Proton-n.m.r. study of interaction of myelin basic protein with a monoclonal antibody

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Proton n.m.r. at 400 MHz has been applied to study the interactions of bovine or porcine myelin basic protein (b- or p-MBP) with a monoclonal antibody to human (h-) MBP. The antibody, an IgG immunoglobulin that contains a sequential epitopic region, cross-reacts with b-MBP but not with p-MBP, the presumed epitope being identical in h- and b-MBP. N.m.r. spectra were recorded from the Fab fragment of the antibody and for mixtures of Fab and MBP at various molar ratios. The n.m.r. spectrum of MBP in the mixture consists mostly of well resolved peaks against a broad background due to the Fab. With b-MBP, but not p-MBP, specific interactions are observed at the residue tyrosine-135, which is part of the epitopic sequence. Other interactions occur between the Fab and both b- and p-MBP at residues distant from the epitopic region. Standard radioassay techniques were employed to calculate the binding constants of both basic proteins with the immunoglobulin. The binding constant, K_b , for IgG to column-immobilized b-MBP at 298 K is $(0.95 \pm 0.07) \times 10^7 \text{ dm}^3/\text{mol}$. The value of K_b decreases with the ionic strength of the medium, suggesting a coulombic interaction between antigen and antibody. N.m.r. spectra were also measured for mixtures of the Fab fragment and peptides containing the epitopic site, with results in agreement with those for the whole protein.

Three laboratories have prepared monoclonal antibodies to human myelin basic protein (h-MBP) (Sires *et al.*, 1981; Fritz & Chou, 1983; Carnegie *et al.*, 1983) by the hybridoma technique of Kohler & Milstein (1975). Two of the monoclonal antibodies described react with an epitope consisting of a linear sequence of residues in the region 130–139 of MBPs from h- and b-MBP, but they do not react with p-MBP. This result is of special interest in view of the use of the porcine protein in an extensive trial of MBP in treatment of multiple sclerosis (Romine & Salk, 1983). In the present study, high-resolution proton n.m.r. and c.d. are used to investigate the interaction of purified Fab fragments from one of the monoclonal antibodies (Carnegie *et al.*, 1983) with bovine and porcine basic proteins and polypeptides derived by cleavage of these proteins. The present study delineates aspects of the antigen–antibody reaction that are of interest not only in regard to the antigenicity of MBP but also

in more general aspects of physical immunochemistry.

A preliminary account of some of this work was presented at the 15th Meeting of the American Society for Neurochemistry, Portland, OR, U.S.A., on 12 March 1984.

Materials and methods

Monoclonal antibody to h-MBP was prepared from a freeze-dried powder of mouse hybridoma ascites fluid, from which the γ -globulins were precipitated twice with 50%-satd. $(\text{NH}_4)_2\text{SO}_4$. The freeze-dried material was dissolved in 0.01 M-phosphate buffer/0.15 M-NaCl, pH 7.6, and applied to a column (5 cm $\times$ 1 cm) of Sepharose 6B-CL to which b-MBP had been covalently linked by the method of Cuatrecasas & Anfinsen (1971). Unbound protein was eluted from the column with the loading buffer. Bound antibody was eluted with 0.1 M-glycine/HCl buffer, pH 2.5. The antibody solution was then dialysed against 0.01 M-phosphate buf-

Abbreviations used: b-, p- or h-MBP, bovine, porcine or human myelin basic protein.

fer/0.15M-NaCl, pH 7.6. The antibody was designated as 'mouse monoclonal #66(2)-2', an IgG-2B light chain with an M_r of 160000. The Fab fragment of this monoclonal antibody was prepared, by papain digestion of the affinity-purified material, as described by Mage (1980). The Fab fragment of freeze-dried mouse IgG (Sigma Chemical Co.) was prepared in the same way. The IgG and Fc fragments were separated from the Fab by adsorption on a DEAE-cellulose column.

Bovine MBP was prepared from white matter of fresh brain by the method of Eylar *et al.* (1969). It was purified by passage twice through a column of Sephadex G-75 eluted with 0.2M-acetic acid. Porcine MBP was donated by Dr. Max Marsh of Eli Lilly and Co., Indianapolis, IN, U.S.A. Polypeptide fragments of b-MBP and p-MBP comprising residues 1-117 and 118-179 were prepared by the method of Burnett & Eylar (1971).

