteins and also allows greater penetration of solvent. In protein XRPC 25 a positively charged residue was located at the entrance to the site, similarly positioned to that reported for protein MOPC 315 [Sutton, Gettins, Givol, Marsh, Wain-Hobson, Willan & Dwek (1977) *Biochem. J.* **165**, 177–197]. All three proteins possess lanthanide-binding sites, but only in protein MOPC 315 is there antagonism between lanthanide and hapten binding. However, the effects of the diamagnetic La(III) on the electron-spin-resonance spectra of bound Dnp spin labels in both proteins MOPC 460 and XRPC 25 suggest an interaction between the two sites. Comparison of this effect with that caused by the addition of the paramagnetic Gd(III) enables the distance between the lanthanide- and hapten-binding sites to be calculated. In both proteins MOPC 460 and MOPC 315 the metal site is approx. 1.0nm from the nitroxide moiety of the spin-labelled hapten, but in protein XRPC 25 this distance is at least 2.0nm.

Part of our programme to determine the solution structure of the combining site of the mouse myeloma protein MOPC 315 is concerned with analysing the structural basis of antibody specificity.

A comparison of the mouse myeloma IgA§ proteins MOPC 315, MOPC 460 and XRPC 25, which all bind dinitrophenyl haptens (Eisen *et al.*, 1968; Jaffe *et al.*, 1971; Sharon & Givol, 1976), has been made. The binding affinity for ε-Dnp-L-lysine is in the order protein MOPC 315 > XRPC 25 > MOPC 460, and differences exist in the specificity for some other ligands. For protein MOPC 460 the order is 2,4-dinitronaphthol > ε-Dnp-L-lysine > menadione, whereas for protein MOPC 315 it is ε-Dnp-L-lysine > menadione > 2,4-dinitronaphthol.

A series of Dnp-spin-labelled haptens was used to map the dimensions of the entrance to the combining site of protein MOPC 315 (Sutton *et al.*, 1977). We have extended the binding studies by Jaffe *et al.* (1971) to include protein XRPC 25, and the spin-label mapping method has been applied to the three myeloma proteins.

Materials and Methods

The IgA and Fab fragment of protein XRPC 25 were prepared as described in Sharon & Givol (1976). The Fab fragment of protein MOPC 460 was pre-

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§ Abbreviations: IgA, immunoglobulin A; Fab fragment, *N*-terminal half of heavy chain and light chain; Fv fragment, variable region of heavy and light chain; e.s.r., electron spin resonance; Dnp, dinitrophenyl; Pipes, 1,4-piperazinediethanesulphonic acid; n.m.r., nuclear magnetic resonance.

pared as described by Lancet & Pecht (1976). Dinitronaphthol was obtained from Koch–Light Laboratories, Colnbrook, Bucks. SL3 0BZ, U.K., and was recrystallized from ethanol. ε-Dnp-L-lysine was obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., and menadione (2-methyl-1,4-naphthaquinone) was obtained from Eastman Organic Chemicals, Kirkby, Liverpool, U.K.

Fluorescence measurements were performed at 25°C as described by Dwek *et al.* (1976), and measurements of the water-proton relaxation rates were carried out as described by Dower *et al.* (1975). The basis of the e.s.r. spin-label methods and the mode of analysis have also been presented by Sutton *et al.* (1977). Particular reference will be made to Tables 1 and 2 of that paper, which tabulate the same series of spin labels as used in the present study.

The binding of the haptens to the proteins was followed by fluorescence titrations in which samples of ethanolic hapten solutions (5 μl) were added to the protein solutions (3 ml, 1 μM-protein), to give hapten concentrations in the range 0–30 μM for dinitronaphthol and the dinitrophenyl derivatives, and 0–200 μM for menadione. The results were used to determine the values of the dissociation constant (K_D) and the limiting fluorescence of the protein in the presence of saturating ligand concentrations relative to that of the free protein (E_b). In those cases showing weak binding, i.e. $K_D \gg$ [protein], these parameters were determined from plots of the reciprocal of the change in fluorescence versus the reciprocal of the total ligand concentration. Where the weak binding condition did not hold, E_b was determined directly from a plot of fluorescence versus hapten concentrations, and a Scatchard plot constructed, assuming one binding site for hapten per molecule of protein.

