Identification of the epitopes of calcitonin gene-related peptide (CGRP) for two anti-CGRP monoclonal antibodies by 2D NMR

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Abstract

The interactions between calcitonin gene-related peptide and FAB fragments prepared from two different high-affinity anti-CGRP monoclonal antibodies (CB3 and CD1) have been studied at physiological pH using the ability of ¹H NMR to detect selectively regions of dynamic flexibility. The 37-residue peptide retains considerable flexibility in regions of its sequence when bound to both antibodies; in each case, more than half of the residues can be seen to have linewidths little perturbed from those of the free peptide. However, the regions where substantial broadening of resonances occur, attributed to substantially reduced motional freedom of the peptide resulting from interactions within the antibody combining site, differ greatly in the two cases. In the complex with CB3 the results indicate that the restricted residues lie exclusively within the C-terminal half of the peptide, and include residues 25 to 32 and the terminal two residues (36 and 37). By contrast, in the complex with CD1, the conformationally restricted residues appear to lie predominantly within the N-terminal half of the CGRP molecule, particularly residues 4–16, although several residues in the middle section of the sequence (22–31) have reduced conformational freedom. These findings, consistent with the results from immunological assays, add considerably to our knowledge of the epitopes.

Keywords: antibody peptide interactions; calcitonin gene-related peptide; epitope mapping; NMR

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide with potent vasodilatory and cardioexcitatory properties whose effects are receptor mediated (Abello et al., 1991; Raynaud et al., 1994). Little is known about the active conformation of the peptide. The N-terminal region, which is constrained by a disulphide bond, is required for activity, although N-terminal fragments by themselves have only low activity (Maggi et al., 1990). The struc-

ture of the peptide is flexible and disordered in solution, although an amphiphilic helix is formed in the middle of the peptide between residues 8 and 18 in trifluoroethanol/H₂O mixtures (Breeze et al., 1991; Hubbard et al., 1991). There are some indications of a preference for a turn-type conformation in the C-terminal half of the peptide in the TFE-induced conformation and also in C-terminal fragments of CGRP (Sagoo et al., 1991). The requirement for activity, if any, for these regions of secondary structure remains to be determined.

Eighteen monoclonal antibodies have been raised to CGRP using either the whole peptide or peptide fragments as the immunogen (Andrew et al., 1990). The smaller peptides produce antibodies of lower affinity than those produced by the whole molecule, and are not able to function well in two-site enzyme-linked immunoassays.

The influence of the monoclonal antibodies, which all have high binding constants for the peptide of 10⁸ M⁻¹, on the vasodilatory effect of CGRP has been investigated by Shaw et al. (1992). A range of effects were observed; some monoclonal antibodies have no effect on the CGRP response, despite their size (150 kDa) compared to CGRP (3.7 kDa), while others inhibited the vasodilatory effects of CGRP, for example, the CD1 antibody that is studied in this paper. A third group of monoclonal antibodies enhanced the vasodilatory effects of CGRP. One of the antibodies (CB3) that

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Abbreviations: CGRP, calcitonin gene-related peptide; CD, circular dichroism spectroscopy; CPMG, Carr Purcell Meiboom Gill; COSY, correlated spectroscopy; DQF, double quantum filtered; ELISA, enzyme linked immunoassay; FAB, fast atom bombardment mass spectrometry; HOHAHA, homonuclear Hartmann-Hahn; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser spectroscopy; RELAY COSY, two-dimensional relayed coherence transfer spectroscopy; TOCSY, total correlation spectroscopy; 1D, one-dimensional; 2D, two-dimensional.

is in this last group, and potentiates the response of CGRP *in vitro*, is also studied in this paper. The epitopes of the monoclonal antibodies have been mapped by using enzyme-linked immunoassays by screening a variety of CGRP peptide fragments for antibody binding. This information was used to identify the region of the peptide that interacts with the monoclonal antibody. No information is available on the conformation recognized or induced by the antibody, or indeed of the detailed residues involved in the interactions, which would be of value in the design of peptide agonists or antagonists of CGRP (Cooper et al., 1989).

The structures of a number of peptides bound to monoclonal antibodies have recently been determined by X-ray crystallography (Churchill et al., 1994; Wilson & Stanfield, 1994; Stanfield & Wilson, 1995). Groove type antibody combining sites are seen for peptides, as opposed to planar sites in the case of proteins and cavities for haptens. The conformations of the free peptides are not known in any defined detail in this case and are assumed to be largely disordered, although in the case of V3 loop peptide NMR studies showed there was a significant population of conformations containing a β -turn (Rini et al., 1993). It is thought that this flexibility may impose thermodynamic constraints on the maximum attainable affinity (Rees et al., 1994). The X-ray studies have demonstrated that the peptides take on a restricted conformation on binding and a range of different conformations have been observed. Several have been found to be bound in extended conformations or with β -type turns (Stanfield et al., 1990; Schulze-Gahmen et al., 1993; Shoham et al., 1993) but some have been seen to take on a helical conformation in the binding pocket (Tsang et al., 1992). In some instances where there is crossreactivity to a protein from which the peptide used as the immunogen was derived, the bound conformation is similar to the same sequence in the folded protein (Rini et al., 1992, 1993; Tsang et al., 1992) but in others a different conformation is found and the origins of crossreactivity are unclear (Stanfield et al., 1990). It is difficult to generalize regarding the nature of interactions of the various peptides in the antibody combining sites. In general, the surface area of interaction is smaller with peptides than with proteins (Wilson & Stanfield, 1994) but for the range of peptide antigens studied so far there seems to be no obvious correlation among surface area, number of contacts, and affinity. The interactions that have been observed include van der Waals contacts, hydrogen bonds, salt bridges, and hydrophobic interactions (Rees et al., 1994 and references therein).

