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SUMMARY

The conformation of a synthetic undecapeptide derived from the *Escherichia coli* tryptophan synthase β_2 subunit was studied by NMR spectroscopy when bound to a monoclonal antibody (mAb 164-2) Fab' fragment directed against the native protein. The peptide ^{1}(H-G-R-V-G-I-Y-F-G-M-K)^{11}, peptide 11, was recognized by the antibody and its corresponding Fab' fragments with high affinity ($K_D = 1.1 \pm 0.2^{*} 10^{-8}$ M). Peptide 11 was labelled with ^{15}N and its structure at the binding site of the Fab' 164-2 fragment was studied by isotope-editing techniques. ^{1}H-^{15}N heteronuclear spectra indicated the presence of two Fab'-peptide 11 complexes with two different conformations in slow chemical exchange on the chemical shift time scale.

Key-words: Peptide, Antibody; Interaction, High-affinity complex, Heteronuclear ^{1}H-^{15}N NMR; Fab' fragment; Tryptophan synthase, β_2 subunit.

INTRODUCTION

A common view that has emerged from structural and kinetic data on antibody-antigen interactions is that an antibody and an antigen adopt a single conformation in the immune complex. Many antigen-antibody structures have been solved to date by X-ray crystallography (for reviews see Wilson and Stanfield, 1993; Webster *et al.*, 1994; Wilson and Stanfield, 1994). In addition, the solution structure of peptides (Anglister *et al.*, 1988; Scherf *et al.*, 1992; Tsang *et al.*, 1992; Tsikaris *et al.*, 1993; Seelig *et al.*, 1995) or other antigens of small size (Glaudemans *et al.*, 1990; Bundle *et al.*, 1994) associated with antibodies or Fab fragments has been determined by NMR. Independently of the type of antigen (peptide, protein, hapten) used in each particular study, a single structure of the antigen bound to an antibody has been found.

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Comparison of structures of free and bound antibodies (Stanfield and Wilson, 1994, and references cited therein) has established that antibodies can exist in multiple conformational states. Conformational adaptation occurring upon complex formation has been observed for many antibody-antigen pairs (Kranz *et al.*, 1982; Friguet *et al.*, 1989). Moreover, it has been demonstrated that free antibodies can show conformational isomerism. Indeed, Foote and Milstein (1994) used kinetic data to show that three antibodies that interact with the hapten 2-phenyl-5-oxazolone present a conformational equilibrium between at least two different conformations, and that the hapten preferentially associates with one of these forms. In their article, the authors proposed that conformational isomerism of a free antibody was a common phenomenon, and that about 10% of the antibodies might exist in alternative conformations. In addition, it has been reported that a monoclonal antibody (mAb) can recognize with high affinity very similar molecules, and yet the conformation of these molecules can be quite different within the complex: the X-ray structure of 5 complexes of closely related steroid hormones bound to the Fab fragment of mAb DB3 showed that the conformation of the Fab was essentially the same in the five structures and that each steroid was bound in a unique conformation (Arevalo *et al.*, 1993). Yet, in spite of the strong structural similarities of the five hormones, two different types of antigen conformations were observed depending on the steroid. In summary, several mechanisms have been described for antibody/antigen interactions. Here we will present a new possibility: an antigen that shows two different conformations when it is complexed to a mAb.

The mAb used in this work is IgG 164-2, elicited against the β_2 subunit of the *Escherichia coli* tryptophan synthase (Djavadi-Ohaniance *et al.*, 1984). It recognizes the hinge region between the F1 (residues 1-272) and F2 (residues 284-397) proteolytic domains of the β chain (Friguet *et al.*, 1989). Binding experiments with synthetic peptides of different length showed that the epitope of mAb 164-2 corresponds to the segment ^{273}-HGRVGIYFGMK^{283} of the β chain. The antibody 164-2 interacts with high affinity with the undecapeptide (from now on referred to as peptide 11 or p11) corresponding to the latter sequence, and with the β_2 subunit. The affinity of mAb 164-2 for p11 ($K_D = 7.5^{*}10^{-9}$ M) and for the β_2 subunit ($K_D = 10^{-9}$ M) are close, indicating that the epitope displays a similar structure within the protein and in the isolated peptide (Larvor *et al.*, 1991). Furthermore, the measured equilibrium dissociation constant (K_D) of the p11-mAb 164-2 complex is, within experimental error, equal to the ratio of the dissociation (k_{off}) and association rate (k_{on}) constants. These results were in agreement with a one-step binding mechanism and suggested that no significant conformational adaptation occurs upon complex formation (Larvor *et al.*, 1991). In the published structure (Hyde *et al.*, 1988), as well as in the refined structure of the tryptophan synthase (Hyde, C.C., personal communication), the segment corresponding to p11 forms a β-hairpin with a β_1 turn at residues ^{279}YFGM^{282}. An NMR study of isolated p11 has shown that its C-terminal part presents some structure centred around residues FGM that are implicated in the β-turn in the β_2 subunit (Delepierre *et al.*, 1991). Within this context, and with the aim of shedding light on how mAb 164-2 can recognize with high

βSH = β-mercaptoethanol.
DTT = dithiothreitol.
EDTA = ethylenediaminetetraacetic acid.
Fab and Fab' = monovalent antigen-binding fragment obtained after, respectively, papain and pepsin proteolysis.
$F(ab')_2$ = divalent antigen-binding fragment.
FID = free induction decay.
IgG = immunoglobulin G.
HMQC = heteronuclear multiple-quantum correlation (spectroscopy).
HPLC = high-performance liquid chromatography.
HSQC = heteronuclear single-quantum correlation (spectroscopy).
mAb = monoclonal antibody.
NMR = nuclear magnetic resonance.
p11 = peptide 11 (peptide HGRVGIYFGMK).
SDS-PAGE = sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

affinity p11 and its cognate within the β_2 subunit, we undertook the characterization of the structure of p11 associated with mAb 164-2. Attempts parallel to the work presented here were made in order to crystallize the Fab' fragments of mAb 164-2 either in the free state or complexed to p11 or to the β_2 subunit, but these were unfortunately unsuccessful (Larvor, M.-P., personal communication). This paper deals with the study of the conformation of p11 bound to the Fab' fragments of mAb 164-2 using high resolution NMR. Peptide 11 was labelled with ^{15}N and isotope-edited experiments, in which only the correlations of the hydrogens attached to labelled nitrogens of the peptide are detected, were performed on p11-Fab' 164-2 complexes. We will report that the Fab'-associated peptide displays two different conformations. Reasons for this unexpected observation will be discussed.