The binding of Gd(III) to the proteins was followed by the enhancement of the solvent water-proton-relaxation rate. Titrations were carried out by adding increasing concentrations of Gd(III). The data were fitted by theoretical curves constructed by assuming two metal-binding sites per Fab fragment (Dower *et al.*, 1975) to give values of the dissociation constant (K_D) and the paramagnetic contribution to the proton-relaxation rate when the Gd(III) is bound to the protein relative to that observed when the Gd(III) is free in solution (E_b).

Results and Discussion

Binding studies

The binding of several Dnp-haptens to the three myeloma proteins was studied by fluorescence quenching at pH5.5 and 25°C. The binding constants and quenchings are presented in Table 1. All five haptens (*a–e*) show a higher affinity for protein MOPC 315 than for either proteins XRPC 25 or MOPC 460 and, except for hapten (*c*), they also show a higher affinity for protein XRPC 25 than for MOPC 460. The binding of the diamagnetic analogue (*b*) of hapten (*a*) is between 5- and 10-fold weaker, which may be due to the slightly different conformation of the nitroxide ring (>N–O is planar but >N–H is pyramidal), or the presence of a stabilizing interaction between a residue in the combining site and the nitroxide group.

The binding of menadione and 2,4-dinitronaphthol was similarly studied, and shows quite different affinities [haptens (*f*) and (*g*), Table 1] to the three myelomas. Menadione binds more strongly to protein MOPC 315 than to protein MOPC 460, but the converse is the case for 2,4-dinitronaphthol, in agreement with the results of Jaffe *et al.* (1971). With protein XRPC 25 neither menadione nor 2,4-dinitronaphthol can be shown, by fluorescence quenching, to bind.

The values ($1-E_b$) of the limiting quenching show a large variation for the binding of different dinitrophenyl derivatives to a single myeloma protein (e.g. protein MOPC 315 shows E_b values ranging from 0.1 to 0.4). However, this does not imply that the dinitrophenyl moieties bind in different ways, since a series of seven different dinitrophenyl derivatives all give very similar n.m.r. difference spectra on binding to the Fv fragment of protein MOPC 315 (Dower *et al.*, 1977), whereas the fluorescence E_b values vary from 0.1 to 0.45. Clearly the E_b value carries no simple structural significance.

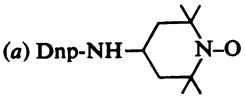
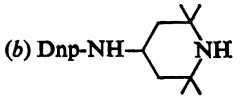
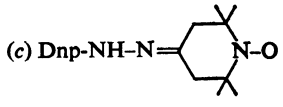
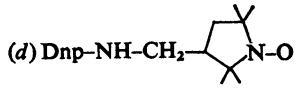
The binding constants of Gd(III) to the three myeloma proteins have also been included in Table 1. The values for proteins XRPC 25 and MOPC 460 were determined by measurements of the water-proton-relaxation rates as described by Dower *et al.* (1975). The binding of Gd(III) to these two myelomas is significantly weaker than in protein MOPC 315. For protein MOPC 315, Gd(III) weakens the binding of ε-Dnp-L-lysine and vice versa (Dwek *et al.*, 1976), whereas no such antagonism is detectable with either protein MOPC 460 or XRPC 25. The binding of ε-Dnp-L-lysine at pH5.5 to either of these two myeloma proteins, as measured by fluorescence quenching, is unaffected in the presence of Gd(III) (≤1.3 mM).