The interactions of peptides with monoclonal antibodies have been studied by NMR spectroscopy as well as X-ray diffraction. In the event of weak binding ($K_d \leq 1 \times 10^6 \text{ M}^{-1}$ and more importantly fast off rates, $>1 \text{ s}^{-1}$, Anglister et al., 1988), methods based on the transferred nuclear Overhauser effect have enabled details of residues involved in close contact with the protein to be identified (Anglister et al., 1990, 1995), along with conformational changes in the peptides induced by binding to antibodies (Cung et al., 1991; Scherf et al., 1992). In the case of peptides that bind with high affinity, this procedure cannot be used and the complex must be studied directly. Given the size of the complex (55 kDa upwards), this is a difficult task. One approach to studying these tight complexes has involved isotope-edited NMR techniques using ^{15}N -labeled peptides (Tsang et al., 1988, 1990, 1992; Wright et al., 1990; Bax et al., 1994), and more recently using both ^{13}C and ^{15}N labeled proteins in antibody interaction studies (Lian et al., 1994; Wikstrom et al., 1995). Hydrogen exchange techniques have also been used to identify protein epitopes in antibody complexes (Pater-

son et al., 1990) and also more long-range effects of antibody binding (Benjamin et al., 1992; Mayne et al., 1992).

These techniques would not be appropriate in the current study as they rely on an intrinsically slowed rate of hydrogen exchange in the free antigen. An alternative strategy has been to exploit the ability of NMR to detect flexible regions of even large proteins by virtue of the narrow resonance lines that characterise such regions in comparison to more rigid regions. This approach has been used to identify those residues of a peptide fragment of lysozyme bound to a monoclonal antibody that are in the epitope region of the antigen (Cheetham et al., 1991a, 1991b). In this paper we describe the use of this approach to examine the interaction of CGRP with two monoclonal antibodies (CB3 and CD1). Both these antibodies have high affinities for CGRP (10^8 M^{-1} (Andrew et al., 1990)) although serological studies indicate that different residues are involved in the interactions between the two molecules.

Results

Assignment of the NMR spectrum of free and bound CGRP

Pre-TOCSY COSY spectra of the complexes of CGRP with the FAB fragments of CB3 and CD1 exhibit many well-resolved cross-peaks (Fig. 1). By contrast the spectra of the isolated FAB components show few crosspeaks (data not shown). FAB fragments (molecular mass 50 kDa) have an overall rotational correlation time of around 20 ns at room temperature (Anglister et al., 1984; Tsang et al., 1990). In the absence of extensive independent motion of regions of the protein, this is expected to give rise to line widths of 30 Hz or greater for the resonances of essentially all protons in the protein, and hence to effective cancellation of antiphase multiplets in the COSY spectrum. The NMR spectra are consistent, therefore, with the FAB fragments studied here having compact structures and few regions of high mobility; the latter are likely to be restricted to the C- and N-termini and some residues on exposed loops in the structure. In accord with this result, 1D spectra recorded using the CPMG sequence show very few resonances having detectable intensity after dephasing times in excess of 60 ms.

The narrow resonances in the spectrum of the complexes therefore can be identified as arising from the bound CGRP. As in the case of the peptide bound to the anti-lysozyme FAB (Cheetham et al., 1991a), this can be associated with the existence of mobile regions of the bound peptide. Again, as in the previous case, the crosspeaks in the spectra of both complexes observed here have counterparts in closely similar positions to those of the isolated peptide.

Sequence specific assignments of isolated CGRP were made using a combination of standard 2D NMR techniques, and a titration from 0 to 50% (v/v) trifluoroethanol/water mixtures making use of the previous assignments of the peptide in 50% trifluoroethanol/ H_2O mixtures (Breeze et al., 1991). The peptide is considerably more soluble at pH 3.7 than at pH 6.0 and initial experiments with the free peptide were conducted at this pH. This also allows a direct comparison with the results of Breeze et al. (1991). Additional experiments were performed in 90% H_2O /10% D_2O at pH 6.0 in order to obtain assignments under conditions identical to those used for the antibody binding experiments. The spectra of the uncomplexed peptide exhibit a large number of well-resolved resonances, as expected for a molecule of this molecular weight (3.7 kDa). Identification of most of the side chains was possible through correlated spectroscopy and then sequential

Fig. 1. Upfield region of the DQF COSY spectra of (A) CGRP, (B) a 0.8 to 1 mixture of CGRP and CB3 FAB, (C) a 0.7 to 1 mixture of CGRP and CD1 FAB. The concentration of FAB was 1 mM. All samples were pH 6.0, 50 mM potassium phosphate, 150 mM sodium chloride and the temperature was 25 °C.

assignment was achieved using the procedure of Wuthrich et al. (1986). Strong α to NH ($i, i + 1$) are observed throughout the sequence, which, with the lack of medium- or long-range NOEs, indicates that the peptide does not adopt a unique structure. Approximately 70% of the ^1H spectrum could be unambiguously assigned using these conditions, the remaining residues being ambiguous because of chemical shift degeneracy of the alpha and amide protons of Ser 34 and Leu 16, Ala 36 and Ala 13, and Thr 30 with Thr 9. This was resolved by recording spectra in a series of trifluoroethanol/ H_2O mixtures which alleviated the spectral overlap problem. The complete assignments in H_2O , pH 3.7, are included in Table 1. These assignments, in conjunction with additional spectra recorded at pH 6.0, were used to compile the pH 6.0 assignments.

