Interactions of the Lanthanide- and Hapten-Binding Sites in the Fv Fragment from the Myeloma Protein MOPC 315

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1. The interactions of lanthanide metals and dinitrophenyl spin-label haptens with the Fv fragment of the mouse myeloma protein MOPC 315 were investigated by the techniques of fluorescence, e.s.r. (electron spin resonance) and high-resolution n.m.r. (nuclear magnetic resonance). 2. The protein fluorescence of Fv fragment at 340nm is quenched by the haptens (fluorescence enhancement, $\epsilon = 0.15$) and enhanced by Gd(III) ($\epsilon = 1.14$) and other lanthanides. The binding of the haptens studied here is insensitive to pH in the range 5.5–7.0 (dissociation constant $K_H = 0.3$ – $1.0 \mu\text{M}$) and shows 1:1 stoichiometry. The binding of Gd(III) also shows 1:1 stoichiometry, but is pH-dependent; the binding constant (K_M) varies from $10 \mu\text{M}$ at pH 7.0 to $700 \mu\text{M}$ at pH 4.8. La(III) binding is less sensitive to pH. The pH-dependences of the metal-binding constants imply that a group in the protein with $pK_a \geq 6.2$ is involved in the binding, and probably also other groups with lower pK_a values. 3. The apparent binding of the haptens is weakened about 20-fold by Gd(III), and vice versa. An equilibrium scheme involving a ternary complex with an interaction between the two binding sites is derived in Appendix I to explain the experimental results at two pH values. 4. Time-dependent fluorescence changes are observed in the presence of Gd(III) at pH 5.5. A two-state kinetic scheme involving a 'slow' conformational change in the Fv fragment is derived in Appendix II to explain this time-dependence. This scheme is consistent with the antagonistic equilibrium behaviour. 5. The e.s.r. changes in the spin-label haptens on binding to Fv fragment and on the subsequent addition of lanthanides are consistent with the binding scheme for haptens and lanthanides proposed from the fluorescence studies. A difference between the limiting quenching of the e.s.r. signal from the bound haptens in the presence of saturating concentrations of Gd(III) and La(III) is attributed to dipolar interactions between bound Gd(III) and the nitroxide moiety of the bound hapten. The residual quenching with Gd(III) allows an estimate of 1.2nm to be made for the distance between the two paramagnetic centres. 6. The 270 MHz proton difference spectrum of the Fv fragment resulting from the addition of La(III) suggests that any metal-induced conformational changes are small and involve relatively few amino acid residues on the Fv fragment. 7. The titration curves of chemical shift versus pH for the three histidine residues in the Fv fragment show that one histidine residue has its pK_a lowered from 6.9 to 6.4 in the presence of La(III). The decrease in pK_a does not arise from direct competition with a proton for a metal-binding site on the histidine residue. The change in pK_a and chemical shift of this residue together with the observation that a small number of residues are perturbed on La(III) binding suggest that the histidine residue is in the vicinity of the metal ion. 8. The addition of Pr(III) results in chemical shifts of the aliphatic proton resonances of the Fv fragment and also broadening of some aromatic resonances. This broadening is unexpected and possible reasons for it are discussed.

Much of our initial programme to determine in solution the amino acids and their orientations in and near to the combining site of the Fv fragment† from the Dnp (dinitrophenyl)-binding IgA (immuno-

† Abbreviation: Fv fragment, variable region of heavy and light chain.

globulin A) myeloma protein MOPC 315 has involved finding suitable paramagnetic centres that would bind to the protein (Dwek *et al.*, 1975a). Such centres provide reference points for high-resolution (n.m.r.) (nuclear-magnetic-resonance) mapping studies (Dwek, 1973). In a previous paper (Dwek

et al., 1975a) we showed how a Dnp-spin-labelled hapten could be used to perturb the proton high-resolution n.m.r. spectrum of the Fv fragment, and by using difference spectroscopy the n.m.r. spectrum of the amino acid residues in and around the combining site was obtained. We assigned resonances in this spectrum to histidine-97 in the light chain and histidine-102 in the heavy chain. However, the fact that a particular nuclear resonance is perturbed by the spin-labelled hapten and consequently 'appears' in the difference spectrum can only place that nucleus in a sphere of given radius from the spin-label grouping. The actual orientation of a nucleus can be obtained from the lanthanide perturbations of the nuclear resonances (Dwek, 1973), and the finding of one specific lanthanide-binding site on the Fv fragment was thus most fortunate (Dwek *et al.*, 1975a).

Our preliminary data (Dwek *et al.*, 1975b) suggested that there was an antagonistic effect between hapten and metal binding, each weakening the other's binding about 20-fold. It is, however, a prerequisite for high-resolution n.m.r. mapping studies to have a full knowledge of all the equilibria existing in solution, and this gave the impetus to the present work. In this paper we consider a number of aspects of the binding of haptens and lanthanides to the Fv fragment of MOPC 315 myeloma protein with particular reference to possible conformational changes induced by binding, and the apparent interaction between the two binding sites.

Materials and Methods

Materials

Fv fragment from the mouse myeloma protein, MOPC 315, was prepared as described previously (Hochman *et al.*, 1973) and then freeze-dried and stored at -20°C . No decrease in hapten-binding capacity or any other changes in properties were detectable after 3 months. Frozen solutions in 0.15M-NaCl (approx. 200 μM -Fv fragment, based on a mol.wt. of 25000) were stable for at least 2 weeks. Protein concentrations were determined by weighing and by absorbance ($\epsilon_{1\text{cm}}^{1\text{mg/ml}} = 1.5$ at 280 nm). The two methods agreed within $\pm 5\%$. Unless otherwise stated, all fluorescence and e.s.r. (electron-spin-resonance) experiments were carried out in 0.15M-NaCl/0.05M-Pipes [piperazine-*NN'*-bis-(2-ethanesulphonic acid)] buffer (analytical grade) adjusted to a pH value in the range 5.0–7.0 by addition of 5M-NaOH.

Oxides of the lanthanides (Koch-Light Ltd., Colnbrook, Bucks., U.K.) were dissolved in 5–10M-HCl and the resulting chlorides evaporated to dryness by gentle heating. The residues were dissolved in water to give 100mM stock solutions (unbuffered; pH approx. 6.0), which were stored frozen for several weeks. Precipitation of lanthanides was negligible at

(I)

(II)

pH 6. Metal concentrations were checked by titration with EDTA, by using Eriochrome Black T as an indicator, and agreed with weighing to $\pm 5\%$.

The spin-label haptens studied were *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-2,4-dinitrobenzene (I) and *N*-(1-oxyl-2,2,5,5-tetramethyl-3-methylamino-pyrrolidinyl)-2,4-dinitrobenzene (II): They were synthesized as described previously (Dwek *et al.*, 1975a). Stock solutions (2mM) were prepared by dissolving the desired amount in ethanol and were stable for 2 months at -20°C and showed no loss of the nitroxide e.s.r. signal. ϵ -Dnp-lysine (BDH Chemicals Ltd., Poole, Dorset, U.K.) was chromatographically pure. Stock solutions were prepared in water.

Methods

Fluorescence measurements. These were made on a Perkin-Elmer/Hitachi MPF-2A spectrofluorimeter with the cell compartment thermostatically maintained at 25°C . Typical protein concentrations were 0.5 μM . Protein fluorescence was excited at 295 nm and observed at 330 nm. A correction for attenuation of incident and emitted light by haptens free in solution was made from a calibration curve obtained at the same excitation and emission wavelengths from a tryptophan blank of similar fluorescence intensity to the protein solution. The correction factor at each hapten concentration was almost independent of the hapten used or the intrinsic tryptophan fluorescence. No corrections were necessary for metals up to a concentration of 3 mM or for the ethanol in which haptens were added.