MATERIALS AND METHODS

Preparation and purification of Fab' and Fab fragments

Murine mAb 164-2 was obtained from ascites. Hybridoma cell culture and ascites production were performed as described (Djavadi-Ohaniance *et al.*, 1984). After incubating the ascitic fluid for 1 h at room temperature with 0.2% of dextran-sulphate, $CaCl_2$ was added up to a 10% final concentration in order to precipitate lipids and dextran. The precipitate was eliminated by centrifugation at 4°C (3,000 *g*, 30 min). Antibodies in the supernatant were precipitated at pH 7 with $(NH_4)_2SO_4$ saturated at 40%. After centrifugation at 4°C (12,000 *g*, 30 min), the pellet was solubilized in buffer A (Tris-HCl 20 mM pH 8) and extensively dialysed against this buffer. Antibodies were further purified by medium pressure anion-exchange chromatography using a "Mono Q HR 16/10" column (Pharmacia) equilibrated with buffer A. Elution was performed with a linear gradient (0-20%) of NaCl 2 M in buffer A. Antibody 164-2 was then concentrated by precipitation at pH 7 with $(NH_4)_2SO_4$ saturated at 50%. The pellet obtained after centrifugation (4°C, 12,000 *g*, 30 min) was solubilized in buffer A for further proteolysis with pepsin or in buffer B (KH_2PO_4 0.1 M, EDTA 2 mM, βSH 2 mM, pH 6.8) for proteolysis with papain, and extensively dialysed against the appropriate buffer.

Purified IgG was proteolysed with pepsin or papain. Cleavage was followed by SDS-PAGE. After cleavage, proteolysis mixtures were processed using the same protocol. For pepsin cleavage, the pH of an IgG solution at 5 mg/ml in buffer A was adjusted to 3.5 with CH_3COOH 1 M. IgG was then incubated for 80 min at 37°C with pepsin (Sigma) 1/400 (w/w). Pepsinolysis was stopped at 4°C by means of a pH jump to 8 with Tris-HCl 3 M pH 12. Pepsin cleavage yielded $F(ab')_2$ fragments with an apparent molecular weight of ~110 kDa. For papain proteolysis, IgG at 5 mg/ml in buffer B was incubated for 30 min at 37°C with papain (Worthington Biochemicals) 1/20 (w/w). The reaction was stopped with iodoacetamide at a final concentration of 57 mM. Papainolysis of mAb 164-2 (subclass 1) resulted in a heterogeneous mixture of $F(ab')_2$ and several Fab (~50 kDa) fragments with slightly different molecular weights.

The proteolysis mixture was loaded onto a gel filtration column containing "Sephacryl S-200 HR" (Pharmacia) in order to eliminate the proteases. The column was equilibrated and run at 4°C with H_3BO_4 0.16 M, NaCl 0.13 M, NaN_3 0.05%, pH 8.0, for pepsinolysis mixtures and with KH_2PO_4 0.1 M NaN_3 0.05% pH 6.8 for papainolysis mixtures. Eluted fractions containing antigen-binding fragments were collected, pooled, concentrated with "Centriflo" cones (Amicon), dialysed at 4°C against buffer A and loaded onto a "Mono Q HR 16/10" column to eliminate Fc fragments and uncleaved antibodies. Antigen-binding fragments were eluted with a linear gradient of NaCl 2 M from 0 to 20% in buffer A. Fractions containing antigen-binding fragments were pooled and incubated for 30 min at room temperature with DTT 5 mM to reduce inter-heavy-chain disulphide bridges and thereby produce monovalent Fab' fragments. Reduction was stopped by adding iodoacetamide in excess (45 mM) and incubating the reaction mixture for 30 min in the absence of light. This reactant acetamidates exposed free thiols and renders the reduction irreversible. Finally, Fab' were concentrated with "Centriflo" cones and dialysed against the NMR buffer (see below).

Purity of Fab' fragments was verified by SDS-PAGE with and without βSH using gradient gels 8-25% or 10-15% (Phast System, Pharmacia).

Peptides

Peptide 11 was labelled with ^{15}N at 4 positions (^2G, ^5G, ^9G and $^{10}M - p11^{4N}$) or at 8 positions (^2G, ^4V, ^5G, ^6I, ^7Y, ^8F, ^9G and $^{10}M - p11^{8N}$). Nitrogen 15-labelled amino acids were purchased from the Commissariat à l'Énergie Atomique (CEA, Saclay). Peptides were synthesized following the solid phase method of Merrifield (1963) using an "Applied Biosystems 430 A" synthesizer and purified by gel filtration and reverse phase medium pressure liquid chromatography. Final purity (>98% for $p11^{4N}$ and >96%

for p11^{8N}) was checked by reverse phase HPLC and amino acid analysis. Sequential assignment of proton signals in NMR spectra confirmed the amino acid composition of the peptides.

Protein and peptide concentration

Fab', Fab and mAb concentrations were determined using a colourimetric assay (Bradford, 1976) or by reading the absorbance at 280 nm considering an extinction coefficient of 1.5 $(mg/ml)^{-1}/cm$ (Onoue *et al.*, 1965). Peptide concentration was determined by measuring the absorption at 280 nm using an extinction coefficient of 1,280 $M^{-1}.cm^{-1}$ for the single tyrosine residue.