Comparison with the spectrum of free CGRP shows that the spectra of both the CGRP complexes exhibit a substantially reduced number of crosspeaks. The positions of the observed crosspeaks are virtually the same as those found in spectra of the free peptide although, in some cases, small shifts in resonance fre-

quency are observed. This allows the spectra of the peptide FAB complexes to be assigned by comparison with the assignments of the free peptide. In a few cases (for example, Val 22, Val 23, and Val 32), the small shifts lead to potential ambiguities in the assignments.

Analysis of the spectra of CGRP FAB complexes

The CGRP peaks remaining visible in the spectra of the peptide FAB complexes are largely different in the two cases; only a few crosspeaks are common to the spectra of the two complexes. This indicates that the regions of CGRP with narrow resonances, attributed to mobile regions of the peptide, are substantially different in the two cases. This is illustrated in Figure 1, showing the regions where crosspeaks due to 92% of the expected α/β correlations and all the β/γ correlations of the threonine side-chain protons are anticipated; in this region some 29 crosspeaks are observable for CGRP itself. The crosspeaks of all resonances in the complex experience some degree of line broadening and, as the crosspeaks due to the three leucines and two lysines are poorly dispersed, they cannot be unambiguously distinguished by their α/β correlations; this leaves 24 α/β correlations that could, in principle, be identified. However, in both complexes of CGRP with CD1 or CB3, the number that can be observed is reduced to 11. Interestingly, there is little overlap between the two groups of resonances remaining, and only four are observed in both spectra. This is particularly clearly seen by comparing resonances of the same type of residue. In the case of the α/β correlations of the five valines, for example, those of Val 8, 22, and 23 are seen in the spectrum of the CB3 complex while the peaks due to Val 28 and Val 32 are absent. In contrast, in the spectrum of the CD1 complex, the crosspeak due to Val 28 is detected, while the crosspeak due to Val 8 and two of the three crosspeaks due to Val 22, Val 23, and Val 32 are absent. Based upon the measured chemical shifts, the detected crosspeak is likely that of Val 32 and the missing crosspeaks are those of Val 22 and Val 23. In the case of the leucine residues, however, α/β crosspeaks of none of the three residues Leu 12, 15, and 16 are seen in either spectrum. Before interpreting this result, however, the fact that the crosspeaks' structure in these two types of residue in the free peptide are very different, those of leucine being very weak, must be considered; this is discussed further below. Overall, however, the data clearly indicate that different regions of the polypeptide chain give rise to narrow resonances in the two cases. Of the α/β crosspeaks, taken together, none of those arising from the 14 residues at the C-terminus can be seen in the spectrum of the CB3 complex, except Ser 34. By contrast, only one (Ala 1) of the 12 residues at the N-terminus of CGRP can be seen in the spectrum of the CD1 complex. The α/β crosspeaks of Thr 6 and Thr 9 appear to be present but are substantially broadened upon binding.

A similar picture emerges when a wider range of crosspeaks is examined. Analysis of resonances of aromatic side chains shows that crosspeaks of Phe 27 and 37 are visible in the spectrum of the CD1 antibody but not in that of CB3. Conversely, those of His 10 are seen in the spectrum of CB3 but not CD1. This result is again in accord with flexibility in the bound state existing in the N-terminal half of CGRP with CD1 and the C-terminal half of the molecule with CB3. The observation, or the lack of observation, of resonances in the complexes of a peptide with a large molecule such as a FAB will depend not just on the extent of line broadening associated with the binding of the peptide to the FAB but on the intensities of individual crosspeaks and their sensitivity to line

Table 1. Proton chemical shifts of free CGRP and changes in linewidth upon antibody binding^a