In the titration of Tb(III) fluorescence by monitoring the 540 nm emission band, a filter with a cut-off at 430 nm was placed in the emission beam to avoid second-order detection of the 280 nm radiation from the exciting radiation. Also, a correction was applied

for the large baseline change in the presence of protein, caused by scatter and tryptophan fluorescence.

Electron-spin-resonance measurements. These were made on a Jeol JES-PE e.s.r. spectrometer operating at X-band (9.25 GHz). Titrations were performed at 20°C on samples of Fv fragment (10–40 μM) of volume 0.3–0.4 ml contained in a flat aqueous sample cell with a syringe attachment for mixing. This arrangement obviated disturbing the instrumental settings during a titration. All measurements were corrected for dilution (maximum 10–20%). It was usually also necessary to apply a small baseline correction to signals from bound haptens owing to the signals from free haptens and free Gd(III).

Nuclear-magnetic-resonance studies. High-resolution n.m.r. spectra were recorded at 270 MHz by using a Bruker spectrometer with an Oxford Instrument Co. (Oxford, Oxon., U.K.) superconducting magnet. The methods used have been described previously (Dwek *et al.*, 1975a). Measurements were made at $30 \pm 1^\circ\text{C}$. Chemical shift values are reported as p.p.m. downfield from the sodium salt of 3-(trimethylsilyl)propanesulphonic acid, used as an external standard.

Ultracentrifuge studies. These were carried out at 20°C in a Beckman model E analytical ultracentrifuge by using the schlieren optics with a standard cell and AnD rotor operating at 52000–56000 rev./min.

Results and Discussion

Fluorescence

Binding of haptens and lanthanides to Fv fragment studied by using protein fluorescence. Protein fluorescence quenching is an established method for determining the binding of Dnp haptens to antibodies (Velick *et al.*, 1960) and to the Fv fragment (Hochman *et al.*, 1973; Haselkorn *et al.*, 1974). The same technique has also been used here to detect the binding of lanthanides to the Fv fragment, although the relative

changes are much smaller. In the following sections the terminology is as defined in Appendix I. All results refer to Gd(III) and spin-label hapten (I) unless otherwise indicated.

(a) Haptens. The binding of hapten (I) to Fv fragment was studied in the pH range 5.5–7.0 by using protein concentrations ($[A]$) of about 0.5 μM (based on mol.wt. 25000) and hapten concentrations ($[H]$) of 0–30 μM . The fluorescence intensity was corrected for Dnp absorption as indicated in the Materials and Methods section. In the presence of saturating hapten the fluorescence is strongly quenched (enhancement, $\epsilon_{\text{AH}} = f_{\text{AH}}/f_{\text{A}} = 0.15$), suggesting that a large perturbation occurs in one or more tryptophan residues. The binding curve could be readily analysed on the assumption of 1:1 stoichiometry. Initial estimates of the dissociation constant, K_{H} , were therefore obtained by using the formula, $K_{\text{H}} = (1-f)^2[H]_{\text{T}}/f$, where f is the ratio of the change in the fluorescence at the equivalence point to the total change in fluorescence at saturation. Estimates of K_{H} and ϵ_{AH} were then used in a curve-fitting program, which gave the final values $\epsilon_{\text{AH}} = 0.15$, $K_{\text{H}} = 0.3\text{--}0.6 \mu\text{M}$. Both ϵ_{AH} and K_{H} are almost pH-independent in the range 5.5–7.0.

The behaviour with hapten (II) and with Dnp-lysine was very similar to the above, and their dissociation constants, K_{H} , are given in Table 1.

The low solubility of haptens (I) and (II) in water means that it is necessary to add them to aqueous solutions in a suitable solvent, in this instance ethanol. However, the apparent binding constant of Dnp-nitroxide (I) to Fv fragment at pH 6.8 ($K_{\text{H,app}}$) is very sensitive to small concentrations of ethanol in the solution, although the limiting fluorescence (f_{AH}) is not significantly altered. Ethanol alone causes a small enhancement in protein fluorescence. The effects of ethanol appear to approximate to those of a competitive inhibitor, since they are reversed by a suitably high hapten concentration. They might simply arise from non-specific binding of ethanol in the hydrophobic hapten-binding pocket, but the

Table 1. Binding constants (μM) of haptens and lanthanides to Fv fragment in 0.15M-NaCl/0.05M-Pipes buffer

Fluorescence experiments and e.s.r. experiments were performed at 25°C and 20°C respectively. The binding constants are defined according to the scheme of Appendix I.

Binding constant	pH 5.5	pH 6.8			Technique
K_M for Gd(III)	140	11			Protein fluorescence
	Hapten (I)	Hapten (I)	Hapten (II)	Dnp-Lys	
K_H	0.3	0.3	0.55 0.3–1.0	0.6	Protein fluorescence
K_{HM} for Gd(III)	3800	500	280		Hapten e.s.r.
	4000	400–800	600–1200		Protein fluorescence
K_{MH} for Gd(III)	6.5	4.0	5.0	10	Hapten e.s.r.
		5–10	5–10		Protein fluorescence
K_{MH} for La(III)		4.0	5.5		Protein fluorescence

possibility of reversible denaturation cannot be ruled out. A 10% (v/v) ethanol solution raises $K_{H,app}$ from $0.3\mu\text{M}$ to $6\mu\text{M}$, but below 2% ethanol the effects on hapten binding are minimal. This restriction is easily met for fluorescence experiments (total volume 3.0ml), but may inconvenience hapten titrations using e.s.r. or n.m.r. where the total volume is much smaller, if artifacts are to be avoided.

(b) Lanthanides. Protein fluorescence was monitored as for hapten binding. The fluorescence changes on adding lanthanides were slow (see below) and 2–4min was required to reach a steady value.

(i) Gd(III). The fluorescence enhancement is fairly constant ($\epsilon_{AM} = f_{AM}/f_A = 1.14 \pm 0.03$) over the pH range 5.0–7.0, although there are moderate changes (approx. 20%) in the intrinsic protein fluorescence in this pH range. Since the lanthanides precipitate above pH 7.0, and the fluorescence enhancement on the addition of Gd(III) declines rapidly below pH 5.0, the investigation of the pH variation of Gd(III) bind-

ing is limited to the range shown in Fig. 1 (pH 4.8–7.0).

The Gd(III)-binding constant, K_M , was estimated from the concentration for half-maximal change in fluorescence. K_M is very sensitive to pH (varying from $700\mu\text{M}$ at pH 4.8 to $10\mu\text{M}$ at pH 7.0), implicating an ionizable group on the protein in the lanthanide binding. The data for Gd(III) in Fig. 1 (lower curve) imply that at least one group with $pK_a \gtrsim 6.2$ is involved in the lanthanide binding. Since the observed slope is greater than 1, the data would also be consistent with other groups having pK_a values between 5.0 and 6.0 being involved. The data do not, however, distinguish between two possibilities, i.e. direct competition of the protons and the Gd(III) for the same site(s) in the protein or an indirect effect of an ionizable group in the Gd(III)-binding site (i.e. transmitted through a conformational change in the protein).

At pH 6.8, K_M is invariant ($\pm 10\%$) over the protein-concentration range 0.26 – $4.2\mu\text{M}$. Thus any protein-protein interactions would appear to be unimportant as regards lanthanide binding. This is supported by ultracentrifuge studies in the same buffer at $80\mu\text{M}$ (2.0mg/ml) protein concentration. Exposure to 9mM -Gd(III) for 12h had no significant effect on the sedimentation coefficient at 20°C ($s = 2.3$ – 2.6S) and no heterogeneity in the sample could be detected. At pH 7.0, the use of Fv-fragment concentrations comparable with K_M ($7.5\mu\text{M}$ and $15\mu\text{M}$) has permitted the stoichiometry of binding to be determined by means of Scatchard plots. The fractional change in protein fluorescence was used as a measure of free and bound protein, which thus assumes that Gd(III)-binding sites not producing a fluorescence change can be neglected. With this limitation, the analysis indicates that the fluorescence enhancement is due to Gd(III) binding at a single site [$K_M = 8$ – $12\mu\text{M}$, n (number of binding sites) = $1.0 \pm 0.2/\text{mol}$].