Equilibrium measurements

The equilibrium dissociation constant (K_D) of antibody-peptide and Fab-peptide complexes was determined in solution at pH 6 in a 50 mM sodium acetate buffer by the ELISA competition technique described by Friguet *et al.* (1985). Errors shown correspond to the root mean square deviation of at least 10 independent K_D determinations.

NMR samples

NMR samples were prepared in 650 µl of CD_3COONa 50 mM, D_2O 5 or 10%, EDTA 10 µM, PMSF 10 µM, NaN_3 0.05 %, pH 6 (buffer C). CD_3COONa and D_2O were from the CEA (Saclay). The concentration of the sample of p11 was 2 mM and that of the complexes ranged from 0.6 to 1 mM. Complexes were formed by adding the appropriate amount of a 20 mM peptide solution in buffer C to the Fab' (also in buffer C).

Active sites of Fab' (or of Fab) preparations were determined following the quenching of the fluorescence of the Fab' tryptophans upon addition of p11 labelled with dinitrophenol on ^{1}His (excitation at 280 nm, emission at 340 nm) (Larvor *et al.*, 1991). The stability of the Fab' was assessed by determining the affinity of the Fab' for p11 using the ELISA technique described above on an aliquot of Fab' that was subjected to the same conditions of temperature as the NMR sample. SDS-PAGE and NMR spectra were also used to evaluate the stability of Fab'. Preparations were stable for at least 8 weeks, though very slow aggregation was observed.

NMR spectroscopy

Spectra were acquired on a "Varian Unity 500" spectrometer operating at 500.0 MHz for ^{1}H nuclei and at 50.7 MHz for ^{15}N nuclei. The spectrometer was equipped with a ^{1}H-multinuclei inverse detection probe. Data were processed on "Sun Sparc" stations using the software Vnmr (Varian Inc.). Spectra were obtained at 40°C. The sodium salt of 3-(trimethylsylil)propane and $^{15}N\ H_4Cl$ were used, respectively as external reference for ^{1}H and ^{15}N chemical shifts.

Spectral width was 5,800, 6,000 or 6,500 Hz for protons in experiments with Fab' fragments and 6,000 Hz in experiments with peptide only. Spectral width for nitrogen was 2,000 Hz in all cases. Two-dimensional experiments were acquired in the phase-sensitive mode and using the hypercomplex method in the f_1-dimension (States *et al.*, 1982). The water line was reduced by selective irradiation during the preparation delay or using the jump-return sequence ($90°_x$-τ-$90°_x$) (Plateau and Guéron, 1982) as the read-pulse (the τ delay was adjusted to 111 µs to obtain a maximal intensity at the centre of the amide proton resonance region). When appropriate, the residual water signal was eliminated after acquisition by means of the method proposed by Sodano and Delepierre (1993).

Pulse sequences used to record two-dimensional heteronuclear ^{1}H-^{15}N spectra of the antigenic complexes correspond to modified versions of the heteronuclear multiple quantum correlation (HMQC) experiment (Summers *et al.*, 1986; Marion *et al.*, 1989) and of the heteronuclear single-quantum correlation (HSQC) experiment (Bodenhausen and Ruben, 1980; Marion *et al.*, 1989). Heteronuclear decoupling during the acquisition delay was achieved with the pulse sequence WALTZ-16 (Shaka *et al.*, 1983). Depending on the sample concentration, between 960 and 1,536 accumulations were acquired for each of the 32 complex points in the f_1-dimension. In the f_2-dimension, 2 k points were recorded. Relaxation delay was 1 or 1.5 s. For the heteronuclear spectrum of the peptide, 320 FIDS were acquired for each point in the f_1-dimension and the relaxation delay was 2 s, the rest of the parameters being the same as for the spectra of the peptide-Fab' complexes. Free peptide resonances were assigned using standard techniques (Wüthrich, 1986).

RESULTS

Antibodies 164-2 were obtained from ascites, purified and proteolysed with pepsin to generate $F(ab')_2$ fragments. These were further purified, reduced with DTT and free cysteine thiol groups

were blocked with iodoacetamide to obtain Fab' fragments. A SDS-PAGE was carried out to investigate the integrity and purity of the Fab' (see fig. 1). Only two bands at a position corresponding to about 25 kDa can be seen in the SDS-PAGE of the Fab' under reducing and non-reducing conditions (lanes 1, 2, 4 and 5), as expected for the heavy and light chains of an IgG, which indicates that the Fab' is pure. In figure 1, a single band at the expected position of ~110 kDa can be seen for the $F(ab')_2$ before reduction with DTT (lane 3). When the reducing agent βSH is added to the $F(ab')_2$ sample, inter-chain disulphides are reduced and two bands of apparent molecular weight ~25 kDa appear in the gel (lane 6). The fact that, in the absence of βSH, two bands at a position of ~25 kDa (lanes 1 and 2) are observed for the Fab' sample instead of a single band at a position of ~50-55 kDa, indicates that during the reduction of $F(ab')_2$ with DTT (see "Materials and Methods"), the disulphide bridges between heavy and light chains are reduced. By isoelectrofocalization on polyacrylamide gels (not shown) an apparent heterogeneity can be detected, as is often reported for purified Fabs (Harlow and Lane, 1988; Stura *et al.*, 1993). The two major species observed have isoelectric points of 5.6 and 6.1 and those of the four minor forms are comprised between 5.4 and 6.0.