Chemical Shifts (ppm) of free peptide					Increase in linewidth (Hz) upon binding			
Residue	NH	α H	β H	Others	CD1 complex		CB3 complex	
					$\alpha\beta$	SC ^b	$\alpha\beta$	SC
Ala-1		4.06	1.52		<1		1-2	
Cys-2	8.80	4.75	3.22		n.d. ^c		1-2	
Asp-3	8.74	4.82	2.82		n.d.		1-2	
Thr-4	7.64	4.49	4.50	γ CH ₃ 1.29	n.d.	>10	n.d.	1-2
Ala-5	8.63	4.18	1.49		>10		1-2	
Thr-6	7.89	4.22	4.18	γ CH ₃ 1.19	n.d.	2-5	n.d.	1-2
Cys-7	7.98	4.49	3.23		>3		1-2	
Val-8	8.12	3.98	2.10	γ CH ₃ 0.80	>3		<1	
Thr-9	8.13	4.18	4.21	γ CH ₃ 1.10	n.d.	2-5	n.d.	1-2
His-10	8.27	4.66	3.32	2H 8.60	>1		n.d.	
Arg-11	8.26	4.27	1.82	4H 7.30				
				γ CH 1.70	>3		<1	
				δ CH 3.18				
				ϵ NH 7.10				
Leu-12	8.27	4.30	1.64	γ CH 1.76	>2	>5	<1	<1
				δ CH ₃ 0.87				
Ala-13	8.16	4.27	1.41		1-2		1-2	
Gly-14	8.21	3.92			n.d.		n.d.	
Leu-15	7.99	4.31	1.72	γ CH 1.83	>2	>5	>2	>5
Leu-16	8.18	4.26	1.75	δ CH ₃ 0.88				
				γ CH 1.76	>2	>5	<1	<1
				δ CH ₃ 0.87, 0.93				
Ser-17	8.30	4.41	3.88		1-2		1-2	
Arg-18	8.15	4.41	1.79	γ CH 1.70	>3		<1	
				δ CH 3.18				
				ϵ CH 7.15				
Ser-19	8.29	4.47	3.84, 3.88		1-2		1-2	
Gly-20	8.40	3.98			n.d.		n.d.	
Gly-21	8.22	3.96			n.d.		n.d.	
Val-22	8.02	4.12	2.00	γ CH ₃ 0.80	>2	>5	<1	1-2
Val-23	8.25	4.07	1.96	γ CH ₃ 0.85	>2	>5	<1	1-2
Lys-24	8.40	4.31	1.67	γ CH 1.45	n.d.	<3	n.d.	<1
				δ CH 1.70				
				ϵ CH 2.97				
				ϵ NH ₃ ⁺ 7.35				
Asn-25	8.41	4.64	2.67	δ NH 7.43, 6.75	>3		>3	
Asn-26	8.38	4.66	2.68	δ NH 7.37, 6.69	>3		>3	
			2.73					
Phe-27	8.14	4.60	3.05	2,6 7.14	<2		>5	
Val-28	7.95	4.32	1.90	γ CH ₃ 0.80	1-2	1-2	>2	>5
Pro-29		4.38	2.33	γ CH 1.90	>1		>1	
			2.20	δ CH 3.60				
Thr-30	8.14	4.22	4.21	γ CH ₃ 1.11	n.d.	<2	n.d.	>10
Asn-31	8.43	4.75	2.82	δ NH 7.50, 6.80	>3		<2	
			2.77					
Val-32	8.08	4.10	2.13	δ CH ₃ 0.86	<2		<2	
Gly-33	8.46	3.97			n.d.		n.d.	
Ser-34	8.15	4.41	3.82		1-2		1-2	
Lys-35	8.30	4.28	1.67	γ CH 1.45	<2	2-3	1-2	1-2
				δ CH 1.70				
				ϵ NH ₃ ⁺				
Ala-36	8.14	4.21	1.18		<2		>5	
Phe-37	8.04	4.55	3.04	2,6 7.16	<2		>3	
CO-NH ₂	7.80							

^aAssignments are given for pH 3.70 in 90% H₂O/10% D₂O at 29°C and are quoted relative to dioxan at 3.743. The change in linewidth is calculated from comparison of the spectrum of the complex to the spectrum of free CGRP (recorded at pH 6.0) multiplied by various values of line broadening. Either the minimum amount of line broadening required to cause the indicated peak to disappear is listed or, if the peak is still visible, the actual increase in linewidth is listed.

^bSC, side-chain crosspeak other than $\alpha\beta$.

^cn.d., not determined because of spectral overlap or because the indicated crosspeak was too close to the diagonal or was obscured by solvent.

broadening. In order to assess the significance of the presence or absence of individual resonances, a series of simulations were carried out, examples of which are shown in Figure 2. In these, the experimental spectrum of the free peptide was examined following the application of different extents of simulated line broadening to mimic the line broadening experienced by the resonances of CGRP on binding to the FAB. By comparing the crosspeaks in the experimental spectrum of the complexes with the appearance of the corresponding crosspeaks in the simulation, the degree of line broadening of peaks of CGRP bound to the FAB was estimated, along with a lower limit for the degree of line broadening experienced by crosspeaks not observed in the spectrum.

The simulations indicate that the extent of line broadening on the resonances of CGRP in the complex varies from <1 Hz, in-

dicating very extensive flexibility, to >10 Hz, indicating restricted motion. The results are tabulated in Table 1 and are displayed in Figure 3. Few resonances are intense enough in the spectrum of the complex to be observed if line broadening exceeds 5 Hz. The simulations also show that the intensities of different crosspeaks have dramatically different dependencies on linewidth. This arises because of the differences in multiplet structure and coupling constants for the crosspeaks between different chemical groups. Thus, for example, crosspeaks arising from the α and β protons of Cys 2 and Val 28 and Pro 29 are very sensitive to line broadening and are not observable under the conditions of these experiments when the linewidth of the peaks in the spectrum of the free peptide are broadened by 1 Hz. In contrast, many crosspeaks are still visible in the simulations when the added line broadening is 5 Hz or more. These include those arising from the α and β protons of alanine and the β and γ protons of threonine and leucine residues. It is clear that the effects of binding of CGRP to the antibodies cannot be attributed to non-specific broadening of the peptide in the presence of FAB, as different regions of the peptide are affected for the two antibodies. Furthermore, some crosspeaks that are sensitive to small amounts of line broadening are still observable, for example, Val 22, Val 23, Val 8, and Cys 7 in the CB3-CGRP complex and Val 32 in the CD1 complex, while others that require much higher levels of line broadening to be rendered unobservable are absent from the spectrum, for example, Phe 27, Thr 30, and Ala 36 in the CB3-CGRP complex and Ala 5 in the CD1 complex.