(ii) Other metals. Tb(III) produces similar changes in protein fluorescence to Gd(III) at pH 5.6. La(III) also produces comparable enhancements at pH 6–7, but the limiting enhancement falls to an unsuitable value (approx. 5%) for carrying out titrations below pH 5.1. With Gd(III) this degree of enhancement (approx. 5%) occurs at $\text{pH} \leq 4.6$. The upper curve of Fig. 1 was obtained by direct measurement of the fluorescence enhancement on titrating with La(III), which varied from 1.05 at pH 5.1 to 1.18 at pH 6.8. La(III), normally considered a suitable diamagnetic control for experiments with Gd(III), apparently binds to Fv fragment with a similar affinity to Gd(III) at pH 5.1 ($K_M \approx 500\mu\text{M}$), but binds an order of magnitude more weakly than Gd(III) at pH 6.8 ($K_M \approx 100\mu\text{M}$). This implies some slightly different mechanism of binding for the two metals. From the slope of the Gd(III)-binding data it seems likely that two or more groups with overlapping ionizations are involved in the binding. To explain the discrepancy

Fig. 1. pH-dependence of the binding of Gd(III) and La(III) to Fv fragment

The binding was monitored by the enhancement of protein fluorescence of Fv fragment (1 – $2\mu\text{M}$) at 340nm , in 0.05M -Pipes buffer/ 0.15M -NaCl, at 25°C . $\circ$, Gd(III); $\bullet$, La(III).

with La(III), a possibility is that some parameter such as ionic radius of the lanthanide is critical for binding at the multiply ionized site (i.e. at high pH) but less critical for binding in the partly ionized site (i.e. at pH 5.1). Nevertheless, a group of $pK \gtrsim 6.2$ seems to be implicated in the binding of both lanthanides.

Eu(III) gave no detectable fluorescence change on binding at pH 5.9, which was therefore studied by measuring the apparent binding of Gd(III) in the presence of Eu(III) or by reversing the Gd(III) enhancements by Eu(III). By assuming purely competitive behaviour, the binding constant was obtained from the formula:

$$K_{M,app.} = K_M(1 + [I]/K_I)$$

where $[I]$ and K_I are the concentration and dissociation constant of the competing lanthanide. At pH 5.9, Eu(III) was found to have a very similar binding constant ($50\text{--}80\mu\text{M}$) to that of Gd(III) ($40\mu\text{M}$).

Ca(II), which is an important factor in complement fixation (Lepow *et al.*, 1963), does not apparently compete for the lanthanide-binding site. At pH 6.8, 2.0mM -Ca(II) has no effect on protein fluorescence and does not alter the Gd(III)-binding constant ($K_M = 20\mu\text{M}$).

(c) Binding studies in the presence of both hapten and lanthanide. At both pH 5.5 and 6.8 the binding of hapten (I) is substantially affected by the presence of Gd(III), and vice versa. Fig. 2 shows that the presence of Gd(III) both weakens the apparent binding constant of hapten and decreases the apparent fluorescence change on hapten binding. At the highest metal concentrations, a limit is approached with a characteristic apparent binding constant and a

Fig. 2. Antagonism of hapten (I) and Gd(III) binding to Fv fragment at pH 6.8

The apparent binding of hapten (I) to Fv fragment ($0.38\mu\text{M}$) was monitored by the protein fluorescence quenching at 340nm at several fixed Gd(III) concentrations [value (mM) beside each line], in 0.05M -Pipes buffer/ 0.15M -NaCl at 25°C .

characteristic fluorescence change. This is inconsistent with simple displacement of hapten by metal and suggests that a ternary complex is formed. The data may be analysed on this basis according to the theory developed in Appendix I to give the dissociation constants of hapten and Gd(III) to Fv fragment in the absence (K_H and K_M) and the presence (K_{MH} and K_{HM}) of the other ligand. In the analysis the assumptions $[H]_{free} \approx [H]_{total}$ and $[M]_{free} \approx [M]_{total}$ have been made, which are valid since for most of the data $[M]_{total}, [H]_{total} \eta[A]_{total} = 0.37\mu\text{M}$.

Appendix I predicts that the decrease in protein fluorescence (ΔF) as a function of hapten concentration for different (constant) Gd(III) concentrations will give a set of hyperbolae (Appendix I, eqn. 7). This is verified by the double-reciprocal plots derived from Fig. 2 (Fig. 3). The apparent binding constants $[K'_D(M)]$ for each different Gd(III) concentration are replotted in the inset as a function of the Gd(III) concentration. The inset also shows a plot of total fluorescence at infinite hapten concentration $[F'(M)]$ [obtained from the extrapolated values of $\Delta F_0(M)$ in the double-reciprocal plots] as a function of Gd(III) concentration. This secondary plot of $F'(M)$ may be analysed according to eqn. (15) of Appendix I to give K_{HM} . The secondary plot of $K'_D(M)$ may be analysed according to eqn. (11) of Appendix I to give L_H , K_{MH} and K_{HM} . The values of K_{HM} determined from the two methods, i.e. from the Gd(III) concentration for half-maximal change in $K'_D(M)$ (0.50mM) and for half-maximal change in $F'(M)$ (0.80mM), agree fairly well and give some indication of the errors in the methods. The value for the fluorescence enhancement in the ternary complex may also be obtained from the replot of $F'(M)$ and is $\epsilon_{AMH} = f_{AMH}/f_A = 0.71$. The calculated values of the binding constants are summarized in Table 1.

Two points are apparent from the data in Table 1. First, since the model predicts that the four binding constants should be related according to the equation $K_M K_{MH} = K_H K_{HM}$, and all four binding constants may be independently determined, the data may be shown to be reasonably consistent with the assumed model, noting that the experimental determinations of the binding constants may show as much as 50% scatter. (This is particularly true for values of K_M and K_{HM} , since the observed changes in fluorescence on addition of metal are small.) Second, the presence of hapten is seen to weaken the binding of Gd(III) by a factor of 10–20-fold and vice versa. The analysis confirms that this antagonism is an indirect effect, i.e. not a direct competition of hapten and Gd(III) for the same site. This implies that the hapten and the Gd(III) must have distinct binding sites on the Fv fragment. This is consistent with previous water proton-relaxation-rate studies on the effect of Dnp-aspartate on the binding of Gd(III) to Fv fragment (Dwek *et al.*, 1974). The interaction between the two distinct

Fig. 3. Graphical treatment of data for the antagonism of hapten (I) and Gd(III) binding to Fv fragment at pH 6.8

Double-reciprocal plots of the curves in Fig. 2 for the apparent binding of hapten (I) to Fv fragment ($0.38 \mu\text{M}$) at several fixed Gd(III) concentrations [value (mM) beside each line]. The inset shows replots from the intercepts of the apparent dissociation constant for hapten (I) [$\circ$, $K'_D(\text{M})$] and the total fluorescence change [Δ , $F'(\text{M})$] as a function of Gd(III) concentration.

Fig. 4. Antagonism of hapten (I) and Gd(III) binding to Fv fragment at pH 5.5

Double-reciprocal plots of the apparent binding of hapten (I) to Fv fragment ($0.57 \mu\text{M}$) were determined by protein-fluorescence quenching at 340 nm at several fixed Gd(III) concentrations [value (mM) beside each line] in 0.05 M-Pipes buffer/0.15 M-NaCl at 25°C . The inset shows a replot of the apparent dissociation constant for hapten (I) [$K'_D(\text{M})$] as a function of Gd(III) concentration.

binding sites could be a direct interaction between two adjacent sites (i.e. partial overlap of the sites) or an indirect interaction (mediated by the protein) between two widely separated sites.