Titration of antigen-binding sites monitored by the quenching of Fab' tryptophan fluorescence upon binding of p11 labelled with dinitrophenol, indicates that all the Fab' molecules are active and that one mole of p11 is bound per mole of Fab'. Equilibrium dissociation constant measurements were performed in the same buffer and pH conditions used for NMR. That the experimental data can be fitted to a single straight line in a Scatchard plot (fig. 2) shows that all the antibody molecules recognize p11 with the same affinity. The equilibrium dissociation constant obtained from this straight line for the Fab'-p11 complex ($K_D = 1.1 \pm 0.2 * 10^{-8}$ M) is close to that of the whole mAb 164-2 ($K_D = 4.4 \pm 0.9 * 10^{-8}$ M). These results indicate that the Fab' preparation is homogeneous and that the binding site has not been altered by proteolysis or downstream purification steps.

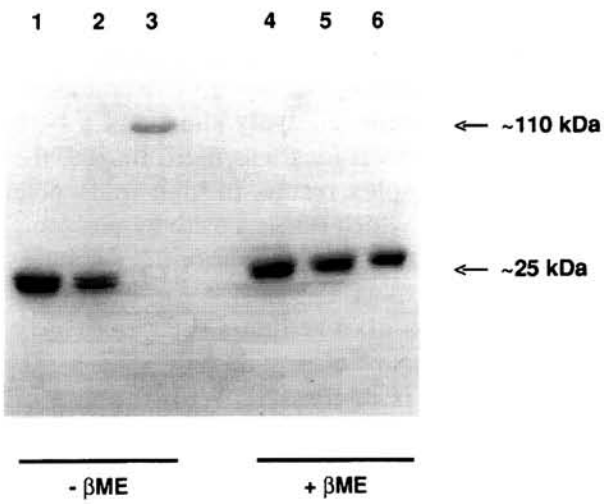

Fig. 1. SDS-PAGE of the purified Fab' and $F(ab')_2$.

Electrophoresis was performed on a "Phast Gel" 8-25% (Pharmacia). The samples contained Fab' at 0.8 and 0.4 mg/ml without βSH (lanes 1 and 2) and with βSH (lanes 4 and 5) or $F(ab')_2$ at 0.4 mg/ml without βSH (lane 3) and with βSH (lane 6).

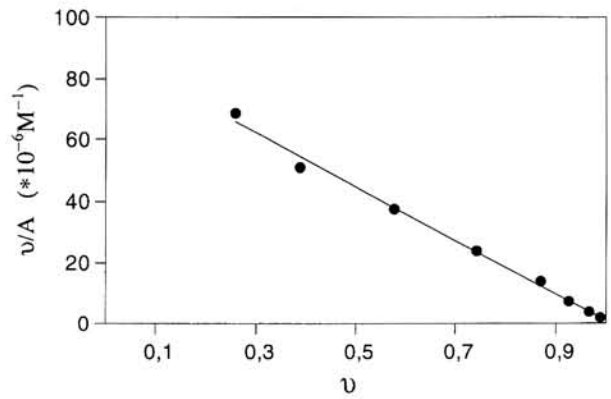

Fig. 2. Scatchard representation of the binding in solution of p11 to Fab' 164-2.

Binding was measured by the ELISA competition method in CH_3COONa 50 mM pH 6 at 22°C. υ represents the fraction of bound Fab' and A the concentration of free antigen.

Fab'/p11 interaction studied by isotope-edited experiments

Isotope-editing techniques such as HSQC and HMQC performed on samples with ^{15}N-labelled peptide selectively detect the correlations of the peptide's amide protons attached to the ^{15}N atoms. The results of the ^{1}H-^{15}N isotope-edited experiments carried out on the peptide and on the Fab'-p11 complex are summarized in figure 3. The spectrum of the peptide p11^{8N} labelled at 8 positions contains 7 signals (fig. 3a). The signal corresponding to the amide group of ^{2}G is absent because at pH 6 and 40°C, the amide proton exchanges very rapidly with the solvent due to the electronic effects of the N-terminal amino group. In the spectrum of a mixture of p11^{4N} (^{15}N-labelled on ^{2}G, ^{5}G, ^{9}G and ^{10}M) with the Fab' fragments (fig. 3b), there are three signals at the frequencies corresponding to the amide groups of ^{5}G, ^{9}G and ^{10}M of the free peptide. As the peptide is in excess relative to the Fab', these signals can be assigned to the amide groups of the free peptide. The signals of the free peptide coexist with at least four other signals arising from the bound peptide. That different signals are observed for the free and bound peptide indicates that free and bound forms of the peptide are in slow chemical exchange in the chemical shift "time" scale. In the spectrum of the complex of the peptide labelled at 8 positions (p11^{8N}) and the Fab' (fig. 3c), the signals of the bound peptide observed for the complex of p11^{4N} (fig. 2b) are also present. The former spectrum was obtained on a sample in which there was no excess of peptide; therefore, all the signals belong to the bound peptide. The signal at 7.99-96.8 ppm in the spectrum of the complex p11^{8N}-Fab' (fig. 3c) is also seen in the spectrum of the complex of p11^{4N} (fig. 3b) and corresponds to the chemical shifts of the free peptide ^{10}M signal. Thus, in the spectrum of p11^{4N}-Fab', there are two signals superimposed: one belongs to the amide group of the residue ^{10}M of the free peptide and the other is due to the bound peptide. Consequently, the bound p11^{4N} generates 5 signals, even though, at most, four signals (and perhaps only three if the amide proton of ^{2}G were not protected against exchange in the complex) from the bound peptide are expected. Likewise, p11^{8N} associated with the Fab' gives rise to 14 reproducible signals when, at most, 8 signals would be expected. As peptides p11^{4N} and p11^{8N} are pure to >96% and Fab' preparations seem to be homogeneous, we can conclude from these observations that p11 adopts two different conformations when bound to the Fab'. Quantification of the different conformers was not possible due to the low signal-to-noise ratio of the spectra (even with 1,536 accumulations per FID) and because the exchange rate of the different amide protons which may influence signal intensity is not known. Even so, the population of both conformers appears to be rather similar.