E.s.r. results

The values of the maximum hyperfine-splitting constant $A_{z'z'}$ for the series of spin-labelled haptens bound to myeloma proteins MOPC 460 and XRPC 25 are presented in Table 2; those of protein MOPC 315 are also included. The effect of slow molecular 'tumbling' on these values must be considered (Sutton *et al.*, 1977) in order to carry out

Table 1. *Comparison of the binding of ligands to three myeloma proteins*

All experiments were carried out in 50mM-Pipes buffer/0.15M-NaCl, pH5.5. The protein concentration was approx. 1 μM and $T = 25°C$. Parameters were obtained from fluorescence titrations.

Ligand	Protein XRPC 25 Fab fragment		Protein MOPC 460 Fab fragment		Protein MOPC 315 Fv fragment	
	Binding constant K_D (μM)	Fluorescence quenching, E_b	Binding constant K_D (μM)	Fluorescence quenching, E_b	Binding constant K_D (μM)	Fluorescence quenching, E_b
(*a*) 	7.4	0.38	32	0.30–0.50	0.30	0.07–0.10
(*b*) 	35–50	0.40–0.60	50–80	0.25–0.45	4.0	0.20
(*c*) 	14	0.63	7.5	0.53	5.0	0.40
(*d*) 	6.9	0.54	33	0.45–0.55	0.60	0.10
(*e*) ε-Dnp-L-lysine	5.4	0.54	15	0.50	0.42	0.24
(*f*) Menadione	⩾200	—	100	0.50–0.60	6.1	0.31
(*g*) 2,4-Dinitronaphthol	⩾30	—	2.9	0.48	15	0.55
Gd(III)	500*	—	500*	0.33*	150	1.14

* These parameters were obtained from titrations of the solvent water relaxation rates in solutions containing Gd(III).

the mobility analysis and compare results from different fragments. Where this is necessary, the new calculated values are presented in the text below.

The magnitude of the corrected $A_{z'z'}$ values will depend on motional averaging brought about by movement of the nitroxide relative to the protein, and also on the polarity of the nitroxide environment. The difficulty involved in separating these two effects is discussed in the preceding paper (Sutton *et al.*, 1977).

Analysis of the six-membered nitroxide ring haptens

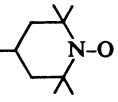
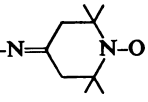
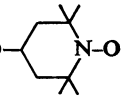
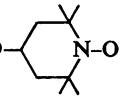
(*a*) *Depths of the sites.* For all three proteins, the $A_{z'z'}$ values of haptens (I) and (II) are much larger than those of haptens (III) and (IV). Whereas the former indicate some motional restriction, the latter are typical of near-isotropic motion, showing that all three sites have a depth less than 1.2nm. For protein MOPC 460, as for protein MOPC 315 (Sutton *et al.*, 1977), the motion of hapten (II) defines the depth of the site as 1.1–1.2nm.

(*b*) *Lateral dimensions.* The $A_{z'z'}$ value of hapten (I) bound to protein MOPC 315 can be accounted for solely by assuming that the only possible motion of the label is flexing of the nitroxide ring (Sutton *et al.*, 1977). The same is true for protein MOPC 460, the slightly lower value indicating rather greater mobility of the nitroxide moiety. In contrast, the $A_{z'z'}$ value for protein XRPC 25, allowing for molecular tumbling, is 3.54mT, implying total immobilization. The dimensions of the site around the six-membered nitroxide ring must be less than 0.6nm×0.9nm (Table 2; Sutton *et al.*, 1977). This result also shows that the dinitrophenyl ring must be rigidly held and the site also rigid. Hapten (II) is also immobilized in protein XRPC 25, whereas the combining site of protein MOPC 460 allows the same motion as in protein MOPC 315.