Discussion

The results in this paper show that many resonances of CGRP bound to two monoclonal antibodies can still be resolved in the ^1H NMR spectra. Simulations show that in order for this to arise, the majority of these must have experienced line broadening of less than 5 Hz on formation of the complexes. The degree of broadening expected for the resonances of a residue in the peptide bound tightly to a FAB fragment is in excess of 30 Hz. The existence of resonances with narrow linewidths can be associated with the retention of extensive flexibility and freedom of motion in regions of the peptide when bound to the FAB molecule. This result is in accord with the findings for a 29-residue peptide corresponding to a loop region of lysozyme when bound to an anti-peptide FAB (Cheetham et al., 1991a). Here, 14 residues were found to be very mobile with the rest restricted to such an extent that they were not visible in the ^1H NMR spectrum. The flexibility of residues not within the epitope has also been inferred from several X-ray studies of FAB-peptide complexes, where long peptides have been used (generally greater than 10 residues), by the fact that some regions of the peptide were not visible in the electron density of the complex, for example, the first nine residues in a 19-residue complex (Stanfield et al., 1990), and seven residues in a 16-residue complex (Rini et al., 1993).

The most striking result from the present study is that the two antibodies differ almost completely in the regions of the CGRP molecule that retain flexibility in the complex. This at once gives confidence that the findings are not a consequence of non-specific interaction between the FAB molecule and the peptide. The regions of the peptide where line broadening of resonances is extreme can reasonably be supposed to include all those interacting strongly with the antibody, and hence forming the epitope region located in the binding site. It is, however, inappropriate to interpret the extent of line broadening in anything other than a qualitative

Fig. 2. Upfield region of the DQF COSY spectrum of CGRP after multiplication with different values of line broadening, (A) 0 Hz, (B) 2 Hz, and (C) 4 Hz.

Residue Number	A1	C2	D3	T4	A5	T6	C7	V8	T9	H10	R11	L12	A13
CGRP bound to CB3	○	○	●	○	○	○	○	○	○	○	○	○	○
CGRP bound to CD1	○	†	†	●	●	*	●	●	*	●	●	●	*

Residue Number	G14	L15	L16	S17	R18	S19	G20	G21	V22	V23	K24	N25	N26
CGRP bound to CB3	†	○	○	○	○	○	†	†	*	*	○	●	●
CGRP bound to CD1	†	●	●	○	●	○	†	†	●	●	○	●	●

Residue Number	F27	V28	P29	T30	N31	V32	G33	S34	K35	A36	F37
CGRP bound to CB3	●	●	●	●	●	●	†	○	○	●	●
CGRP bound to CD1	○	○	●	○	●	○	○	○	○	○	○

Fig. 3. A diagrammatic representation of the regions of CGRP that are affected by binding to FAB fragments of the CB3 antibody and the CD1 antibody as determined by 2D NMR (○) unaffected residues, (●) immobilized residues, (*) residues showing line broadening but still retaining considerable flexibility, (†) not determined because the peaks are too close to the diagonal (glycines) or because the resonances are obscured by solvent. The crosspeaks affected are for the α and β protons, or β and γ protons, except for the aromatic residues in the case of binding by CD1 where the effects on the ring protons are stated as the side-chain crosspeaks occur close to the solvent. The effects on lysine, leucine, and arginine are on the γ/δ crosspeaks as the α/β crosspeaks are very sensitive to line broadening and are too overlapped to be clearly distinguished. In the case of CD1 the solvent has also obscured signals from Cys 2 and Asp 3.

manner, given the complex dependence of proton relaxation on motional averaging and the environment of the proton, and the possible contribution of exchange effects. Furthermore, it is possible that resonances in addition to those highly immobilized in the binding site are broadened by interaction with the FAB, for example, if the binding induces persistent structure in regions not in the binding site. However, the lack of change in chemical shifts and linewidth suggests that there appear to be few long-range effects on the conformational properties of the flexible regions of CGRP resulting from binding in either antibody complex. It is, also, unlikely that such regions of structure will be persistent unless they are closely associated with the FAB or regions of the bound peptide. It is, nevertheless, of considerable interest to examine the NMR findings in the light of serological typing.

The epitope mapping of CGRP to monoclonal antibodies was done by using ELISAs to assay the crossreactivity of the monoclonals with various peptides. It is difficult, however, for these experiments to identify precise residues involved unless many different peptide fragments and analogues are used. For CB3, seven different peptide fragments of human α -CGRP were used and the only region that was clearly identified as essential for binding was the C-terminal Phe-amide. This residue is also implicated in binding to CB3 by the NMR studies described here. Both the α/β and the ring protons of Phe 37 are sufficiently broadened not to be visible in the spectrum of the complex. NMR experiments have also identified several other residues in the C-terminus that interact strongly with the CB3 antibody.