Binding of IgG to b-MBP and p-MBP

The monoclonal IgG was labelled to 2.1×10^6 c.p.m./ μ g with ^{125}I with Enzymobeads (Bio-Rad) according to manufacturer's instructions. ^{125}I -labelled IgG was incubated with b-MBP coupled to CNBr-activated Sepharose 6B-CL for 30 min, at 310 K, and the radioactivity associated with the Sepharose determined. Bovine serum albumin (0.5%, w/v) was included in the incubation buffers in order to minimize non-specific interactions between the ^{125}I -labelled IgG and the Sepharose. The binding constant K_b of b-MBP was determined by first binding the ^{125}I -IgG to the covalently linked MBP-Sepharose column and then determining the competitive release of label by unlabelled IgG.

In preparation for n.m.r. runs, protein samples were freeze-dried from 99.6% $^2\text{H}_2\text{O}$ and dissolved in 99.96% $^2\text{H}_2\text{O}$ (Merck, Sharp and Dohme) at concentrations up to 1.0 mM in 0.01 M-phosphate buffer, pH 7.6. N.m.r. spectra were obtained at 310 K with a Bruker WM-400 spectrometer, with samples in 5 mm-outer-diameter precision tubes. For MBP samples a 90° radiofrequency pulse (7-9 μ s) was used to accumulate 1024 transients over 16000 data points, with a spectral width of 4800 Hz and a repetition time of 1.7 s. Spectra of the Fab antibody fragment were obtained at 1.0 mM under similar conditions but with 4000 transients. Spectra of mixtures of Fab and MBP (or MBP peptides) were obtained under the same conditions as the MBP runs with (Fab) = 0.50 mM and (MBP)/(Fab) from 1:0 to 1:1. C.d. spectra were measured in 0.5 mm-path-length quartz cells (Markson Scientific Co., Del Mar, CA, U.S.A.) at 298 K with a JASCO-500C spectropolarimeter. MBP and Fab fragment concentrations varied from 0 to 13.3 μ M.

Results

Binding of ^{125}I -IgG to b-MBP-Sepharose

The binding of ^{125}I -labelled IgG to b-MBP coupled to CNBr-activated Sepharose 6B-CL is dependent on the ionic strength of the incubation buffer. The percentage of bound antibody decreases from 70% at 0M-NaCl to 32% at 0.2M-NaCl. The enhanced binding at low ionic strength does not appear to arise from non-specific interactions between the IgG and the Sepharose, since ^{125}I -labelled IgG did not bind to ovalbumin coupled to the same batch of CNBr-activated Sepharose 6B-CL.

Unlabelled IgG inhibited the binding of ^{125}I -labelled IgG to b-MBP-Sepharose. The dependence of this inhibition on the concentration of unlabelled IgG is determined from a Scatchard analysis (1949) in which the ratio of bound IgG to free IgG (y) is a linear function of the concentration of bound IgG (x). At 20°C the data fitted the line $y = ax + b$ and with $a = (-3.55 \pm 0.24) \times 10^{-2}$, $b = 5.01 \pm 0.21$ and the binding constant was calculated as $(0.95 \pm 0.07) \times 10^7 \text{ dm}^3 \cdot \text{mol}^{-1}$. At physio-

Fig. 1. Inhibition of the binding of ^{125}I -labelled IgG to b-MBP-Sepharose by soluble MBP

The binding of ^{125}I -labelled IgG (2.1×10^6 c.p.m./ μ g; 0.27 μ g/ml) to a 17% (v/v) suspension of b-MBP-Sepharose equilibrated with 0.01 M-phosphate/0.5% bovine serum albumin, pH 7.6, was determined at the indicated b-MBP concentrations (○). The binding of ^{125}I -labelled IgG (2.1×10^6 c.p.m./ μ g; 0.53 μ g/ml) to a 17% (v/v) suspension of b-MBP-Sepharose equilibrated with 0.01 M-phosphate/0.15M-NaCl/0.5% bovine serum albumin, pH 7.6, was determined at the indicated concentrations of b-MBP (●) and p-MBP (■). The data shown were obtained from two separate experiments.