Analysis of the five-membered nitroxide ring haptens

(*a*) *Protein XRPC 25.* When bound to protein XRPC 25, both haptens (V) and (VI), which contain a

Table 2. *Maximum hyperfine splittings, $A_{z'z'}$ (mT), in the e.s.r. spectra of spin-labelled haptens bound to myeloma proteins MOPC 315, XRPC 25 and MOPC 460*

Hapten	Protein	MOPC 315	Protein	XRPC 25	Protein	MOPC 460
(I) Dnp-NH– 	Fab	2.67±0.01	Fab	3.18±0.07	Fab	2.45±0.07
(II) Dnp-NH-N= 	Fab	2.30±0.05	Fab	2.93±0.10	Fab	2.39±0.07
(III) Dnp-NH-CH_2-CO-O- 	Fv	1.59±0.10	Fab	1.59±0.10	Fab	1.60±0.10
(IV) Dnp-NH-[CH_2]$_3$-CO-O- 	IgA	1.60±0.02	Fab	1.59±0.10	Fab	1.60±0.10
(V) Dnp-NH-CH_2-	Fab	3.20±0.03 2.85±0.03	Fab	3.45±0.03 3.20±0.03	Fab	⩽2.0
(VI) 5-F-Dnp-NH-	Fab	3.00±0.03 2.63±0.02	Fab	3.00±0.03 2.73±0.03	Fab	2.55±0.03 2.43±0.03
(VII)† Dnp-NH-N=	IgA	3.12±0.03		—	IgA	3.12±0.03

* Denotes chiral centre. In the following data the two sets of values refer to the two enantiomers.
† From Hsia & Little (1973).

chiral centre, exhibit the two signals from the two enantiomers, and all four $A_{z'z'}$ values, 3.84 and 3.56mT [hapten (V)], 3.43 and 3.04mT [hapten (VI)] are greater than those for protein MOPC 315. These values have been corrected to allow for the slow molecular tumbling by the same method that was applied for the Fab fragment of protein MOPC 315 (Sutton *et al.*, 1977). It is assumed that all haptens are sensitive to the slow tumbling of protein XRPC 25. Since the value of $A_{z'z'}$ for the fully immobilized spin labels has been shown to be between 3.2 and 3.4mT, as discussed in Sutton *et al.* (1977), values much higher than this [e.g. for the 'immobilized' enantiomer of hapten (V)] are attributed to polarity effects. Only one enantiomer of hapten (VI) shows evidence of any motional averaging, and it is concluded that the combining site of protein XRPC 25 is asymmetrical and rather narrower than that of protein MOPC 315. However, the width must still be at least 0.7nm to allow any motion at all (Table 3; Sutton *et al.*, 1977). It follows that the height must be less than 0.9nm from the values for the six-membered nitroxide ring spin labels above.

(*b*) *Protein MOPC* 460. Hapten (V) behaves quite differently when bound to protein MOPC 460 compared with the other two myeloma proteins (Fig. 1). It is clear that the value of $A_{z'z'}$ is very much decreased in protein MOPC 460 and indicates considerable mobility. The enantiomers of hapten (VI) also have greater mobility in protein MOPC 460 compared with the other myeloma proteins (Table 2). However, the mobility of hapten (VI) in protein MOPC 460 is

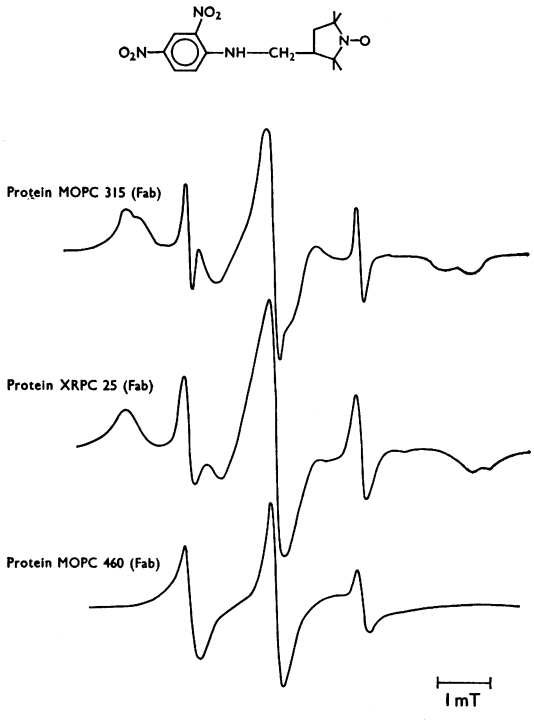


Fig. 1. *E.s.r. spectra of hapten (V) bound to the three myeloma proteins*
Protein concentration was 100μM in 0.15M-NaCl/0.05M-Pipes buffer, pH6.4.