The analysis was repeated at pH 5.45 (see Fig. 4). A comparison of the calculated binding constants at the two pH values (see Table 1) shows two interesting results. The binding constant of hapten [either in the presence or in the absence of Gd(III)] is insensitive to pH in the range 5–7. However, the binding constant of metal (in both the presence and the absence of hapten) is quite sensitive to pH. This is consistent with the results of the previous sections.

La(III) gives similar effects to those of Gd(III) at pH 6.9 (see Table 1), except that a higher concentration of La(III) is necessary, because of the intrinsically weaker binding constant of La(III) to Fv fragment. The behaviour of hapten (II) is also similar to that of hapten (I) (see Table 1).

(d) Time-dependent changes on binding lanthanides and haptens to Fv fragment. In the absence of Gd(III) the fluorescence quenching on addition of haptens occurs rapidly on the time-scale required for a measurement (approx. 10s). However, in the presence of Gd(III), the fluorescence quenching on the addition of hapten is markedly slower and re-

quires a period of minutes to reach a new equilibrium. Similarly, the fluorescence change on the addition of Gd(III) requires minutes to equilibrate. This is shown in Fig. 5 for the consecutive addition of Gd(III) (different concentrations) and hapten ($3.3\ \mu\text{M}$) at pH 5.5. In each case, there is a rapid fall of the fluorescence on the addition of hapten, followed by a further slow decrease. The slow decrease is always characterized by a single exponential half-life (τ) as indicated by the semi-log plots shown in the inset to Fig. 5. The addition of EDTA completely reverses the Gd(III)-induced changes (in both the presence and the absence of hapten), indicating that the reactions are reversible. This is further substantiated by the observation that τ is a function only of the final metal and hapten concentration and is not affected by adding metal and hapten in the reverse order (see Fig. 6).

Since the binding equilibria involving the hapten and the metal would be expected to be rapid (Eigen & Hammes, 1963; Hammes, 1968), the slow changes suggest that in the presence of metal a slow equilibrium between two or more different conformations of the protein is displaced. The rapid changes in the presence of hapten [but not Gd(III)] suggest that the hapten binds preferentially to a conformational

Fig. 5. Relaxation of Fv-protein fluorescence in the presence of Gd(III) at pH 5.5

The time-dependence is shown of the fluorescence intensity at 340 nm on addition of Gd(III) and the further change on addition of a fixed concentration of hapten (I) at several Gd(III) concentrations in 0.05 M-Pipes buffer/0.15 M-NaCl at 25°C. Fv fragment (Fv; $0.5\ \mu\text{M}$) was added at the time indicated by the arrow. Gd(III) was added at the following concentrations: (1), 0.13 mM; (2), 0.27 mM; (3), 0.53 mM; (4), 1.06 mM; (5), 2.10 mM. Hapten (I) ($3.3\ \mu\text{M}$) was added at $t = 0$ for part (b). The inset shows semi-log plots of the second change (ΔF) after addition of hapten.

Fig. 6. Dependence of the relaxation of Fv-fragment fluorescence on (a) Gd(III) and (b) hapten (I) concentrations at pH 5.5

The reciprocal of the half-time for the fluorescence change (τ) is plotted against Gd(III) concentration in the presence (-----) and absence (—) of 3.3 μM -hapten (I) (a) and against hapten concentration in the presence of 1.06 mM-Gd(III) (b). The curves are computed fits (see the text). Protein concentrations were 0.3–0.5 μM (●) or 1–3 μM (○). Relaxation times in the presence of both ligands were determined with the hapten added last, except for one measurement where the addition was reversed (Δ).

state that predominates in the solution. The mixture of slow and fast changes observed in the presence of Gd(III) after the addition of hapten (see Fig. 5) can then be qualitatively understood in terms of the hapten binding rapidly to one conformation and then displacing the equilibrium between the two conformations. Adding metal to a solution containing hapten and Fv fragment gives only the slow change, as might be expected, since the starting equilibrium then lies well to one side.

τ is independent ($\pm 10\%$) of protein concentration in the range 0.3–3.0 μM both with Gd(III) alone and with Gd(III) plus hapten, indicating that polymerization is unlikely to be an important factor in the equilibria. In addition, ultracentrifuge studies with 80 μM -Fv fragment indicate that there is negligible polymerization in the presence of Gd(III), hapten, or both together. Hence the changes probably represent an isomerization of the protein. This isomerization

must, however, be pH-dependent, since τ is much smaller at pH 7 than at pH 5.5.

Appendix II shows a method for the quantitative analysis of this slow relaxation based on a two-state model. The analysis confirms that, for a given hapten and metal concentration, the relaxation processes can always be described by a single τ value (Appendix II, eqn. 8). In principle, a detailed treatment of the fluorescence changes and their half-times as a function of metal and hapten concentrations could give estimates of the intrinsic dissociation constants K_m and K'_m for binding to the two states of the protein separately, for which the constants derived in Appendix I (K_M etc.) are weighted averages. The forward and backward velocity constants of the isomerization k_{+1} and k_{-1} could also be obtained. Thus the kinetic treatment can potentially give more information than the equilibrium treatment. Here we consider only a limited amount of data, sufficient to establish the validity of the two-state treatment as a first approximation; to determine the constants of the system uniquely rapid reaction techniques, such as temperature jump, will probably be required in addition.

Fig. 6(a) shows the dependence of τ^{-1} (see Appendix II) on Gd(III) concentration in the presence and absence of hapten. Fig. 6(b) shows the dependence of τ^{-1} on hapten concentration in the presence of Gd(III). Eqn. (5) of Appendix II predicts that the change in τ^{-1} (relative to the value at zero metal concentration) should be hyperbolic. For the case with no hapten present, eqn. (5) simplifies to:

$$\Delta(\tau^{-1}) = [\tau^{-1}(M) - \tau^{-1}(0)] = \frac{k_{+1} \left(\frac{K_m}{K'_m} - 1 \right) [M]}{K_m + [M]}$$

The metal concentration for half-maximal change gives K_m directly, and the starting and final values of τ^{-1} give $(k_{+1} + k_{-1})$ and $k_{-1} + k_{+1} (K_m/K'_m)$ respectively. Eqn. (10) enables us to relate these constants to the already determined statistical dissociation constant K_M . Putting $k_{+1}/k_{-1} = L$, the isomerization constant, and $K_m/K'_m = c$, the ratio of intrinsic dissociation constants for the two states, it can readily be seen that:

$$\frac{\tau^{-1}(M=\infty)}{\tau^{-1}(M=0)} = \frac{1 + Lc}{1 + L}$$

From the data of Fig. 6, it is difficult to obtain accurate estimates of $\tau^{-1}(0)$ and K_m . The continuous line is computed for $K_m = 80 \mu\text{M}$, $\tau^{-1}(0) = 0.15 \text{ s}^{-1}$, $\tau^{-1}(M = \infty) = 0.015 \text{ s}^{-1}$, but it is possible to vary K_m and $\tau^{-1}(0)$ over a twofold range and still obtain a fit within the experimental limits of the data. It should be noted that the errors are particularly large at low metal concentrations both because τ is short

and also because the fluorescence enhancement (maximum 15%) is considerably decreased. L and c cannot be determined independently from Fig. 6, but by using the estimate $(1+Lc)/(1+L) \approx 0.1$, the limits can be placed as $0 < c < 0.1$, with the corresponding limits for L of $9 < L < \infty$. Physically, this suggests that in the absence of hapten the protein is predominantly ($\geq 90\%$) in one form (A'), and that $Gd(III)$ then binds almost exclusively (at least a factor of 10 more strongly) to the other form (A). The lack of time-dependence in the absence of $Gd(III)$ suggests that the hapten preferentially binds to the form that predominates in free solution (A').