The serological mapping of CD1 for CGRP used only five peptide fragments and indicated that the epitope involves a region around residues 13 to 19. At least four of these residues are seen in the NMR experiments to have broadened resonances and thus potentially are tightly bound. Ala 13 and Ser 19, however, give rise to narrow resonances even for the α/β crosspeaks and must retain considerable flexibility implying they do not themselves interact strongly with the antibody. These residue-specific interactions cannot be determined by the ELISA techniques, which only determine broad regions of peptide where an overall interaction takes place.

Additional residues also interact strongly with the CD1 antibody, most notably those near the N-terminus of the peptide. Thus, in both cases the epitope, as defined by NMR, corresponds broadly to serological typing (Andrew *et al.*, 1990), although NMR has also identified residues involved in the interaction with the FAB in addition to those seen in the serological typing.

Taking both sets of data (NMR and ELISA) together we can conclude that the epitopes for the monoclonal antibodies appear linear in nature and consist of a continuous stretch of about eight or more residues in both peptides, with several residues close to, but outside, this region where resonances are also influenced by the binding. This is in contrast to the case with the lysozyme peptide where a discontinuous epitope was found involving two sets of residues at least 15 residues apart in the peptide sequence. In the latter example, however, antibodies were raised against peptides with a disulphide bridge and regions linked by the bridge form the epitope. Thus, although this bridge was not present in the peptide when binding was monitored, it is likely that in the bound state these regions of the peptide chain are close together. In the majority of NMR and X-ray studies, linear epitopes have been identified for peptides, but this is not necessarily the case in protein complexes. It is interesting that residues within the linear epitopes for CB3 and CD1 appear to differ in the extent to which conformational mobility is restricted. Thus, for example, in CD1 Thr 6 and Thr 9 are broadened but still detectable, but residues to either side are broadened more severely. This result is, however, consistent with previous X-ray and transferred NOE (NMR) studies of peptides bound to antibodies, which show that, even in the epitope, individual residues may be involved in binding to different extents. Thus, in the complex of a FAB with a seven-residue peptide from influenza virus, hemagglutinin residues that are essential for binding were found to be interspersed with those whose role is much weaker and may be replaced with little effect on binding (Schulz-Gahmen *et al.*, 1993). Similarly, the magnitude of the observed NOEs in an NMR study of the binding of an acetylcholine receptor monoclonal antibody with a decapeptide has been shown to vary

depending upon the restriction of mobility experienced, which was different for each residue (Cung et al., 1991).

The size of the epitope as defined by NMR is in good agreement with other X-ray and NMR studies that suggest epitopes of nine or 10 residues are involved in tight binding of peptides to monoclonal antibodies. The majority of studies have used, however, much smaller peptides of up to 10 residues, in which case 60 to 65% of the antigen has been found to be buried. Peptides have been seen to adopt a range of conformations in the binding pocket. The ^1H NMR methods described here selectively detect flexible regions of the bound peptide in the complex but are unable to detect resonances from strongly immobilized portions of the bound ligand among the mass of protein resonances and therefore cannot yet define the bound conformation. Nevertheless, the data have given insight into the regions of CGRP interacting with the different monoclonal antibodies and into the nature of the bound complexes.

Materials and methods

Analysis of CGRP

Synthetic human α -CGRP was purchased from Bachem Feinchemikalien (Budendorf, Switzerland). Amino acid analysis after acid hydrolysis gave the expected molar proportions. Fast atom bombardment mass spectrometry gave molecular ions in agreement with calculated values.

Preparation of monoclonal FAB fragments

High-affinity monoclonal antibodies to epitopes on CGRP were obtained as described previously (Andrew et al., 1990). Tissue cultures from Sp/20 myeloma cell lines producing CB3 or CD1, which are both mouse IgG isotype 1, were concentrated 10-fold and stored frozen at -20°C in 100 mL batches. Antibody (approximately 100 mg) was purified from 4×100 mL batches via affinity chromatography using protein A-sepharose 6 Fast Flow as described by Harlow and Lane (1988). The antibody samples were kept frozen at -80°C until used for preparation of FAB fragments. FAB fragments from CB3 and CD1 antibodies were prepared by papain digestion as described by Harlow and Lane (1988). The resulting solutions were dialyzed overnight against 50 mM potassium phosphate, 150 mM sodium chloride, pH 6.0, at 4°C , and then concentrated to approximately 1 mM using an Amicon concentrator. Labile amide hydrogens were exchanged for deuterons by 10-fold dilution of the samples into deuterated buffers followed by reconcentration to approximately 1 mL. The dilution/reconcentration steps were repeated three times. The purity ($>90\%$) of the antibody and FAB fragments was confirmed by gel electrophoresis and HPLC gel filtration using a TSK 2000 column.

NMR data collection

^1H NMR spectra were recorded at 25, 27, or 29°C , on either a home-built 500 MHz spectrometer comprising an Oxford Instrument Company magnet and a GE/Nicolet 1280 computer, or a Bruker AM series 500 MHz instrument. Phase-sensitive detection was achieved on the former by the States method (Marion et al., 1989) and on the Bruker instrument by using time-proportional phase incrementation of the first pulse (TPPI; Marion & Wuthrich, 1983). Pre-TOCSY COSY spectra (Otting & Wuthrich, 1987) were recorded with 2048 data points and 512 complex increments, using

a 26 ms MLEV-17 spin lock period. 2D DQF COSY spectra were recorded with 4096 data points and 1K t1 increments. Residual HOD was suppressed by presaturation. For some experiments solvent suppression was achieved via the 1-1 pulse sequence (Hore, 1989). NOESY spectra (Jeener et al., 1979) were recorded using mixing times of 150, 200, 250, 300, and 400 ms. A RELAY experiment was collected with a mixing time of 18 ms, and phase-sensitive homonuclear Hartmann-Hahn (HOHAHA) experiments were recorded with spin locking time of 45 and 65 ms. 1D NMR spectra, using the CPMG sequence, were recorded with dephasing times of between 1 and 60 ms.