far less than that of hapten (V). This is noteworthy, since hapten (V) has the greater intrinsic mobility. This means that the constraints of the geometry of the sites of proteins MOPC 315 and XRPC 25 must allow hapten (VI) greater mobility than hapten (V). The binding site of protein MOPC 460 imposes no such constraints and the motion of the haptens, as reflected in their lower values of $A_{z'z'}$, is limited only by their own geometry. Inspection of Table 3 of Sutton *et al.* (1977) shows that the observed values of $A_{z'z'}$ can be accounted for by full rotation about the NH–CH_2 bond in hapten (V) and the bond to the nitroxide ring in hapten (VI). This is confirmed by the observation of an 'inner' ($A_{x'x'}$) splitting for hapten (VI). The calculated $A_{z'z'}$ value (Table 3, Sutton *et al.*, 1977) for hapten (V), assuming full rotation, is 1.90mT and is consistent with the observed value ($\leqslant$2.0mT). For hapten (VI), there is also good agreement between the calculated values ($A_{z'z'} = 2.34$mT; $A_{x'x'} = 1.02$mT) on the basis of rotation about the bond to the nitroxide ring (Sutton *et al.*, 1977) and the observed values ($A_{z'z'} = 2.55$ and 2.43mT; $A_{x'x'} = 1.04$mT). However, in view of the similar $A_{z'z'}$ values for the two enantiomers of hapten (VI) and the small degree of anisotropy of the spectrum for hapten (V), there is no evidence for any asymmetry of the entrance to the combining site in protein MOPC 460, in contrast with the sites of proteins XRPC 25 and MOPC 315.

In contrast with haptens (V) and (VI), hapten (VII) behaves identically when bound to proteins MOPC 315 and MOPC 460, with an $A_{z'z'}$ value indicating almost complete immobilization. Conformational analysis (Sutton *et al.*, 1977) shows that this hapten has little intrinsic mobility, so that, if the dinitrophenol ring were rigidly held, then, even with no other contact between hapten and protein, the large $A_{z'z'}$ value would be expected. This shows that the dinitrophenyl ring must be rigidly held. This unusual result for hapten (VII) shows the advantage of using a series of structurally related spin labels. Alone, the rigid immobilization of hapten (VII) might be considered an artifact resulting from a property of the molecule itself, but the results for hapten (II) especially show this to be meaningful.

The conclusions about the combining sites of proteins XRPC 25 and MOPC 460 together with the picture of protein MOPC 315 derived from the earlier studies (Sutton *et al.*, 1977) are shown in Fig. 2.

The choice of enantiomers shown bound to proteins XRPC 25 and MOPC 460 in Fig. 2 is arbitrary.

It is noteworthy that when hapten (VI) was bound to whole IgA MOPC 460 protein (rather than the Fab fragment) the same $A_{z'z'}$ values were observed. It appears that in this sytem, where there is considerable motion of the nitroxide ring relative to the site and little or no contact between them, the $A_{z'z'}$ value is independent of the slower rotational motion of the fragment, a result which has been discussed with regard to the combining site of protein MOPC 315 (Sutton *et al.*, 1977).

Polarity of the combining sites

A polarity profile of protein MOPC 315 has been reported (Sutton *et al.*, 1977) constructed from values of A_0, the isotropic hyerpfine-splitting constant, which is an index of polarity. A_0 may be calculated for a rigidly immobilized spin label from the correlation with $A_{z'z'}$ (Griffith *et al.*, 1974), where there is sufficient axial motion for the observation of an inner splitting, ($A_{x'x'}$), from the equation:

$$A_0 = \tfrac{1}{3}(A_{z'z'} + A_{y'y'} + A_{x'x'})$$

where $A_{x'x'} = A_{y'y'}$.