The conclusions are obviously consistent with the conclusions from the equilibrium-binding studies, but provide much more insight into the apparent antagonism between the two binding sites. In terms of the above model, the Fv fragment exists free in solution predominantly in the form that binds the hapten tightly and the metal ion weakly. The addition of metal ion then shifts the equilibrium towards the conformation that binds the metal ion tightly and the hapten weakly, producing an apparent weakening of the hapten binding constant. The addition of hapten would have a reciprocal effect. Thus, whereas the physiological importance of the lanthanide binding to Fv fragment remains to be determined, the binding properties of the lanthanide may be used as a monitor of subtle conformational changes in the Fv fragment that occur on hapten binding.

Binding of Tb(III) to Fv fragment by Tb(III) studied by fluorescence enhancement. The addition of Fv fragment to Tb(III) solutions causes little change in the fluorescence-excitation spectrum (emission at 540nm) except for a large increase in a band centred on 280nm. Titrations of the 540nm emission band (excitation 280nm) with Tb(III) ($0-500\mu M$) were carried out in the presence and absence of protein ($2\mu M$) at pH 5.6. The results suggest that Tb(III) binding ($K_M \approx 300\mu M$) is substantially weaker than $Gd(III)$ binding and that the fluorescence of the bound Tb(III) is enhanced by a factor of 100–200 (assuming a 1:1 stoichiometry). The excitation spectrum suggests that the mechanism of enhancement is energy transfer from tryptophan to the metal.

Electron spin resonance

Binding of spin-label haptens, and the binding of lanthanides in the presence of spin-label haptens. (a) Haptens. The e.s.r. signals of haptens (I) and (II) change on binding to Fv fragment from the narrow lines characteristic of a mobile label to broader lines characteristic of a less mobile label, particularly for hapten (II). The use of e.s.r. spectra of a series of haptens to probe the geometry, rigidity and polarity of the combining site has been described (Dwek *et al.*, 1975a,b). Because of the very high intensity of

the free-label signal in comparison with the bound-label signal it is impossible to observe the latter directly except at the extremes of magnetic field. For this reason label II, which gives a greater peak-to-peak separation, is more convenient to monitor than label I. The intensity of the narrow high-field (right-hand) line can, on the other hand, be used directly as a measure of the free hapten concentration in the presence of bound hapten, since the contribution of immobilized labels to this region of the spectrum is negligible. Fig. 7 illustrates the method for simultaneously monitoring the free and bound hapten in a titration of a fixed total concentration of hapten (II) ($20\mu M$) with an increasing concentration of Fv fragment ($0-60\mu M$) in $0.1M$ -Pipes buffer/ $0.15M$ -NaCl, pH 6.9. The inset shows the decrease in signal from the free hapten as the protein concentration is increased. The data are consistent with 1:1 stoichiometry and a dissociation constant (K_H) of $0.3-1.0\mu M$, in good agreement with fluorescence titrations (Table 1). In this titration the concentration of ethanol was constant and as low as possible for addition of the hapten (approx. 1%).

If the titration is performed in reverse (increasing the hapten concentration at fixed protein concentration) or the signal from bound hapten is monitored, small but distinct apparent deviations from the expected stoichiometry occur. They may partly be connected with the need to add relatively large concentrations of ethanol (see above) or, for hapten (I) only, with the limited solubility in aqueous solutions (approx. $30\mu M$), but could also indicate heterogeneity of the bound hapten with regard to spectral characteristics. This might arise if the hapten bound weakly at a second site or could bind to two conformational states of the protein and displace the equilibrium between them. These complications stress that changes in the signal of the bound hapten must be interpreted with caution.

(b) Lanthanides. (i) $Gd(III)$. When $Gd(III)$ is added to solutions of Fv fragment ($10-30\mu M$) at pH 6.9 in the presence of a moderate concentration of hapten (I) or (II) ($25\mu M$) changes in intensity of the signal from both bound and free hapten occur. The signal of bound hapten decreases hyperbolically as a function of $Gd(III)$ concentration with an apparent K_M of $600-800\mu M$ and a limiting intensity of ≤ 0.2 of the starting value. The signal of free hapten increases over the same range of metal concentrations, then falls steadily at higher concentrations of metal ($>2mM$). We interpret the initial rise as being due to displacement of hapten from the protein as the metal binds. The subsequent fall is probably due to dipolar interactions between the two paramagnetic species in free solution, since a control containing no protein gives a very similar decrease. The decrease in signal from the bound hapten may be due to other causes than displacement, and this is discussed below.

Fig. 7. Changes in the e.s.r. spectrum of hapten (II) binding to Fv fragment

The e.s.r. spectrum [(a) bound-hapten signals, (b) free-hapten signals] of a fixed total concentration of hapten (II) ($20\mu\text{M}$) is recorded at increasing concentrations of Fv fragment ($0\text{--}60\mu\text{M}$) in 0.05M -Pipes buffer ($\text{pH } 6.9$)/ 0.15M -NaCl at 20°C . The inset shows the binding of hapten (II) to Fv fragment as monitored by the e.s.r. signal of the free hapten. The bar represents a magnetic field of 1mT .

The displacement can be explained in terms of the above fluorescence analysis. Monitoring the high-field line provides estimates of the concentrations of free hapten and (by difference from the total hapten added) of bound hapten. The amount of hapten displaced from the protein by a saturating concentration of Gd(III) can similarly be estimated. The protein is then in only two forms (AM and AMH) and so the dissociation constant, $K_{\text{MH}} = [\text{AM}][\text{H}]/[\text{AMH}]$, can be estimated. The values for K_{MH} for haptens (I) and (II) are $5\text{--}10\mu\text{M}$, in agreement with the results from fluorescence measurements (Table 1).

With hapten (I) it is impossible to ensure 100% saturating conditions with metal present because of the limited solubility ($30\mu\text{M}$), but this difficulty can be avoided with the five-membered-ring hapten (II), which is soluble in aqueous solutions to greater than $100\mu\text{M}$. Under these conditions there is no detectable displacement of hapten from the protein when Gd(III) binds, but there is still a decrease in signal from the bound hapten, which is hyperbolic and characterized by limiting relative amplitude of 0.4 and $K_{\text{M,app.}} = 1100\mu\text{M}$ at $\text{pH } 6.9$. This must reflect an intrinsic change in the AH complex on binding Gd(III). The value $1100\mu\text{M}$ is of the same order as the dissociation constant $K_{\text{HM}} = [\text{AH}][\text{M}]/[\text{AHM}]$ determined by fluorescence (Table 1). The e.s.r. results

thus appear to support the binding scheme proposed above.

Effect of pH on Gd(III) titrations. A series of four titrations of Fv fragment with Gd(III) were performed in the pH range $5.9\text{--}7.0$ in the presence of concentrations of free hapten (I) near to its solubility limit (approx. $30\mu\text{M}$). The bound and free hapten were both monitored as above. The changes in free hapten were used to estimate the decrease expected in the signal of bound hapten at infinite metal concentration due purely to displacement. In every case the displacement of hapten from the bound site was no more than 30% of the total bound hapten, whereas the observed signal decrease was much larger ($50\text{--}80\%$). After correction for this displacement, the relative amplitude of the e.s.r. spectrum for the bound site (with metal present) was found to vary from $0.3\text{--}0.4$ at $\text{pH } 7.0$ to 0.7 at $\text{pH } 5.9$. Thus there must be a pH-dependent change affecting the signal from the immobilized hapten in the bound site in the presence of metal. $K_{\text{M,app.}}$ for Gd(III) increases from 0.8mM at $\text{pH } 7.0$ to 4.0mM at $\text{pH } 5.9$, which corresponds satisfactorily to the variation in K_{HM} detected by fluorescence.