NMR data analysis

Data were processed on a GE/Nicolet 1280 data station or a Sun 4/110 computer using the FTNMR software package (Hare Research, Inc.). All spectra were zero filled once in t1, and apodized with a trapezoidal and double exponential function prior to the first Fourier transformation. For the second transformation the data were zero filled twice and multiplied by only a trapezoidal apodization function. Chemical shifts were referenced to dioxan (3.745 ppm). 2D data sets of the unbound peptide were processed with a number of different line-broadening functions to simulate the effect of increasing linewidth on the intensity of crosspeaks in the COSY spectrum of the peptide. The resulting spectra were contoured at identical levels and compared to the spectra of the bound peptide.

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References

- Abello J, Kaiserlian D, Cuber JC, Revillard JP, Chayrial JA. 1991. Characterization of calcitonin gene related peptide receptors and adenylate cyclase response in the murine macrophage cell line P388 D1. *Neuropeptides* 19:43-49.
- Andrew DP, Bidgood TD, Bose C, Brown D, Galfre G, Sherwood M. 1990. Monoclonal antibodies distinguishing alpha and beta forms of calcitonin gene-related peptide. *J Immunol Methods* 154:87-94.
- Anglistter J, Frey T, McConnell M. 1984. NMR study of the complexes between a synthetic peptide derived from the B subunit of cholera toxin and three monoclonal antibodies. *Biochemistry* 23:1138-1142.
- Anglistter J, Jacob C, Assulin O, Ars G, Pinker R, Arnon R. 1988. NMR study of the complexes between a synthetic peptide derived from the B subunit of cholera toxin and three monoclonal antibodies against it. *Biochemistry* 27:717-724.
- Anglistter J, Scherf T, Zilber B, Levy R. 1995. Two-dimensional NMR studies of the interactions between a peptide of cholera toxin and monoclonal antibodies. *Biopolymers* 37:383-389.
- Anglistter J, Scherf T, Zilber B, Levy R, Jacob C, Assulin O, Ast G, Pinker R, Arnon R, Frey T, McConnell HM. 1990. Use of deuterium labelling in NMR studies of antibody combining site structure. *Q Rev Biophys* 23:175-203.
- Bax A, Grzesiek S, Gronenborn AM, Clore GM. 1994. Isotope-filtered 2D HOHAHA spectroscopy of a peptide-protein complex using heteronuclear Hartmann-Hahn dephasing. *J Magn Reson Ser A* 106:269-273.