Protein XRPC 25 has a similar profile to that of protein MOPC 315. The argument used for protein MOPC 315 in relation to the magnitude of the higher $A_{z'z'}$ value for one enantiomer of hapten (V), in terms of a nearby positive charge, is also applicable to protein XRPC 25. The charge must be in a very similar position at the entrance to the site (Fig. 2), but the higher $A_{z'z'}$ values for protein XRPC 25 in

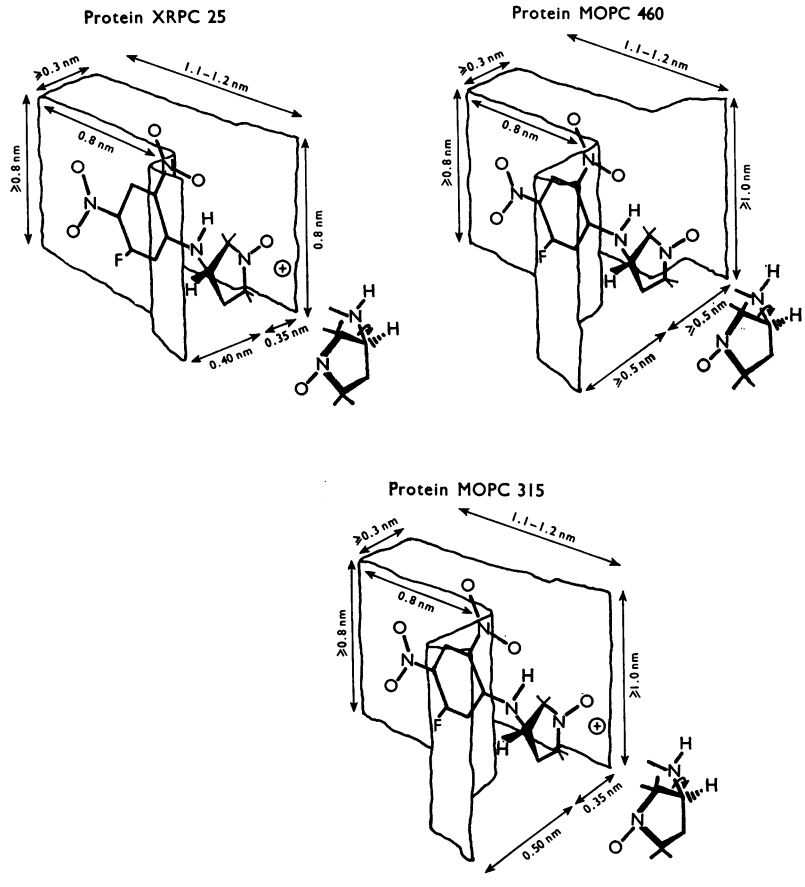


Fig. 2. *Comparison of the combining sites of three different myeloma proteins as determined by spin-label mapping* The positions of the two enantiomers of hapten (VI) are shown for each combining site. The assignment of the absolute configuration of the hapten shown in the site is arbitrary and is intended solely to illustrate the different dimensions of the sites.

comparison with protein MOPC 315 might be accounted for either by closer proximity of the nitroxide groups to this polar residue in the narrower combining site, or by a more hydrophobic environment due to the more effective exclusion of water. In contrast, the enantiomers of the shorter hapten (VI) show that the sites of proteins MOPC 315 and XRPC 25, in the region probed by these labels, are apparently identical.

The combining site of protein MOPC 460, however, is rather different. The extensive mobility of hapten (VI) will obscure any small contribution to the hyperfine interaction from a localized positive charge. The observation of an inner splitting with this hapten allows the polarity index A_0 to be calculated. Since the corresponding enantiomers are rigidly held in the other two proteins, A_0 values for these can be estimated from the linear correlation with $A_{z'z'}$ (Sutton *et al.*, 1977) and compared; A_0 is 1.40mT for proteins MOPC 315 and XRPC 25 and 1.54mT for protein MOPC 460.