logical ionic strength, b-MBP, but not p-MBP, inhibited the binding of ^{125}I -labelled IgG to b-MBP-Sepharose (Fig. 1, ●). This inhibition was not apparent at low ionic strength (Fig. 1, ○). It is possible that the specific inhibition of binding of IgG to b-MBP-Sepharose by soluble b-MBP is obscured by non-specific ionic interactions between the Sepharose 6B-CL (which is known to bear sulphate and carboxy groups) and cationic b-MBP and b-MBP-IgG complexes. The binding of the latter complexes, formed with increasing concentrations of soluble b-MBP, could mask the inhibition of the b-MBP-Sepharose-IgG interaction by soluble b-MBP. This hypothesis is consistent with the inhibition of the b-MBP-Sepharose-IgG interaction by soluble b-MBP at high ionic strength, under conditions where non-specific ionic interactions would be minimized. This inhibition appeared to be specific, in that p-MBP at concentrations up to $38\text{ }\mu\text{g/l}$ ($\sim 20\text{ }\mu\text{M}$) did not inhibit the binding.

N.m.r. spectra

The ^1H spectrum of the Fab in phosphate buffer (1.0 mM at pH 7.6) is shown in Fig. 2 (bottom). As expected for a globular protein of molecular mass $\sim 50\text{ kDa}$, the spectral lines are broadened and resolution is low, especially in the downfield (aro-

matic) region of the spectrum. Some upfield (aliphatic) peaks, however, are quite sharp and reasonably well resolved. Their chemical shifts indicate that they are probably resonances of side-chain methyl groups that are highly mobile. The complete spectrum of b-MBP is shown in Fig. 2 (top) under conditions that are the same as those for the Fab fragment, except that only 640 transients were accumulated as compared with 4000. It will be appreciated, therefore, that few detailed features of the Fab spectrum would be observed in the spectrum of an equimolar mixture of Fab and MBP under these conditions. The Fab fragment is used in the n.m.r. studies in order to ascertain fine details in the spectrum of MBP.

The spectrum of the basic protein has been analysed in a series of papers (Mendz *et al.*, 1982, 1983a,b) in which assignments of resonances to specific amino acid residues are reported. These assignments are employed in the present n.m.r. study of the interaction of MBP with monoclonal antibody.

Fig. 3 shows the downfield region of the spectra of b-MBP and p-MBP and of mixtures of these proteins with the Fab at molar ratios (Fab/MBP) = 0–1. The first important point to be noted is that the spectra of both p-MBP and b-MBP are sharp and well-resolved against a broad background pre-

Fig. 2. ^1H n.m.r. spectra of (a) b-MBP (1.0 mM in 10 mM-phosphate buffer, pH 7.6) and (b) Fab fragment of monoclonal antibody (1.0 mM in 10 mM-phosphate buffer, pH 7.6)

Fig. 3. Downfield regions of ^1H n.m.r. spectra of p-MBP (a) and b-MBP (b) and of mixtures of these proteins with Fab fragments of monoclonal antibody to h-MBP at molar ratios (Fab/MBP) of 0:1–1:1 in 10 mM-phosphate buffer, pH 7.6 'Y-135- $\epsilon_{1,2}$ ' refers to ϵ -CH resonances of tyrosine-135.

sumably due to Fab. Even at an equimolar ratio of Fab to MBP, many regions of the MBP spectrum are only moderately broadened and, for most spectral lines, no significant chemical-shift differences are observed compared with the basic proteins alone. The most likely explanation of this result is that most of the MBP molecule retains its high mobility even while bound to the much larger Fab molecule. Except, perhaps, for the immediate neighbourhoods of localized binding sites, the polypeptide chain of MBP is thus freely mobile, so that the effective correlation times of the observed protons are not markedly increased from those in unbound MBP in aqueous solution.