- Benjamin DC, Williams DC Jr, Smith-Gill SJ, Rule GS. 1992. Long-range changes in a protein antigen due to antigen-antibody interaction. *Biochemistry* 31:9539-9545.
- Breeze AL, Harvey TS, Bazzo R, Campbell ID. 1991. Solution structure of human calcitonin gene-related peptide by ^1H NMR and distance geometry with restrained molecular dynamics. *Biochemistry* 30:575-582.
- Cheetham JC, Raleigh DP, Griest RE, Redfield C, Dobson CM, Rees AR. 1991a. Antigen mobility in the combining site of an anti-peptide. *Proc Natl Acad Sci USA* 88:7968-7972.
- Cheetham JC, Redfield C, Griest RE, Raleigh DP, Dobson CM, Rees AR. 1991b. Use of two-dimensional ^1H nuclear magnetic resonance to study high-affinity antibody-peptide interactions. *Methods Enzymol* 207:202-228.
- Churchill ME, Stura EA, Pinilla C, Appel JR, Houghten RA, Kono DH, Balderas RS, Fieser GG, Schulze-Gahmen U, Wilson IA. 1994. Crystal structure of a peptide complex of an anti-influenza peptide antibody FAB 26/9. Comparison of two different antibodies bound to the same peptide antigen. *J Mol Biol* 241:534-556.
- Cooper GJ, Greene HE, Cooper GJS. 1989. Amylin antagonists and treatment of Type-2 diabetes and alleviation of insulin resistance or insulin suppression in pancreatic tissue, and screening or assaying for amylin antagonists. Patent W089/06135.
- Cung MT, Demange P, Marraud M, Tsikaris V, Sakarellos C, Papadoulis I, Kokla A, Tzartos S. 1991. Two-dimensional ^1H -NMR study of antigen-antibody interactions: Binding of synthetic decapeptides to an anti-acetylcholine receptor monoclonal antibody. *Biopolymers* 31:769-776.
- Harlow E, Lane D. 1988. *Antibodies a laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Hore PJ. 1989. Nuclear magnetic resonance. Solvent suppression. *Methods Enzymol* 176:64-77.
- Hubbard JAM, Martin SR, Chaplin, LC, Bose, Kelly SM, Price NC. 1991. Solution structures of calcitonin-gene-related-peptide analogues of calcitonin gene-related peptide and amylin. *Biochem J* 275:785-788.
- Jeener J, Meier BH, Bachmann P, Ernst RR. 1979. Investigation of exchange processes by 2D NMR spectroscopy. *J Chem Phys* 71:4546-4553.
- Lian LY, Barsukov IL, Derric J, Roberts GC. 1994. Mapping the interactions between streptococcal protein G and the FAB fragment of IgG in solution. *Nature Struct Biol* 1:355-357.
- Maggi CA, Rovero P, Ginliani S, Evangelista S, Regoli D, Meli A. 1990. Biological activity of N-terminal fragments of calcitonin gene-related peptide. *Eur J Pharmacol* 179:217-219.
- Marion D, Ikura M, Tschudin R, Bax A. 1989. A rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J Magn Reson* 85:393-399.
- Marion D, Wuthrich K. 1983. Application of phase-sensitive two-dimensional correlated spectroscopy (COSY) for measurements of ^1H - ^1H spin-spin coupling constants in proteins. *Biochem Biophys Res Commun* 113:967-974.
- Mayne L, Paterson Y, Ceasoli D, Englander SW. 1992. Effect of antibody binding on protein motion studies by hydrogen exchange labeling and two-dimensional NMR. *Biochemistry* 31:10678-10685.
- Otting G, Wuthrich K. 1987. Pre-TOCSY, a new experiment for obtaining complete 2D ^1H NMR spectra for proteins in aqueous solution. *J Magn Reson* 75:546-549.
- Paterson Y, Englander SW, Roder H. 1990. An antibody binding site on cytochrome *c* defined by hydrogen exchange and two dimensional NMR. *Science* 249:755-759.
- Raynaud A, Cohen R, Modigliani E. 1994. Calcitonin gene-related peptide. *Presse Med* 23:171-175.
- Rees AR, Staunton D, Webster DM, Searle SJ, Henry AH, Pedersen JT. 1994. Antibody design: Beyond the natural limits. *Trends Biotechnol* 12:199-206.
- Rini JM, Schulze-Gahmen U, Wilson IA. 1992. Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Science* 255:959-965.
- Rini JM, Stanfield RL, Stura EA, Salinas PA, Proffy AT, Wilson IA. 1993. Crystal structure of a human immunodeficiency virus type 1 neutralizing antibody, 50.1, in complex with its V3 loop peptide antigen. *Proc Natl Acad Sci USA* 90:6325-6329.
- Sagoo JK, Bose C, Beeley NRA, Tendler SJB. 1991. Structural studies on the [Bu(t)-Cys18](19-37)-fragment of human beta-calcitonin gene-related peptide. *Biochem J* 280:147-150.
- Scherf T, Hiller R, Naider F, Levitt M, Anglister J. 1992. Induced peptide conformations in different antibody complexes: Molecular modeling of the three-dimensional structure of peptide-antibody complexes using NMR-derived distance restraints. *Biochemistry* 31:6884-6897.
- Schulze-Gahmen U, Rini JM, Wilson IA. 1993. Detailed analysis of the free and bound conformations of an antibody. X-ray structures of FAB 17/9 and three different FAB-peptide complexes. *J Mol Biol* 234:1098-1118.
- Shaw NE, Foulkes R, Andrew DP, Brown DT, Hughes B. 1992. The effect of monoclonal antibodies to calcitonin gene-related peptide (CGRP) on CGRP-induced vasodilation in pig coronary artery rings. *Br J Pharmacol* 106:196-198.
- Shoham M. 1993. Crystal structure of an anticholera toxin peptide complex at 2.3 Å. *J Mol Biol* 232:1169-1175.
- Stanfield RL, Fiescher TM, Lerner RA, Wilson IA. 1990. Crystal structure of an antibody to a peptide and its complex with peptide antigen at 2.8 Å. *Science* 248:712-719.
- Stanfield RL, Wilson IA. 1995. Protein-peptide interactions. *Curr Opin Struct Biol* 5:103-113.
- Tsang P, Fieser TM, Ostresch JM, Lerner RA, Wright PE. 1988. Isotope-edited NMR studies of FAB-peptide complexes. *Peptide Res* 1:87-92.
- Tsang P, Rance M, Fieser TM, Ostresch JM, Houghten RA, Lerner RA, Wright PE. 1992. Conformation and dynamics of an FAB-bound peptide by isotope-edited NMR spectroscopy. *Biochemistry* 31:3862-71.
- Tsang P, Wright PE, Rance M. 1990. Specific deuteration strategy for enhancing direct nuclear Overhauser effects in high molecular weight complexes. *J Am Chem Soc USA* 112:8183-8185.
- Wikstrom M, Sjobring U, Drakenberg TM, Forsen S, Bjorck L. 1995. Mapping of the immunoglobulin light chain-binding site of protein L. *J Mol Biol* 250:128-133.
- Wilson IA, Stanfield RL. 1994. Antibody-antigen interactions: New structures and conformational changes. *Curr Opin Struct Biol* 4:857-867.
- Wright PE, Dyson HJ, Lerner RA, Riechmann L, Tsang P. 1990. Antigen-antibody interactions: An NMR approach. *Biochem Pharmacol* 40:83-88.
- Wuthrich K. 1986. *NMR of proteins and nucleic acids*. New York: Wiley & Sons.