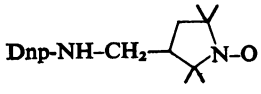
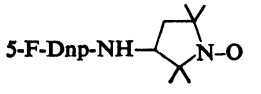
The value for protein MOPC 460 is close to that of a free aqueous environment, showing that the entrance to this combining site must be open to solvent, unlike those of proteins MPOC 315 and XRPC 25. This is consistent with the pictures of the sites presented in Fig. 2.

Distance between lanthanide- and hapten-binding site

All three myeloma proteins possess lanthanide-binding sites (Table 1). The apparent binding of the lanthanides to the Fab and Fv fragments of protein MOPC 315 is weakened about 20-fold in the presence

Table 3. *Distance between lanthanide- and hapten-binding sites determined by paramagnetic quenching of the e.s.r. spectra of spin-labelled haptens*

The limiting quenchings (% decrease of initial peak intensity) are the values obtained from addition of either 5mM-Gd(III) or -La(III). The metal–nitroxide distances (nm) are calculated by assuming a correlation time τ_c of 10^{-10}s (Sutton *et al.*, 1977). Other conditions were: pH6.4; 0.15M-NaCl; 0.05M-Pipes; 100μM-protein. The paramagnetic contribution to the quenching is the difference between the limiting quenchings with Gd(III) and La(III) expressed as a percentage of the signal intensity when maximally quenched by La(III).

Protein	Limiting quenching (%) Gd(III)	Limiting quenching (%) La(III)	Paramagnetic quenching (%)	Distance (nm)
XRPC 25 (Fab)				
	24±2	21±2	4±4	2.0
MOPC 460 (IgA)				
	65±2	12±2	60±3	1.00±.05

of ε-Dnp-L-lysine and the spin-labelled haptens (Dwek *et al.*, 1976), whereas this is not the case for the other two myeloma proteins. An estimate of the distance between the lanthanide- and hapten-binding sites can be obtained from the paramagnetic quenching of the e.s.r. signal of the hapten caused by the presence of Gd(III). La(III) is used as the diamagnetic control (Sutton *et al.*, 1977). Table 3 gives the limiting quenchings caused by Gd(III) and La(III) for the Fab fragment of protein XRPC 25 with hapten (V) and the IgA of protein MOPC 460 with hapten (VI). Since the resolution of the two bound signals is difficult with these proteins, only the combined peak was monitored, providing an average distance for the two enantiomers. The paramagnetic quenching contribution (column 3, Table 3) then allows the distance to be calculated if the electron-spin relaxation time of Gd(III) is known. Assuming a value of 10^{-10}s, which has been shown to be reasonable for protein MOPC 315 (Sutton *et al.*, 1977), the distances shown in Table 3 are obtained. It is assumed that there are two metal-binding sites on the Fab fragments (Dower *et al.*, 1975), but the distances calculated will be heavily weighted towards the nearest site because the dipolar interactions between the spin label and the paramagnetic metal ions resulting in the quenching of the e.s.r. spectrum are proportional to $1/r^6$, where r is the distance between the two paramagnetic centres.

The metal–nitroxide distance in protein MOPC 460, which is 1.0nm, is similar to that obtained for protein MOPC 315, which was 1.0–1.1nm (Sutton *et al.*, 1977). In contrast, protein XRPC 25, which shows little paramagnetic quenching, places the metal at least 2.0nm from the nitroxide groups of hapten (V). Thus, although the metal sites in these proteins have not been located precisely, protein MOPC 460 appears similar to protein MOPC 315, but protein XRPC 25 differs with respect to the relationship between the sites.

In all three cases the effect of the non-paramagnetic lanthanum indicates an interaction between the metal- and hapten-binding sites, which, at least in protein XRPC 25, occurs over a very large distance.