The differences between the interactions of b-MBP and p-MBP with the monoclonal Fab are most clearly seen in the ϵ -CH resonances of tyrosine-135. p- and b-MBP each contain four tyrosine residues at positions 14, 69, 128 and 135. In the whole protein the ϵ -CH resonances of these residues overlap to some extent, but the most upfield resonance of the Tyr- ϵ -CH group has been assigned (Menz *et al.*, 1983b) to one component of

the Tyr-135 doublet. This tyrosine residue has been identified by indirect evidence as part of the antigenic determinant for the monoclonal antibody (Carnegie *et al.*, 1983). The n.m.r. observation is that this resonance decreases in intensity in mixtures of Fab and b-MBP but does not undergo chemical-shift changes. In contrast, in the case of p-MBP, which does not cross-react with the monoclonal antibody, there is a slight broadening of the Tyr-135- ϵ -CH lines, but no decrease in intensity and no change in chemical shift. The resonances of two other residues in this region, Tyr-128 and His-139, have been assigned. Owing to the partial overlap of the aromatic resonances of Tyr-128 with those of Tyr-14 and Tyr-69, only measurements of peaks corresponding to the three residues together are possible. Upon addition of the monoclonal fragment there is no detectable loss of intensity in either the δ -CH or ϵ -CH spectral lines of p- or b-MBP.

Such differential effects can be seen even more clearly in the spectra, in Fig. 4, of mixtures of the Fab with the peptides derived by cleavage of p-

and b-MBP and constituting residues 118–179 of the proteins. These peptides are designated p(118–179) and b(118–179). The only tyrosine residues in these peptides are Tyr-128 and Tyr-135. The Tyr-135- ϵ -CH resonances of b(118–179) lose about 60% of their intensity on interaction with Fab, whereas in the case of p(118–179) there is no such effect on the corresponding resonances. A similar effect can also be seen in the δ -CH resonances of Tyr-135. The resonances of Tyr-128 do not change in intensity in either peptide. Two histidine residues in positions 139 and 145 occur in b(118–179). Their δ_2 lines are at about 6.935 p.p.m. (Fig. 4). Addition of the Fab broadens these peaks without changing their intensities. An explanation would be that the resonance of His-145 remains unchanged, whereas that of His-139 undergoes intermediate exchange broadening between conformations, in the n.m.r. time scale. The residue at position 139, located at one end of the epitopic region, would not be substantially immobilized, but the slightly different chemical shifts in its resonances would reflect the conformational changes experienced between the bound and free forms of the antigen peptide.

Besides the specific interaction with Tyr-135 observed with the bovine protein and peptide, there is spectroscopic evidence for another interaction

involving the sole tryptophan residue of the proteins, namely Trp-117. In the spectra of b- and p-MBP the resonances of the ϵ_3 and ζ_2 protons of tryptophan at 7.61 and 7.40 p.p.m. are resolved. The intensities of these resonances are decreased when Fab is mixed with either b-MBP or p-MBP, so that, at a Fab/MBP molar ratio of ≥ 0.75 , the tryptophan resonances are no longer discernible from background noise in the spectrum of b-MBP and reduced to about 30% in p-MBP.

The upfield aliphatic regions of the spectra in Fig. 5 also show effects of interaction of both p- and b-MBP with Fab. In the case of b-MBP, the γ -CH₃ lines of Thr-66 and Thr-67 are considerably broadened at an equimolar ratio of Fab to MBP. In the case of p-MBP, however, both these doublets are split into a pair of partially overlapping doublets. The methionine methyl resonances of b- and p-MBP are affected in different ways by interaction with Fab. In the spectrum of b-MBP with Fab, the Met-176 resonance is split into a major peak and a minor downfield peak, and the Met-21 resonance is slightly shifted upfield. In the case of p-MBP, the Met-176 resonance is not affected by the Fab, whereas the Met-21 resonance is shifted upfield by about 0.02 p.p.m., i.e., more than in the case of b-MBP. All these effects on aliphatic reson-

Fig. 4. Downfield regions of the ^1H n.m.r. spectra of the peptides p(118–179) (a) and b(118–179) (b) and of mixtures of these peptides with Fab fragment of monoclonal antibody to h-MBP at a molar ratio of Fab to peptide of 0.67

The experimental conditions were the same as those given in Fig. 3. 'Y-135- $\delta_{1,2}$ ' and 'Y-135- $\epsilon_{1,2}$ ' refer to δ -CH and ϵ -CH resonances respectively of tyrosine-135.