(ii) Other lanthanides. The above results indicate that there is a real decrease in the broad e.s.r. signal from bound haptens on the formation of the ternary

complex with Gd(III). This could occur because of magnetic dipolar interactions (Leigh, 1970; Dwek, 1973) between the bound Gd(III) and the bound nitroxide. However, in view of the earlier studies a second possibility, that the metal may induce a change in mobility of the hapten, which is reflected in the shape of the e.s.r. spectrum, must be considered.

The effects of titrating Fv fragment (35 μ M) with La(III), Eu(III) and Gd(III) on the e.s.r. spectrum of bound hapten (II) were compared at pH 6.9 under conditions where no displacement occurs (80–100 μ M-hapten). With the diamagnetic La(III) there is a decrease in signal on binding, though much smaller than with Gd(III) (limiting relative amplitude = 0.65) with $K_{M,app.} \approx 4$ mM. Also, the binding of Eu(III) causes an apparent quenching (limiting amplitude = 0.5, $K_{M,app.} = 2.0$ mM). However, diamagnetic La(III) cannot cause paramagnetic quenching and Eu(III) would be expected to produce a significantly smaller paramagnetic quenching than Gd(III), because of the large difference in their electron-spin-relaxation times (Dwek, 1973).

It would then appear that a significant amount of the apparent quenching of the e.s.r. spectrum from the bound spin-label signal is due to a mechanism distinct from the paramagnetic quenching effect. This could be due to a further immobilization of the nitroxide moiety on binding of the lanthanides, causing a further broadening of the e.s.r. signal of the bound spin label. Because of the partial overlap of the e.s.r. spectra from the bound and free spin-labelled haptens, it is difficult to ascertain directly if the width of the bound nitroxide spectrum alters under these conditions. If, however, the effects of the diamagnetic La(III) are taken as a control for the 'conformational' effect of lanthanide binding on the e.s.r. spectrum of the bound spin label, the extra quenching effect ($0.4/0.65 \approx 0.6$) of the paramagnetic Gd(III) may be used as a first approximation to the paramagnetic contribution to the quenching. If the estimate for the electron-spin-relaxation time of Gd(III) of 0.15 ns (Jones *et al.*, 1974) is assumed, an estimate of 1.2 nm for the distance between the Gd(III) and the nitroxide can be made (Dwek, 1973).

This distance estimate must be considered very tentative because of the small difference in quenching between Gd(III) and the La(III) control and because of the anomalous behaviour with Eu(III). The theory of paramagnetic quenching of the bound nitroxide e.s.r. spectrum by paramagnetic ions (Leigh, 1970) suggests that Eu(III) should produce negligible quenching relative to the La(III) control. That this is not so may mean either that the effects are entirely 'conformational' or that there is some other mechanism of quenching with paramagnetic ions with very short electron-spin-relaxation times, such as Eu(III), that may have to be considered. Such a mechanism may be unimportant for Gd(III), which

has a long electron-spin-relaxation time, but until sufficient model systems have been studied with a variety of lanthanides it is not really possible to evaluate this.

The fluorescence and the e.s.r. results, taken together, confirm the existence of a specific lanthanide-binding site on Fv fragment. This site interacts, either directly or indirectly, with the hapten-combining site. The magnitude of the lanthanide-binding constants in the absence of haptens, and their pH-dependence, suggests that at least two ionizable groups on the protein are involved in binding the metal. The known properties of lanthanides in other systems (Phillips & Williams, 1966; Williams, 1974) further suggest that these groups are probably carboxylate ions.

High-resolution n.m.r. studies

The 270 MHz proton spectra of the Fv fragment (1.2 mM) at pH 4.33 in the presence and absence of La(III) (19 mM) are shown in Fig. 8. The difference spectrum resulting from the presence of La(III) has an intensity less than 5% of that of the Fv fragment spectrum and is also shown in Fig. 8. This suggests that any conformational changes resulting from the addition of metal are relatively small and probably involve few residues. The advantage therefore of n.m.r. studies over the fluorescence studies is clearly seen; n.m.r. gives direct structural information. Although the fluorescence studies allow the time-dependence of the conformational changes in the presence of metal to be monitored, they give no information on the extent of any such changes, which are now shown to be relatively small.

Fig. 9 shows the titration curves of the chemical shift of the C-2 and C-4 protons versus pH for the three histidine residues in the Fv fragment. The titration curves in the absence of La(III) are in good agreement with those previously published (Dwek *et al.*, 1975a,b). The addition of 19 mM-La(III) results in both chemical shifts and a slight decrease in pK_a value (from 6.9 to 6.4) of one of these histidine residues. The high concentration of La(III) used in these experiments is necessary because of the weak metal-binding constants, particularly at low pH. At pH values ≥ 6.5 the binding of La(III) is about five times stronger than at pH 5.0 and a limited titration using 2 mM-La(III) between pH values 6.5 and 8.5 gave results in excellent agreement with those obtained at the higher La(III) concentrations. This shows that any effects caused by a change in ionic strength from the introduction of a relatively high concentration of La(III) are small.

The slight decrease in the pK_a of the histidine residue (0.5 unit) shows that La(III) does not compete with a proton for the nitrogen-binding site on the histidine ring. If that were the case, the addition of La(III) would be expected to decrease the binding

Fig. 8. 270 MHz proton spectrum of Fv fragment from protein MOPC 315

The concentration of the Fv fragment was 1.2 mM and the sample contained 0.15 M NaCl. The spectra were recorded at 30°C and pH 4.33. (a), No addition; (b), in the presence of 19 mM-La(III); (c) (a-b), difference spectrum at normal intensity; (d) (a-b) $\times 4$ enlarged difference spectrum.

constant of the proton by a factor of $\{1 + [\text{La(III)}]/K_{\text{La(III)}}\}$, where $K_{\text{La(III)}}$ is the binding constant of La(III) to the Fv fragment in the high-pH region above the pK_a of 6.2 as determined by fluorescence. Since this binding constant has been determined to be 100 μM , the addition of 19 mM-La(III) would lead to a 191-fold decrease in the proton-binding constant or a decrease in the pK_a value by 2.3 units. This is not consistent with the observed decrease in the histidine pK_a value of only 0.5 unit. This result is not really surprising, since the chemistry of the lanthanides is such that they would not be expected to bind to nitrogen ligands such as histidine (Phillips & Williams, 1966). However, the change in chemical shift and pK_a of this histidine residue and also the small number of residues in the Fv fragment perturbed on adding La(III) would suggest that this histidine residue is in the vicinity of the metal ion. It is also worth noting that the presence of La(III) broadens the C-2(H) resonance of this histidine residue and to a lesser extent the C-4(H) proton. This could result from either a metal-induced conformational change bringing one or more protons near to this histidine residue with con-

sequent dipolar broadening, or from chemical exchange phenomena (Dwek, 1973). In the latter case the addition of La(III) would have to result in the histidine residue existing in two environments with the time-scale for exchange between them being in the slow to intermediate region on the n.m.r. time-scale (Dwek, 1973). This could happen, for instance, if one of the La(III)-chelating groups moves back and forth in a 'breathing' movement with consequent alterations in the environment of the histidine.

A general broadening of the proton n.m.r. spectrum of Fv fragment also results from the presence of Pr(III), which is much more pronounced than with La(III). Titrating Pr(III) at pH 4.8 into Fv fragment (1.1 mM) results initially in shifts in the aliphatic region and $\beta\text{-CH}_2$ region of the proton n.m.r. spectrum of the Fv fragment. The fraction of resonances shifted increases as the Pr(III) concentration is increased to 0.92 mM, consistent with slow exchange (Dwek, 1973). Throughout this range, however, there appear to be few shifts in the aromatic region. As the Pr(III) concentration is further increased there are still detectable shifts in the aliphatic region, but a

Fig. 9. Titration of the histidine chemical shifts for the Fv fragment from protein MOPC 315 in the absence (○) and in the presence (□) of saturating La(III)

For details see the text. The C-4 protons for the histidine residue with pK_a 8.2 were not resolved in the presence of La(III).

general broadening of the whole n.m.r. spectrum is apparent. This broadening increases as the titration continues up to 19mM-Pr(III), and consequently it is very difficult to observe any changes in the histidine-residue resonances. In general Pr(III) is expected to cause shifts in nuclear resonances (Dwek, 1973).