Conclusion

The method of spin-label mapping has shown that there are differences between the combining sites of the three myeloma proteins, although these conclusions are necessarily limited to the regions of the sites probed by the nitroxide groups. The dinitrophenyl ring itself has been shown to be held rigidly in all three proteins. A limitation of the e.s.r. mapping technique is that it gives no information about the dimensions around the dinitrophenyl ring, and the values given in Fig. 2 are those required to fit the dinitrophenyl ring into the site. Although the depths of all three combining sites are very similar (1.1–1.2nm), there are differences in the lateral dimensions at the entrance to the site. Protein XRPC 25 has a smaller combining site than protein MOPC 315 in both the width and height, although it retains the asymmetrical shape of the latter. In contrast, protein MOPC 460 is wider, allowing greater penetration of solvent than protein MOPC 315, and is more symmetrical with respect to the plane of the rigidly held dinitrophenyl ring. The e.s.r. mapping also indicates a positively charged residue at the entance to the site in both proteins XRPC 25 and MOPC 315, similarly

placed in each, but it is not possible to infer either the presence or absence of this residue in the combining site of protein MOPC 460.

Although the modes of binding for menadione and 2,4-dinitronaphthol are uncertain, these molecules are undoubtedly more bulky that the dinitrophenyl group. The weak binding of menadione and 2,4-dinitronaphthol to protein XRPC 25 may reflect a limitation on the possible modes of binding due to the restricted height and width of the entrance to the combining site.

The sites of proteins MOPC 315 and MOPC 460 are sufficiently large to accommodate them without the need to involve distinct binding sites, as has been suggested for protein MOPC 460 (Richards *et al.* 1975). Certainly, for protein MOPC 315, work in this laboratory has demonstrated that all these haptens do bind at the same site (Dwek, 1976), so it may be necessary to re-examine the interpretations of Richards *et al.* (1975).

We thank Professor R. R. Porter, F.R.S., Professor D. C. Phillips, F.R.S., Professor R. J. P. Williams, F.R.S., and Miss Elizabeth Press for their encouragement and interest in this work. We also thank the Medical Research Council and Science Research Council for their support. R. A. D. is a member of the Oxford Enzyme Group. D. G. is the recipient of an EMBO short-term fellowship.

References

Dower, S. K., Dwek, R. A., McLaughlin, A. C., Mole, L. E., Press, E. M. & Sunderland, C. A. (1975) *Biochem. J.* **149**, 73–82

Dower, S. K., Wain-Hobson, S., Gettins, P., Givol, D., Jackson, N. R. C., Perkins, S. J., Sunderland, C. A., Sutton, B. J., Wright, C. E. & Dwek, R. A. (1977) *Biochem. J.* **165**, 207–225

Dwek, R. A. (1976) *Contemp. Top. Mol. Immunol.* **6**, 1–53

Dwek, R. A., Givol, D., Jones, R., McLaughlin, A. C., Wain-Hobson, S., White, A. I. & Wright, C. (1976) *Biochem. J.* **155**, 37–53

Eisen, H. N., Simms, E. S. & Potter, M. (1968) *Biochemistry* **7**, 4126–4134

Griffith, O. H., Dehlinger, P. J. & Van, S. P. (1974) *J. Membr. Biol.* **15**, 159–192

Hsia, J. C. & Little, J. R. (1973) *FEBS Lett.* **31**, 80–84

Jaffe, B. M., Simms, E. S. & Eisen, H. N. (1971) *Biochemistry* **10**, 1693–1699

Lancet, D. & Pecht, I. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3549–3553

Richards, F. F., Konigsberg, W. H., Rosenstein, R. W. & Varga, J. M. (1975) *Science* **187**, 130–137

Sharon, J. & Givol, D. (1976) *Biochemistry* **15**, 1591–1594

Sutton, B. J., Gettins, P., Girol, D., Marsh, D., Wain-Hobson, S., Willan, K. J. & Dwek, R. A. (1977) *Biochem. J.* **165**, 177–197