Fig. 5. Upfield aliphatic regions of ^1H n.m.r. spectra of *p*-MBP (a) and *b*-MBP (b) and equimolar mixtures of these proteins with Fab fragments of monoclonal antibody to *h*-MBP

The experimental conditions were as for Fig. 3. Abbreviations used: M-21- ϵ and M-176- ϵ , ϵ -CH₃ resonances of methionine-21 and -176; N-Ac, CH₃ resonances of *N*-terminal acetyl group; T-66- γ and T-67- γ , γ -CH₃ resonances of threonine-66 and -67.

ances appear to be the result of non-specific weak binding, with consequent changes possibly in conformation or in ring-current effects in different regions of the proteins.

The linewidths of the MBP resonances are a measure of the transverse relaxation times (T_2) of the protons concerned. In the interaction of *p*-MBP with Fab, there is practically no change in linewidth of the aliphatic resonances up to an equimolar ratio of Fab to MBP. In the case of *b*-MBP, the changes in linewidth vary with the resonance concerned. Some of these changes are seen in Fig. 5. We may note particularly the different effects for the CH₃ resonances of the two methionine residues. The linewidth of Met-21 is increased from 2.3 Hz to 5.8 Hz, whereas the linewidth of Met-176 remains unchanged at 2.34 Hz, as the ratio Fab/MBP is increased from 0 to 1.0.

In a control experiment, the n.m.r. spectra of mixtures of the Fab fragment of mouse IgG and *b*-MBP were examined. Ratios of Fab to MBP up to 3.0 were taken, and the spectra obtained under conditions identical with those used for the monoclonal-Fab+MBP studies. No evidence of any specific interaction with the epitopic region of MBP was found, but effects due to non-specific binding were observed that are similar to those in mixtures of monoclonal Fab+*p*-MBP. The resonances of Trp-117 are reduced in intensity and became undetectable at Fab/MBP = 3.0. The resonances of both Met-21 and Met-176 are shifted slightly in a way similar to the effects in the monoclonal-Fab+*p*-MBP spectra.

C.d. spectra

The c.d. spectra of *b*-MBP, the Fab fragment

Fig. 6. C.d. spectra of b-MBP (1:0,), Fab fragment (0:1 —) and antigen-antibody complex (1:1, ----) in 10 mM-phosphate buffer, pH 7.6, and at 298 K

and the antigen-IgG-fragment complex are shown in Fig. 6. The spectrum of MBP corresponds to that of an unordered polypeptide chain. The spectrum of the Fab fragment reflects a high proportion of β -sheet structure. The spectrum of the complex corresponds approximately to the superimposition of the MBP and Fab spectra. This result indicates that the basic protein has not undergone any major conformational changes similar to those observed, for example, in its interactions with lipids (Mendz & Moore, 1983).

Discussion

The present n.m.r. observations are the first direct evidence for binding of the monoclonal antibody to a specific region of the MBP molecule. The epitopic region comprising residues 130–139 in h-MBP and b-MBP contains the sequence of residues Gly-Arg-Ala-Ser-Asp-Tyr-Lys-Ser-Ala-His. In p-MBP, the two serine residues in this sequence have been replaced by proline residues and reactivity with the monoclonal antibody is lost. The n.m.r. data show a distinct difference in the binding of the monoclonal antibody to b- and p-MBP. In the case of the b-MBP, the residue Tyr-135 appears to become immobilized and its resonances reduced in intensity by 70% on interaction with the Fab fragments of the monoclonal antibody, whereas the Tyr-135 in p-MBP is not affected by addition

of the Fab. These observations suggest that the conformations of the two basic proteins in the presence of Fab differ considerably in the region of Tyr-135.