The observed broadenings, particularly in the aromatic region, are more characteristic of those expected for paramagnetic metal ions with long electron-spin relaxation times. One possible explanation, which may account for this broadening, is some chemical exchange phenomenon involving relative motion between Pr(III) and the aromatic residues. The different environments of the aromatic residues experience different pseudo-contact shifts. [Such shifts would, of course, be in addition to any resulting merely from the presence of the metal, for which La(III) should provide the appropriate diamagnetic blank.] Again if the relative motion is slow enough this would lead to broadening of the resonances. Alternatively or additionally, there could be a dipolar interaction between the Pr(III) and nearby residues, which would result in broadening of resonances, in much the same way as has been observed for the spin-label hapten (Dwek *et al.*, 1975a,b). Nevertheless,

the broadenings with Pr(III) are unexpected, and such effects may present a limitation on the use of lanthanide ions to obtain geometrical information in solution in some cases. The mechanism of broadening is unclear and thus further investigations to test the generality of the effects, to find out in which systems they are most likely to be observable, and to test the validity of theoretical models such as the one proposed by Guéron (1975), are merited.

Conclusion

The magnitude of the binding constant of metal to the Fv fragment would, by comparison with model compounds, suggest that the metal is co-ordinated to at least two carboxyl groupings on the Fv fragment. The group with the pK_a value of about 6.2 (determined by fluorescence) associated with metal binding is not a histidine residue and is therefore most probably a carboxyl group of one residue acting as part of a 'dibasic' acid. This situation has been found in lysozyme for the lanthanide-binding site, which is between aspartate-52 and glutamate-35 (Blake & Rabstein, personal communications). The histidine residue which has $pK_a \approx 6.9$ in the absence of lanthanide is most probably in the vicinity of the lanthanide-binding site. The conformational changes on metal binding as detected by fluorescence studies have been shown by high-resolution n.m.r. studies to involve only a few residues, suggesting that they are reasonably localized. The antagonistic effects of hapten and metal binding suggest that the two binding sites are close. Confirmation of this comes from the e.s.r. studies with lanthanides in the presence of a spin-labelled hapten, which give a distance of 1.2nm between the lanthanide-binding site and the nitroxide moiety. The Fv fragment exists in at least two different conformers, each with different affinities for hapten and metal. In the absence of either hapten or metal, the Fv fragment exists predominantly (>90%) in one conformation. The hapten binds preferentially to this form, whereas the metal binds preferentially to the other form. Since the lanthanides and the haptens both bind tightly and specifically, this suggests that the myeloma protein may exist in a multiplicity of forms, each form displaying a specificity for a different class of ligands.

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References

- Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry: Application to Enzyme Systems*, Clarendon Press, Oxford
- Dwek, R. A., Knott, J. A., McLaughlin, A. C., Myatt, R. W., Press, E. M., Price, N. C., Richards, R. E. & White, A. I. (1974) *Proc. Rare Earth Res. Conf. 11th* 1, 184-193
- Dwek, R. A., Knott, J. C. A., Marsh, D., McLaughlin, A. C., Press, E. M., Price, N. C. & White, A. I. (1975a) *Eur. J. Biochem.* 53, 25-39
- Dwek, R. A., Jones, R., Marsh, D., McLaughlin, A. C., Press, E. M., Price, N. C. & White, A. I. (1975b) *Philos. Trans. R. Soc. London Ser. B* 372, 53-74
- Eigen, M. & Hammes, G. G. (1963) *Adv. Enzymol. Relat. Areas Mol. Biol.* 25, 1-38
- Guéron, M. (1975) *J. Magn. Reson.* 19, 58-66
- Hammes, G. G. (1968) *Acc. Chem. Res.* 11, 321-329
- Haselkorn, D., Friedman, S., Givol, D. & Pecht, I. (1974) *Biochemistry* 13, 2210-2222
- Hochman, J., Inbar, D. & Givol, D. (1973) *Biochemistry* 12, 1130-1135
- Jones, R., Dwek, R. A. & Forsén, S. (1974) *Eur. J. Biochem.* 47, 271-283
- Leigh, J. S., Jr. (1970) *J. Chem. Phys.* 52, 2608-2612
- Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J. & Hinz, C. F. (1963) *J. Exp. Med.* 117, 938-1008
- Phillips, C. S. G. & Williams, R. J. P. (1966) *Inorganic Chemistry*, vols. 1 and 2, Clarendon Press, Oxford
- Velick, S. F., Parker, C. W. & Eisen, H. N. (1960) *Proc. Natl. Acad. Sci. U.S.A.* 46, 1470-1482
- Williams, R. J. P. (1974) *Recent Res. Cancer Results* 48, 1-11

APPENDIX I

Derivation of the Equations for the Fluorescence Titrations of Fv Fragment with Haptens and Metal Ions

In the thermodynamic scheme (Scheme 1), A represents the myeloma protein fragment (Fv), H the hapten and M the lanthanide metal. The appropriate dissociation constants are defined as shown. As usual, we have the condition $K_H K_{HM} = K_M K_{MH}$.

From the conservation equation for A and by using eqns. (1)-(4) we obtain

$$[A] = \frac{[A]_T}{1 + \frac{[H]}{K_H} + \frac{[M]}{K_M} + \frac{[H][M]}{K_H K_{HM}}}$$

where $[A]_T$ is the total amount of antibody present.

The total fluorescence, F, in a solution containing a fixed amount of antibody, $[A]_T$, and arbitrary amounts of hapten and metal may be given by

$$F = f_A[A] + f_{AM}[AM] + f_{AH}[AH] + f_{AMH}[AMH] \quad (5)$$

where f_A is the fluorescence per unit concentration of the antibody and f_{AM} , f_{AH} and f_{AMH} are similarly defined. We can therefore write

$$F = \frac{\left(f_A + f_{AM} \frac{[M]}{K_M} + f_{AH} \frac{[H]}{K_H} + f_{AMH} \frac{[H][M]}{K_H K_{HM}} \right) [A]_T}{1 + \frac{[H]}{K_H} + \frac{[M]}{K_M} + \frac{[H][M]}{K_H K_{HM}}} \quad (6)$$

As in any titration, what is usually measured is the difference between the initial fluorescence and the fluorescence after the addition of an arbitrary amount of ligand (either metal or hapten). For our analyses in this paper we consider titrations in which

Scheme 1. Binding scheme for metal, hapten and the antibody fragment

the metal concentration is held constant and the hapten concentration is varied, and define ΔF as

$$\begin{aligned}\Delta F &= F(H, M) - F(0, M) \\ &= \Delta F_0(M) \frac{\frac{[H]}{K_D'(M)}}{\left\{1 + \frac{[H]}{K_D'(M)}\right\}}\end{aligned}\quad (7)$$

where

$$\Delta F_0(M) = [A]_T \frac{\left\{\left(\frac{f_{AH}}{K_H} + \frac{f_{AMH}[M]}{K_H K_{HM}}\right)\left(1 + \frac{[M]}{K_M}\right) - \left(f_A + f_{AM} \frac{[M]}{K_M}\right)\left(\frac{1}{K_H} + \frac{[M]}{K_H K_{HM}}\right)\right\}}{\left(1 + \frac{[M]}{K_M}\right)\left(\frac{1}{K_H} + \frac{[M]}{K_H K_{HM}}\right)}\quad (8)$$

and

$$K_D'(M) = \frac{1 + \frac{[M]}{K_M}}{\frac{1}{K_H} + \frac{[M]}{K_M K_{MH}}}\quad (9)$$

