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A multiply substituted G–H loop from foot-and-mouth disease virus in complex with a neutralizing antibody: a role for water molecules

Wendy F. Ochoa,[1] Susana G. Kalko,[1] Mauricio G. Mateu,[2] Paula Gomes,[3] David Andreu,[3] Esteban Domingo,[2] Ignasi Fita[1] and Nuria Verdaguer[1]

[1] Instituto Biología Molecular de Barcelona, Consejo Superior de Investigaciones Científicas, Jordi-Girona 18-26, 08034 Barcelona, Spain

[2] Centro de Biología Molecular 'Severo Ochoa', Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, 28049 Cantoblanco, Madrid, Spain

[3] Departament de Química Orgánica, Universitat de Barcelona, 08028 Barcelona, Spain

The crystal structure of a 15 amino acid synthetic peptide, corresponding to the sequence of the major antigenic site A (G–H loop of VP1) from a multiple variant of foot-and-mouth disease virus (FMDV), has been determined at 2·3 Å resolution. The variant peptide includes four amino acid substitutions in the loop relative to the previously studied peptide representing FMDV C-S8c1 and corresponds to the loop of a natural FMDV isolate of subtype C_1. The peptide was complexed with the Fab fragment of the neutralizing monoclonal antibody 4C4. The peptide adopts a compact fold with a nearly cyclic conformation and a disposition of the receptor-recognition motif Arg–Gly–Asp that is closely related to the previously determined structure for the viral loop, as part of the virion, and for unsubstituted synthetic peptide antigen bound to neutralizing antibodies. New structural findings include the observation that well-defined solvent molecules appear to play a major role in stabilizing the conformation of the peptide and its interactions with the antibody. Structural results are supported by molecular-dynamic simulations. The multiply substituted peptide developed compensatory mechanisms to bind the antibody with a conformation very similar to that of its unsubstituted counterpart. One water molecule, which for steric reasons could not occupy the same position in the unsubstituted antigen, establishes hydrogen bonds with three peptide amino acids. The constancy of the structure of an antigenic domain despite multiple amino acid substitutions has implications for vaccine design.

Introduction

Foot-and-mouth disease virus (FMDV) is the causative agent of the most economically important disease of cloven-hoofed animals worldwide (Pereira, 1981; Brown, 1994). The virus belongs to the genus *Aphthovirus* of the family *Picornaviridae* (Rueckert, 1996). Control of the disease has been based on large-scale vaccinations with whole-virus inactivated vaccines, limitation of animal movements and destruction of herds exposed to the virus (the 'stamping-out' procedure) (reviews in Bachrach, 1968; Brown, 1994; Domingo *et al.*, 1990; Timoney *et al.*, 1992). The available vaccines show generally good protection against infection with homologous virus and with antigenically related isolates. Difficulties facing the eradication of FMD include the antigenic diversity of FMDV in nature, which has been reflected in the identification of seven serotypes (A, O, C, SAT1, SAT2, SAT3 and Asia1), 65 subtypes, until subtyping was interrupted (Pereira, 1977), and multitudes of antigenic variants that often co-circulate in a given geographical area (Mateu *et al.*, 1988). Antigenic variation imposes a periodic updating of vaccine strains, antigenic properties of which must match those of the circulating viruses. Furthermore, some FMD outbreaks have been traced to vaccine strains of the virus (Beck & Strohmaier, 1987). For these reasons, it would be highly desirable to develop effective, synthetic FMD vaccines.

Neutralizing antibodies are important determinants of

Author for correspondence: Nuria Verdaguer.
Fax +34 93 2045904. e-mail nvmcri@cid.csic.es

0001-6861

Table 1. Amino acid sequences of site A peptide variants

Peptides A15-C_1-Brescia, A15-C-S8c1 and A15-C-S30 represent sequences of FMDV isolates belonging to subtype C_1. MAb 4C4, which was raised against C_1-Brescia, reacts with all three peptides as shown by immunochemical and structural analyses (Mateu *et al.*, 1990, 1992; Verdaguer *et al.*, 1995, 1997). Synthetic peptide A15-C-S8c1/LV presents a decrease in binding affinity for most site A-specific MAbs, including 4C4 (Mateu *et al.*, 1992). −, Less than 20% of the binding observed with the other peptides (+) in a quantitative enzyme immunodot assay described by Mateu *et al.* (1992). No virus has been reported with a site A sequence corresponding to peptide A15-C-S8c1/LV. The one-letter amino acid code is used.