At equimolar ratio of antigen to antibody, practically all the antigen will be in the bound form. The loss in intensity of the Tyr-135 peaks indicates that the b-MBP is tightly bound in the region of this tyrosine residue, which is approximately in the middle of the antigenic determinant, and that the greatly decreased relaxation time of the bound tyrosine residue results in broadening of its resonances. The intensities of the resonances corresponding to two close residues in the primary sequence, Tyr-128 and His-139, do not change in intensity, a result that emphasizes specificity of the binding site. The broadening of the His-139 peak without measurable loss of intensity indicates an intermediate exchange rate between two conformations without appreciable change in the relaxation time.

The picture that thus emerges from the radioassay and n.m.r. data is that of a tight specific binding at Tyr-135 that induces conformational changes in the epitopic region still affecting the spectral line of His-139, located four residues away, but with no observable effects on the resonances of Tyr-128, located seven residues away. As yet, no comments are possible on other residues in this region, such as Arg-131, Asp-134 and Lys-136, because no assignments have been made of their spectral lines.

The n.m.r. data indicate that, in addition to the primary specific antibody-binding site, there are one or more secondary non-specific binding sites. The region about the tryptophan residue is highly conserved in MBP proteins. It was eliminated as a candidate for this antibody-binding site because it has the same sequence in strongly reacting (human, monkey, bovine and rat long-form), weakly reacting (rabbit) and non-reacting (pig and guinea-pig) proteins (Sires *et al.*, 1981; Carnegie *et al.*, 1983). We have observed that these aromatic resonances also decrease in intensity in the interaction of MBP with palmitoyl lysophosphatidylcholine. It is thus probable that the tryptophan residue may be in hydrophobic interaction with the Fab fragment. Theoretical calculations averaging every six residues (Hoop & Woods, 1983) show that, in both MBP proteins, the tryptophan residue is found in a hydrophobic region.

The effects on the Thr-66 and Thr-67 methyl resonances of both proteins may be interpreted as results of non-specific binding, probably of a hydrophobic nature. This pair of residues is in a region with hydrophobicity 40% larger than the region of the tryptophan. The splitting of resonances in the porcine-protein spectrum is indicative

of a slower exchange rate between conformations than the exchange causing the broadening observed in the bovine-protein spectrum.

The Met-176- ϵ -CH₃ resonance remains unchanged in the p-MBP spectrum, indicating that there is no measurable interaction between the residue and the antibody fragment. The splitting of Met-176 CH₃ resonance in the b-MBP spectrum is, on the other hand, evidence for slow exchange between bound and unbound species. Met-176 is found in a region of calculated zero hydrophobicity.

MBP in aqueous solution has the conformation of an extended polypeptide chain with a number of kinks and turns, but practically no α -helical or folded regions. Except for very few regions, the proton resonances of the bound protein remain sharp and do not lose intensity. Thus the bulk of the protein appears to retain its normal mobility, despite the tight binding of the antigenic site to the larger Fab molecule. C.d. data confirm this view, showing that, when MBP is bound to Fab, there is no observable change of either protein. The absence of tertiary structure in MBP facilitates the local mobility of those parts of the polypeptide chain that are not bound to the antibody.

The present n.m.r. study is one of the first to deal with the binding of a large protein antigen to an antibody. In previous n.m.r. studies the antigen has been a small peptide (Geller *et al.*, 1980) or an isolated hapten (Dwek *et al.*, 1977; Dower & Dwek, 1979). MBP is an antigen in which the epitope is a sequential region of amino acid residues. Binding of this region leaves the remainder of the mobile polypeptide chain free to engage in less specific binding or conformational variations. A protein antigen that depended on secondary or tertiary structure to produce an epitopic region might not be able to exhibit such subsidiary features in its reaction with antibody.

The decrease of the antigen-antibody binding constant K_b with increasing ionic strength indicates that electrostatic (coulombic) interactions are involved in the binding reaction. In terms of the theory for the effect of an electrolyte on a macroion equilibrium (Record *et al.*, 1978; Burton *et al.*, 1980), one can estimate that the binding reaction involves the release of one Na⁺Cl⁻ ion-pair. The epitopic sequence contains three residues with charged side chains: Arg-131, Asp-134 and Lys-137. We cannot yet ascertain which one of these may be involved in the binding process, but further n.m.r. studies with synthetic peptide antigens may

elucidate this question, as well as providing evidence concerning the conformation of the epitopic sequence in both free and bound states.

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