Several isotope-edited experiments were performed to obtain scalar and dipolar correlations of the peptide bound to the Fab' in order to assign the resonances to the peptide nuclei and obtain structural information. Indeed, HMQC-TOCSY and HSQC-TOCSY experiments (Norwood *et al.*, 1990, and references cited therein) were performed with 1,024 and 2,016 accumulations per FID and mixing times of 40 and 50 ms; HSQC-NOESY spectra (Norwood *et al.*, 1990) were acquired with 3584 scans per FID and mixing times of 40 and 70 ms, with or without using the jump-return sequence (Plateau and Guéron, 1982) to suppress the water signal. Unfortunately, in the case of the p11-Fab' complexes, no scalar or dipolar correlation was observed even though mixing times were relatively short and a high number of accumulations was used. Indeed, the size of the complex results in high transverse relaxation rates which cause sensitivity problems and impede detecting any scalar or dipolar interaction between spins. The existence of two different complexes also reduces the sensitivity because the amount of peptide producing a particular signal is decreased. With the aim of diminishing the transverse relaxation rate by increasing the rate of molecular tumbling, a sample of the complex was incubated at a higher temperature (50°C) and studied by NMR. However, a massive precipitate formed within a few hours, hindering any further study at this temperature.

Though complete assignment is not possible, the signals labelled with the symbol "#" in

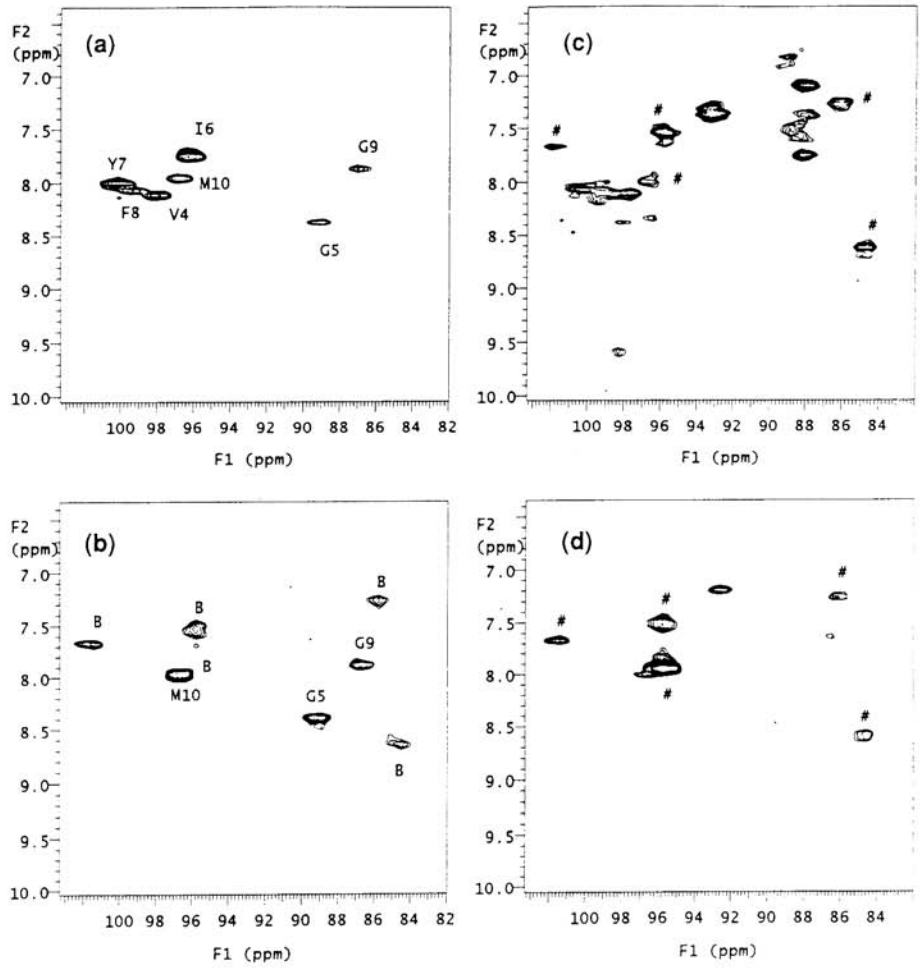

Fig. 3. ^{1}H-^{15}N isotope-edited spectra of peptide 11 and its immune complexes.

The spectra were recorded at 40°C in buffer C at pH 6. The F2 dimension corresponds to proton chemical shifts, while F1 corresponds to nitrogen chemical shifts. Spectra were transformed with a line-broadening constant of 20 Hz in the proton dimension and with a sine bell function shifted by $\pi/2$ in the nitrogen dimension. Base line was corrected for all the spectra.

a) HSQC spectrum of $p11^{8N}$ (2 mM). b) HMQC spectrum of the complex of $p11^{4N}$ (0.75 mM) and Fab' 164-2 (0.6 mM). Peptide is in excess. Assignments for the free $p11^{4N}$ signals are indicated; signals arising from the bound peptide are labelled with the letter B. c) HSQC spectrum of the complex between $p11^{8N}$ (0.7 mM) and Fab' (0.8 mM). d) HSQC spectrum of the complex between $p11^{4N}$ (0.8 mM) and the Fab (1 mM). Fabs used to record this spectrum were obtained by proteolysis of mAb 164-2 with papain. #=signals of the bound peptide that are also present in the spectrum shown in b. The jump-return pulse sequence (Plateau and Guéron, 1982) was used to diminish the water signal intensity in a and b.

figure 3 (c and d) can be ascribed to the amide groups of ^{2}G, ^{5}G, ^{9}G and ^{10}M in the bound forms, because these signals are also present in the spectrum of the complex formed with the peptide labelled at those four positions. Moreover, three signals of the free and bound peptide seem to resonate at the same proton and nitrogen chemical shifts: these signals might be imputed to the amide groups of ^{4}V, ^{7}Y and ^{10}M. However, the coincidence in chemical shifts of

these signals for the bound and free peptide might be fortuitous.