Peptide	Sequence	Binding to MAb 4C4
A15-C_1-Brescia	YT**A**S**T**RGDLAH**L**T**A**T	+
A15-C-S8c1	YT**A**S**A**RGDLAH**L**T**T**T	+
A15-C-S8c1/LV	YT**A**S**A**RGDLAH**V**T**T**T	−
A15-C-S30	YT**T**S**T**RGDLAH**V**T**A**T	+

protection against FMD and other picornavirus diseases (Mateu, 1995; McCullough *et al.*, 1992; Misbah *et al.*, 1992). An understanding of the types of interactions between antibodies and viruses that lead to virus neutralization is essential for vaccine design. One of the major antigenic sites of FMDV is located in the G–H loop of capsid protein VP1 (Bittle *et al.*, 1982; Pfaff *et al.*, 1982; Strohmaier *et al.*, 1982). This loop is disordered on the surface of FMDV particles (Acharya *et al.*, 1989; Curry *et al.*, 1996; Lea *et al.*, 1994, 1995; Logan *et al.*, 1993). For FMDV of serotype C, this antigenic site has been termed site A, and it behaves as an independent unit, with very limited influence of other capsid residues regarding the interaction of site A with antibodies (Hewat *et al.*, 1997; Lea *et al.*, 1994; Mateu, 1995; Verdaguer *et al.*, 1999). The behaviour of this antigenic loop in FMDV particles in its interaction with antibodies can be mimicked faithfully with synthetic peptides that represent the relevant amino acid sequences found in authentic virus (Clarke *et al.*, 1983; Mateu *et al.*, 1989, 1990; Mateu, 1995; Rowlands *et al.*, 1983). Interestingly, antigenic site A includes a highly conserved Arg–Gly–Asp (RGD) triplet that serves as the recognition site of an integrin receptor (Berinstein *et al.*, 1995; Fox *et al.*, 1989; Hernández *et al.*, 1996; Jackson *et al.*, 1997; Mason *et al.*, 1994; Neff *et al.*, 1998).

The structure of a synthetic peptide representing antigenic site A of FMDV C-S8c1 [a biological clone of natural isolate C-Sta Pau Sp70 (Sobrino *et al.*, 1983), a virus representative of the European subtype C_1 FMDVs] in a complex with the Fab of neutralizing monoclonal antibodies (MAbs) raised against the virus has been studied by X-ray crystallography (Verdaguer *et al.*, 1995, 1996, 1998). The synthetic peptide spanned positions 136–150 of capsid protein VP1 (peptide A15, Table 1). Two complexes were studied, involving MAbs SD6 (Verdaguer *et al.*, 1995, 1996) and 4C4 (Verdaguer *et al.*, 1998). The two neutralizing MAbs were raised against two different FMDV type C isolates (Mateu *et al.*, 1990): SD6 against C-S8c1 and 4C4 against C_1-Brescia, a virus that differs from C-S8c1 at two positions (140 and 149) within site A (Table 1). In the two complexes, the peptide antigen acquired a very similar quasi-circular conformation and the RGD motif participated directly in the interaction with residues of the complementarity-determining regions (CDRs) of the antibodies (Verdaguer *et al.*, 1998). The RGD triplet appeared in an open turn conformation, very similar to that of reduced FMDV particles of serotype O (Logan *et al.*, 1993), and also similar to the conformation in RGD-containing integrin ligands (Pfaff, 1997). Remarkably, the Gly-142 and Asp-143 residues of the RGD triplet appear to be critical for interaction both with an integrin receptor and with neutralizing antibodies directed to site A (Mateu *et al.*, 1996; Verdaguer *et al.*, 1995). Both structural and biochemical evidence suggest that MAbs SD6 and 4C4 neutralize by monovalent binding to antigenic site A (Verdaguer *et al.*, 1997, 1998; review in Domingo *et al.*, 1999).

The quasi-circular shape of the peptide antigen in the complexes was stabilized by peptide–antibody interactions and also through intrapeptide hydrogen bonds and van der Waals' interactions (Verdaguer *et al.*, 1995, 1996, 1998). If a structure similar to the one found in these complexes was also maintained with variant antigens (including one or several amino acid substitutions), the structure would gain ground as the basis for the design of new anti-FMD immunogens. Several natural isolates of FMDV of serotype C have been characterized previously with regard to the amino acid sequence at antigenic site A and reactivity with MAbs (Martínez *et al.*, 1991; Mateu *et al.*, 1988, 1989, 1990; Verdaguer *et al.*, 1995, 1996, 1998; Villaverde *et al.*, 1991). One of the isolates analysed, termed C-S30 (or C_1-Barcelona Sp/81), included four replacements within antigenic site A. Two of the changes were coincident with those found in the C_1-Brescia virus (Ala-140 → Thr, Thr-149 → Ala) and two were new (Ala-138 → Thr, Leu-147 → Val) (Table 1). In spite of the presence of four amino acid substitutions in the G–H loop of VP1, the reactivity of FMDV C-S30 with MAb 4C4 was indistinguishable from that of FMDV C-S8c1 (Mateu *et al.*, 1990). Single substitutions at positions 138 and, especially, 140 and 149 did not have a significant effect on the interaction of synthetic peptide antigens with most MAbs, including 4C4, in ELISA (Verdaguer *et al.*, 1998). However, the single replacement of residue Leu-147, highly conserved among FMDVs of serotype C, by valine diminished greatly the reactivity of synthetic peptides with most MAbs, including 4C4, in enzyme immunodot assays and competitive ELISA (Mateu *et al.*, 1992; Verdaguer *et al.*, 1998). That is, the four amino acid replacements found at the antigenic site A of C-S30 relative to C-S8c1 (Mateu *et al.*, 1989, 1990) appear to exert a compensatory effect that restores the affinity of C-S30 (Mateu *et al.*, 1990) and of a synthetic antigen