If the free metal concentration remains constant during the titration with hapten, then eqn. (7) is a normal saturation (Michaelis-Menten) curve with an apparent Michaelis constant $K_D(M)$, given by eqn. (9), and an apparent total change in fluorescence, $\Delta F_0(M)$, given by eqn. (8). Both $K_D(M)$ and $\Delta F_0(M)$ depend on the total metal concentration, and in the limit when $M \rightarrow 0$, $K_D = K_H$. K_{HM} and K_{MH} may be found by a simple replot of the apparent Michaelis constant K_D' , since

$$\Delta K_D' = K_D'(M) - K_D'(0)\quad (10)$$

$$= (K_{MH} - K_H) \frac{\frac{[M]}{K_{HM}}}{1 + \frac{[M]}{K_{HM}}}\quad (11)$$

We note that a double-reciprocal plot of $\Delta K_D'$ versus the metal concentration will give K_{HM} from the extrapolated value on the abscissa and a value of $(K_{MH} - K_H)$ on the ordinate. Thus all four binding constants in Scheme 1 can be unequivocally determined. The fluorescence yields for each of the four complexes may also be easily obtained. f_A is obtained from the fluorescence of an arbitrary concentration of the myeloma fragment (Fv). f_{AH} and f_{AM} are obtained either directly from the fluorescence of an

Fv-fragment solution in the presence of saturating hapten and metal respectively or from the standard extrapolation procedures for binding complexes. f_{AMH} is obtained either in a similar direct way or by an alternative way as follows. We define $F'(M)$ as

$$F'(M) = \Delta F_0(M) + F(0, M)\quad (12)$$

$F'(M)$ is the total fluorescence intensity at infinite hapten concentration for an arbitrary metal ion concentration. Then it can be shown that

$$F'(M) = \frac{f_{AH} K_{HM} + f_{AMH}[M]}{K_{HM} + [M]}\quad (13)$$

Eqn. (13) may be rearranged into a Michaelis-Menten form by analogy with eqns. (9) and (10), i.e.

$$\Delta F'(M) = F'(M) - F'(0)\quad (14)$$

$$= (f_{AMH} - f_{AH}) \left(\frac{\frac{[M]}{K_{HM}}}{1 + \frac{[M]}{K_{HM}}} \right)\quad (15)$$

The usual double-reciprocal plot will then give K_{HM} and $(f_{AMH} - f_{AH})$ from the intercepts.

APPENDIX II

Derivation of the Relaxation Times for the 'Two-State' Model of Fv Fragment

We assume that the Fv fragment exists in two conformations, designated A and A'. Both forms of Fv fragment are assumed to bind hapten (H) and metal ions (M). The reactions in Scheme 1 are considered. The ratio $[A']/[A]$ is given by L. The kinetic equations may be written

$$\frac{d[A']}{dt} = k_{+1}[A] - k_{-1}[A'] - k'_{+h}[H][A'] + k'_{-h}[A'H] - k'_{+m}[M][A'] + k'_{-m}[A'M] \quad (1)$$

$$\frac{d[A'M]}{dt} = k_{+2}[AM] - k_{-2}[A'M] - k'_{-m}[A'M] + k'_{+m}[A'][M] - k'_{+mh}[A'M][H] + k'_{-mh}[A'MH] \quad (2)$$

In principle, we have eight coupled differential equations. Since the observed relaxation times are long (of the order of seconds), we assume that the hapten- and the metal-ion-binding processes occur much more rapidly than the relaxation between the two different forms of Fv fragment. Then the two binding processes will be essentially at equilibrium during the relaxation process. Eqns. (1) and (2) can thus be rewritten

$$\frac{d[A']}{dt} = k_{+1}[A] - k_{-1}[A'] \quad (3)$$

$$\frac{d[A'M]}{dt} = k_{+2}[AM] - k_{-2}[A'M] \quad (4)$$

The conservation equation for the Fv fragment may be written as

$$[A] + [A'] + [AM] + [A'M] + [AH] + [A'H] + [AMH] + [A'MH] = [A]_T$$

Using the following equilibrium conditions

$$K_h = \frac{[A][H]}{[AH]} \quad k'_h = \frac{[A'][H]}{[A'H]}$$

$$K_m = \frac{[A][M]}{[AM]} \quad K'_m = \frac{[A'][M]}{[A'M]}$$

$$K_{hm} = \frac{[AH][M]}{[AMH]} \quad K'_{hm} = \frac{[A'H][M]}{[A'MH]}$$

eqn. (3) may be rearranged to give

$$\frac{d[A']}{dt} = -\tau_1^{-1}[A'] + \frac{k_{+1}[A]_T}{1 + \frac{[M]}{K_m} + \frac{[H]}{K_h} \left(1 + \frac{[M]}{K_{hm}}\right)}$$

where τ_1^{-1} is defined below.

Therefore the relaxation rate for the equilibration between [A] and [A'] is

$$\tau_1^{-1} \equiv k_{-1} + k_{+1} \cdot \frac{\left\{1 + \frac{[M]}{K'_m} + \frac{[H]}{K'_h} \left(1 + \frac{[M]}{K'_{hm}}\right)\right\}}{\left\{1 + \frac{[M]}{K_m} + \frac{[H]}{K_h} \left(1 + \frac{[M]}{K_{hm}}\right)\right\}} \quad (5)$$

Scheme 1

It is important to note that these are not the same binding constants as defined in Appendix I. The binding constants in Appendix I are average values of the binding constants for the two forms of the Fv fragment.

The same procedures can be followed to determine the relaxation rate, τ_2^{-1} , for the equilibration between [AM] and [A'M].

$$\tau_2^{-1} = k_{-2} + k_{+2} \frac{K'_m \left\{ 1 + \frac{[M]}{K'_m} + \frac{[H]}{K'_h} \left(1 + \frac{[M]}{K'_{hm}} \right) \right\}}{K'_m \left\{ 1 + \frac{[M]}{K'_m} + \frac{[H]}{K'_h} \left(1 + \frac{[M]}{K'_{hm}} \right) \right\}} \quad (6)$$

What is the relationship of τ_2 to τ_1 ? By using the equilibrium conditions for the metal-binding reactions and eqn. (4), we can write

$$\frac{d[A']}{dt} = \frac{K'_m}{[M]} \cdot \frac{d[A'M]}{dt} = -k_{-2}[A'] + k_{+2} \frac{K'_m}{K_m} [A] \quad (7)$$

Comparing eqn. (7) with eqn. (3) implies that

$$k_{-2} = k_{-1}$$

$$k_{+2} \frac{K'_m}{K_m} = k_{+1}$$

Inserting these conditions into eqn. (8) gives

$$\tau_2 = \tau_1 = \tau \quad (8)$$

This means that the antibody and the antibody-metal complexes will relax with the same time-constant, τ , given by eqn. (5). Likewise, a similar analysis shows that the other antibody complexes, i.e. the antibody-hapten complexes and the antibody-hapten-metal complexes, will relax with an identical time-constant. This means that the observed relaxation should follow a single exponential decay.

Also, we note that the binding constants in Appendix I may be related to the intrinsic binding constants to the two forms of the antibody fragments. For instance

$$\frac{1}{K_H} = \frac{\frac{1}{K_h} + \frac{L}{K'_h}}{1 + L} \quad (9)$$

$$\frac{1}{K_M} = \frac{\frac{1}{K_m} + \frac{L}{K'_m}}{1 + L} \quad (10)$$

Thus the reciprocal of the apparent binding constant in the fluorescence titrations is simply the weighted average value of the reciprocals of the binding constants to the two different forms of the antibody.