During proteolysis with pepsin, antibodies are incubated with the enzyme at acidic pH. In order to determine whether the acid milieu might cause a modification of the binding region that could explain the formation of two distinct complexes, Fab fragments were prepared by proteolysis of the mAb 164-2 with papain at neutral pH. Papain is not efficient at proteolysing murine antibodies of subclass 1 (Harlow and Lane, 1988), and papainolysis of mAb 164-2 (IgG1) generated Fab fragments presenting different sizes on SDS-PAGE. These Fab fragments bind p11 with the same affinity as the Fab' obtained by pepsin cleavage. The HMQC spectrum of the complex of $p11^{4N}$ with the Fab obtained by proteolysis at neutral pH (fig. 3d) shows 5 signals in common with the spectrum of the complex $p11^{4N}$-Fab'. This indicates that the peptide associated with Fabs that have not been incubated at an acid pH also adopts two conformations, and that the peptide binds the Fab and the Fab' in similar conformations. Nevertheless, an additional peak at 7.20-92.6 ppm in the spectrum of the Fab-associated peptide suggests that the chemical environment of the peptide is different when bound to the fragments obtained after pepsinolysis or after papainolysis.

DISCUSSION

Isotope-edited experiments performed on the complex p11-Fab' 164-2 show that the peptide associated with the Fab' adopts two different conformations. However, the Fab' fragments bind the peptide in solution with a single affinity constant and all the Fab' molecules are active and bind one molecule of p11, as indicated by competition ELISA and site-titration experiments. In addition, Fab' preparations seem pure and homogeneous, as observed by SDS-PAGE. Moreover, previous work has suggested a single-step mechanism for the binding of p11 and mAb 164-2 (Larvor *et al.*, 1991). Two possibilities could account for the observation of two conformers of the peptide complexed to the Fab': (i) the Fab' preparations could be *de facto* heterogeneous, or (ii) the peptide could show two conformational isomers when bound to homogeneous Fab's.

(i) Non-homogeneous Fab' fragments

Cells 164-2 secreting the antibodies were very carefully subcloned three times, and the hypothesis that these might be contaminated with other cells producing an antibody capable of binding the peptide with a very similar affinity must be discarded. It is possible that clone 164-2 synthesizes hybrid antibodies. Indeed, such a phenomenon has already been described (Chaffotte *et al.*, 1985) for antibody D_4-B_6, a mAb that recognizes the β_2 subunit of *E. coli* tryptophan synthase: this antibody contains two different heavy chains with different isotypes (γ_1 and γ_{2b}) (Djavadi-Ohaniance *et al.*, 1984), as well as two types of light chains. Only antibodies carrying a γ_{2b} heavy chain and one type of the light chains can bind the β_2 subunit. Such a clone secreting heterogeneous antibodies might derive from the fusion of two lymphocytes with a myeloma cell (Chaffotte *et al.*, 1985). In the case of mAb D_4-B_6, the differences in isotype, affinity and specificity of the different combinations of heavy and light chains enabled the detection of heterogeneous antibodies. However, if a similar phenomenon had occurred with the clone 164-2, its detection would be very difficult because mAb 164-2 has only one isotype (γ_1, Djavadi-Ohaniance, unpublished result), and its two sites are active and present a single affinity constant with respect to p11 and to the β_2 subunit. Alternatively, the cells 164-2 may have undergone mutations that would result in microheterogeneities and thereby in a loss of the monoclonality of the cells.

Chemical or conformational modifications could take place during the Fab' preparation. For instance, incubation at acidic pH could modify some residues and/or induce an irreversible conformational change, as has been observed for other immunoglobulins incubated at acidic pH (Buchner *et al.*, 1991; Lilie and Buchner, 1995). In order to investigate this possibility, Fab fragments were prepared by cleavage with papain at

neutral pH, thus avoiding the incubation at acidic pH that is necessary for pepsin proteolysis. Two conformers of the peptide bound to the Fab were also detected by NMR. Moreover, Fab' (pepsinolysis) and Fab (papainolysis) show the same affinity for p11 as the intact antibody. These results indicate that incubation at acid pH was not responsible for the eventual modification of the antibody. An alternative source of heterogeneity in the Fab' could be a chemical modification during the reduction of the cystines with DTT or the blocking of the thiols with iodoacetamide. Indeed, it is possible to imagine that iodoacetamide slowly reacts with a relatively exposed cysteine in the paratope or close to it.

With regard to the different isoelectric points detected by electrofocalization of the Fab' 164-2 on polyacrylamide gels, it should be pointed out that this is very often observed for antibodies and Fab fragments. Microheterogeneities caused by oxidation of aspartic and glutamic residues or differences in glycosylation are normally invoked to explain this phenomenon (Harlow and Lane, 1988). Different isoelectric points have been detected even among Fab fragments that have been crystallized and whose structure has been solved by X-ray crystallography (Stura *et al.,* 1993). Thus, the Fab' 164-2 seems as homogeneous as the Fab used for structural studies by X-ray or NMR, as indicated by SDS-PAGE, their isotype and the measure of the association and dissociation rate constants and the affinity constant.

(ii) Two conformations of the peptide associated with homogeneous Fab'

The Fab' 164-2 could exist in an equilibrium between two conformational isomers, with each of these isomers binding the peptide under a distinct form or a single conformation of Fab' could interact with two different forms of the peptide. In the latter case, the two forms of the peptide would compete for two binding sites at least partially superimposed, given that one peptide is bound per molecule of Fab' in the binding experiments. If the Fab' were actually a single molecular species, our experimental results would not discriminate between these two possibilities.

A possible explanation for the formation of two different complexes could be the ionization-deionization equilibrium of histidine residues of the peptide or of the Fab'. This type of equilibrium has already been taken into account to explain the differences between the crystal structure (Shoham, 1993) and the solution structure (Scherf *et al.,* 1992) of the CTP3 peptide bound to mAb TE33, with the crystal and solution structures being determined at different pH. Also, Edmundson *et al.* (1993) have completely changed the conformation of a peptide bound to a dimer of antibody light chains through the modification of the pH.

If the Fab' 164-2 were actually homogeneous, as they seem to be, our work would show for the first time that a mAb can form two different high-affinity complexes with the same antigen. This would add to the diversity of mechanisms that have been found in antigen/antibody interactions and would be in agreement with the proposal of Foote and Milstein (1994) that antibody conformational polymorphism might be a common phenomenon. If, instead, Fab' 164-2 turned out to be heterogeneous, the work presented here would point out that one has to be very cautious before concluding that mAbs produced by a cell line are homogeneous. In fact, mAb 164-2 would be evaluated as homogeneous by all the tests that are typically used to assess the homogeneity of a mAb for thermodynamic or structural studies. Only isotope-edited experiments revealed the existence of two conformers of the bound peptide. If, for certain applications, a mAb can be heterogeneous without causing any problem, for others such as structural studies, epitope mapping, affinity and stoechiometry determinations, homogeneous and monospecific antibodies are needed.

Finally, we would like to point out that the size of the Fab fragments (~50 kDa) renders structural studies of high affinity complexes by NMR a daunting task. To the best of our knowledge, only a qualitative structure of the main chain of a peptide in a high affinity complex with a Fab has been determined by NMR in a pioneering work

by Tsang and co-workers (1992). Genetic engineering techniques leading to the smaller Fv fragments (~25 kDa) that retain the antigen-binding capability should make feasible the structural characterization by NMR of peptides tightly associated with their antigenic sites. The Fv fragment of mAb 164-2 is currently being cloned in our laboratory in order to determine the structure of p11 bound to the Fv fragments and to confirm that the two conformations of the complexed peptide described in this work indeed arise from the binding of the peptide to homogeneous Fab'. In conclusion, although our failure to detect a structural or functional heterogeneity in the Fab' preparation does not entirely rule out the possibility of a heterogeneity, the fact that these Fab' fragments were prepared and purified to the same standards as those used for structural studies by X-ray diffraction or NMR spectroscopy strongly suggests that the preparation was indeed homogeneous, and renders particularly interesting the unexpected observation of two alternating conformations of the bound antigen.

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Un peptide fixé à un anticorps monoclonal adopte-t-il toujours une conformation unique ?

La conformation d'un peptide de 11 résidus issu de la sous-unité β_2 de la tryptophane synthase de *Escherichia coli* en interaction avec le fragment Fab' d'un anticorps monoclonal (mAb 164-2) dirigé contre la protéine native a été étudiée par spectroscopie RMN. Le peptide ^{1}(H-G-R-V-G-I-Y-F-G-M-K)^{11}, peptide 11, est reconnu par l'anticorps et ses fragments Fab' avec une très forte affinité ($K_D = 1,1 \pm 0,2 * 10^{-8}$ M). Le peptide 11 a été marqué par l'azote 15 et sa conformation dans le complexe formé avec les fragments Fab' 164-2 a été étudiée par des techniques d'édition isotopique. Les spectres hétéronucléaires ^{1}H-^{15}N montrent qu'il existe deux complexes Fab'-peptide 11 avec deux conformations différentes en échange lent sur l'échelle des temps des déplacements chimiques.

Mots-clés : Peptide, Anticorps; Interaction, Complexe de haute affinité, Résonance magnétique nucléaire ^{1}H-^{15}N, Fragment Fab', Tryptophane synthase, Sous-unité β_2.

References

Anglister, J., Jacob, C., Assulin, O., Ast, G., Pinker, R. & Arnon, R. (1988), NMR study of the complexes between a synthetic peptide derived from the β subunit of cholera toxin and three monoclonal antibodies against it. *Biochemistry*, 27, 717-724.

Arevalo, J.H., Taussig, M.J. & Wilson, I.A. (1993), Molecular basis of cross-reactivity and the limits of antibody-antigen complementarity. *Nature* (Lond.), 365, 859-863.

Bodenhausen, G. & Ruben, D.J. (1980), Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem. Phys. Lett.*, 69, 185-189.

Bradford, M.M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254.

Buchner, J., Renner, M., Lilie, H., Hinz, H.J., Jaenicke, R., Kiefhabel, T. & Rudolph, R. (1991), Alternatively folded states of an immunoglobulin. *Biochemistry*, 30, 6922-6929.

Bundle, D.R., Baumann, H., Brisoon, J.-R., Gagné, S.M., Zdanov, A. & Cygler, M. (1994), Solution structure of a trisaccharide-antibody complex: comparison of NMR measurements with a crystal structure. *Biochemistry*, 33, 5183-5192.

Chaffotte, A.F., Djavadi-Ohaniance, L. & Goldberg, M.E. (1985), Does a monospecific hybridoma always secrete homogeneous immunoglobulins? *Biochimie*, 67, 75-82.

Delepierre, M., Larvor, M.-P., Baleux, F. & Goldberg, M.E. (1991), ^{1}H NMR conformational analysis of a high-affinity antigenic 11-residue peptide from the tryptophan synthase β_2 subunit. *Eur. J. Biochem.*, 201, 681-693.

Djavadi-Ohaniance, L., Friguet, B. & Goldberg, M.E. (1984), Structural and functional influence of enzyme-antibody interactions: effects of eight different monoclonal antibodies on the enzymatic activity of *Escherichia coli* tryptophan synthase. *Biochemistry*, 23, 97-104.

Edmundson, A.B., Harris, D.L., Fan, Z.-C., Guddat, L.W., Schley, B.T., Hanson, B.L., Tribbick, G. & Geysen, H.M. (1993), Principles and pitfalls in designing site-directed peptide ligands. *Proteins Struct. Funct. Genet.*, 16, 246-267.

Foote, J. & Milstein, C. (1994), Conformational isomerism

and the diversity of antibodies. *Proc. Nat. Acad. Sci.* USA, 91, 10370-10374.

Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. & Goldberg, M.E. (1985), Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Meth.*, 77, 305-319.

Friguet, B., Djavadi-Ohaniance, L. & Goldberg, M.E. (1989), Polypeptide-antibody binding mechanism: conformational adaptation investigated by equilibrium and kinetic analysis. *Res. Immunol.*, 140, 355-376.

Glaudemans, C.P.J., Lerner, L., Daves, D.G., Kovac, P., Venable, R. & Bax, A.D. (1990), Significant conformational changes in an antigenic carbohydrate epitope upon binding to a monoclonal antibody. *Biochemistry*, 29, 10906-10911.

Harlow, E. & Lane, E. (1988), *in* "Antibodies. A laboratory manual" (pp. 628-629). Cold Spring Harbor Laboratory, New York.

Hyde, C.C., Ahmed, S.E., Padlan, E.A., Miles, E.W. & Davies, D.R. (1988), Three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ multienzyme complex from *Salmonella typhimurium*. *J. Biol. Chem.*, 263, 17857-17871.

Kranz, D.M., Herron, J.N. & Voss, E.W., Jr. (1982), Mechanisms of ligand binding by monoclonal antifluorescyl antibodies. *J. Biol. Chem.*, 257, 6987-6995.

Larvor, M.-P., Djavadi-Ohaniance, L., Friguet, B., Baleux, F. & Goldberg, M.E. (1991), Peptide/antibody recognition: synthetic peptides derived from the *E. coli* tryptophan synthase β_2 subunit interact with high affinity with an anti-β_2 monoclonal antibody. *Mol. Immunol.*, 28, 523-531.

Lilie, H. & Buchner, J. (1995), Domain interactions stabilize the alternatively folded state of an antibody Fab fragment. *FEBS Lett.*, 362, 43-46.

Marion, D., Ikura, M., Tschudin, R. & Bax, A. (1989), Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J. Magn. Reson.*, 85, 393-399.

Merrifield, R.B. (1963), Solid phase peptide synthesis. — I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.*, 85, 2149-2154.

Norwood, T.J., Boyd, J., Heritage, J.E., Soffe, N. & Campbell, I.D. (1990), Comparison of techniques for ^{1}H-detected heteronuclear ^{1}H-^{15}N spectroscopy. *J. Magn. Reson.*, 87, 488-501.

Onoue, K., Yagi, Y., Grossberg, A.L. & Pressman, D. (1965), Number of binding sites of rabbit macroglobulin antibody and its subunits. *Immunochem.*, 2, 401-415.

Plateau, P. & Guéron, H. (1982), Exchangeable proton NMR without base-line distortion using new strong-pulse sequences. *J. Am. Chem. Soc.*, 104, 7310-7311.

Scherf, T., Hiller, R., Naider, R., 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 distances restraints. *Biochemistry*, 31, 6884-6897.

Seelig, G.F., Prosise, W.W., Hawkins, J.C. & Senior, M.M. (1995), Development of a receptor peptide antagonist to human γ-interferon, and characterization of its ligand-bound conformation using transferred nuclear Overhauser effect spectroscopy. *J. Biol. Chem.*, 270, 9241-9249.

Shaka, A.J., Keeler, J. & Freeman, R. (1983), Evaluation of a new broadband decoupling sequence: WALTZ-16. *J. Magn. Reson.*, 53, 313-340.

Shoham, M. (1993), Crystal structure of an anticholera toxin peptide complex at 2.3 Å. *J. Mol. Biol.*, 232, 1169-1175.

Sodano, P. & Delepierre, M. (1993), Clean and efficient suppression of the water signal in multidimensional NMR spectra. *J. Magn. Reson.*, 104, 88-92.

Stanfield, R.L. & Wilson, I.A. (1994), Antigen-induced conformational changes in antibodies: a problem for structural prediction and design. *Trends Biotechnol.*, 12, 275-279.

States, D.J., Haberkorn, R.A. & Ruben, D.J. (1982), A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson.*, 48, 286-292.

Stura, E.A., Fieser, G.G. & Wilson, I.A. (1993), Crystallization of antibodies and antibody-antigen complexes. *Immunometh.*, 3, 164-179.

Summers, M.F., Marzilli, L.G. & Bax, A. (1986), Complete ^{1}H and ^{13}C assignments of coenzyme B12 through the use of new two-dimensional NMR experiments. *J. Am. Chem. Soc.*, 108, 4285-4294.

Tsang, P., Rance, M., Fieser, T.M., Ostresh, J.M., Houghten, R.A., Lerner, R.A. & Wright, P.E. (1992), Conformation and dynamics of an Fab'-bound peptide by isotope-edited NMR spectroscopy. *Biochemistry*, 31, 3862-3871.

Tsikaris, V., Detsikas, E., Sakarellos-Daitsiotis, M., Sakarellos, C., Vatzaki, E., Demange, P., Marraud, M., Tzartos, S.J. & Cung, M.T. (1993), Conformational requirements for molecular recognition of acetylcholine receptor main immunogenic region (MIR) analogues by monoclonal anti-MIR antibody: a two-dimensional nuclear magnetic resonance and molecular dynamics approach. *Biopolymers*, 33, 1123-1134.

Webster, D.M., Henry, A.H. & Rees, A.R. (1994), Antibody-antigen interactions. *Curr. Opin. Struct. Biol.*, 4, 123-129.

Wilson, I.A. & Stanfield, R.L. (1993), Antibody-antigen interactions. *Curr. Opin. Struct. Biol.*, 3, 113-118.

Wilson, I.A. & Stanfield, R.L. (1994), Antibody-antigen interactions: new structures and new conformational changes. *Curr. Opin. Struct. Biol.*, 4, 857-867.

Wüthrich, K. (1986), *in* "NMR of proteins and nucleic acids" (pp. 130-161). John Wiley & Sons, New York.