representing its antigenic site A (Mateu *et al.*, 1992) for MAb 4C4. Therefore, the substituted antigen offered an opportunity to investigate the structure that a variant form of antigenic site A could acquire as a complex with antibody. The results described in this report reaffirm the quasi-circular conformation for this antigenic loop and reveal new features about the forces involved in the stability of site A and about antigen–antibody interactions, in particular, key interactions mediated by water molecules.

Methods

■ **MAb and synthetic peptides.** The origin and characterization of the anti-FMDV MAb 4C4 (Mateu *et al.*, 1987, 1989, 1990) and the preparation and purification of the Fab fragment of MAb 4C4 (Verdaguer *et al.*, 1998) have been reported previously. Peptides were synthesized by solid-phase procedures, purified and analysed by using procedures that have been described previously (Carreño *et al.*, 1992; Mateu *et al.*, 1996).

■ **Crystallization and data collection.** Crystals of the complex between the Fab fragment of the MAb 4C4 and the variant peptide A15-CS30 (Table 1) were obtained by the hanging drop-vapour diffusion technique and successive micro- and macroseedings (Stura & Wilson, 1992). Typically small, twined needles, obtained with 18% PEG 4K, were used for microseeding, which produced larger needles at 16% PEG 4K. Finally, these larger needles were used for macroseedings in 2 µl droplets containing 7 mg/ml Fab, 1·8 mg/ml peptide, 6·5% PEG 4K, 0·2 M LiCl with 50 mM Tris–HCl (pH 9), equilibrated against a reservoir containing 13% PEG 4K equally buffered at room temperature. Crystals were orthorhombic, space group $P2_12_12_1$ with unit cell parameters a = 48·2 Å, b = 69·3 Å and c = 146·5 Å, containing one molecule of the complex per asymmetric unit, which corresponds to a solvent content volume of 49%. A data set was collected, at 100 K with 20% glycerol as a cryoprotectant, by using a MarReseach imaging plate on a Rigaku rotating anode. Intensities were evaluated and scaled internally with programs Denzo and Scalepack, respectively (Otwinowski & Minor, 1996). Data were 93% complete at 2·3 Å resolution, with an internal agreement factor (R_{symm}) of 8·5% (Table 2).

■ **Structure solution and refinement.** Crystals of the complex between peptide A15-C-S8c1/LV and the Fab of MAb 4C4 seemed related to crystals formed with the same Fab and peptide A15-C-S8c1 (the names and amino acid sequence of peptide antigens are listed in Table 1), whose structure had been solved previously (Verdaguer *et al.*, 1998). However, the unit cell parameter c differed by about 10 Å and the structure was newly determined by molecular replacement by using the

Table 2. Features of crystals of the antigen–4C4 Fab complex analysed in the present study

Feature	Value	Reference value	Band width
Crystallization and data collection			
Space group	$P2_12_12_1$		
Cell parameters (Å)	48·18, 69·32, 146·54		
Resolution (Å)	20–2·3		
Overall completeness (%)	93·0		
Average I/σ	7·5		
R_{symm} (%)	8·5		
Total no. of residues:			
Fab	429		
Peptide	13		
Total no. of solvent molecules	166		
Volume of solvent (%)	49		
Diffraction agreement			
Resolution (Å)	15–2·3		
Number of reflections	17 943		
R_{free}	0·262		
R-factor	0·248		
RMSD from ideal distance:			
Bond length (Å)	0·009		
Bond angle (°)	1·48		
Average thermal factor (Å^2):			
Fab	23·6		
Peptide	23·2		
Stereochemistry of main chain:			
Omega angle SD (°)	1·2	6·0	3·0
Bad contacts per 100 residues	0·8	7·6	10·0
Zeta angle SD1·3	3·1	1·6	
Stereochemistry of side chain:			
Chi-1 pooled SD	13·3	20·3	5·0

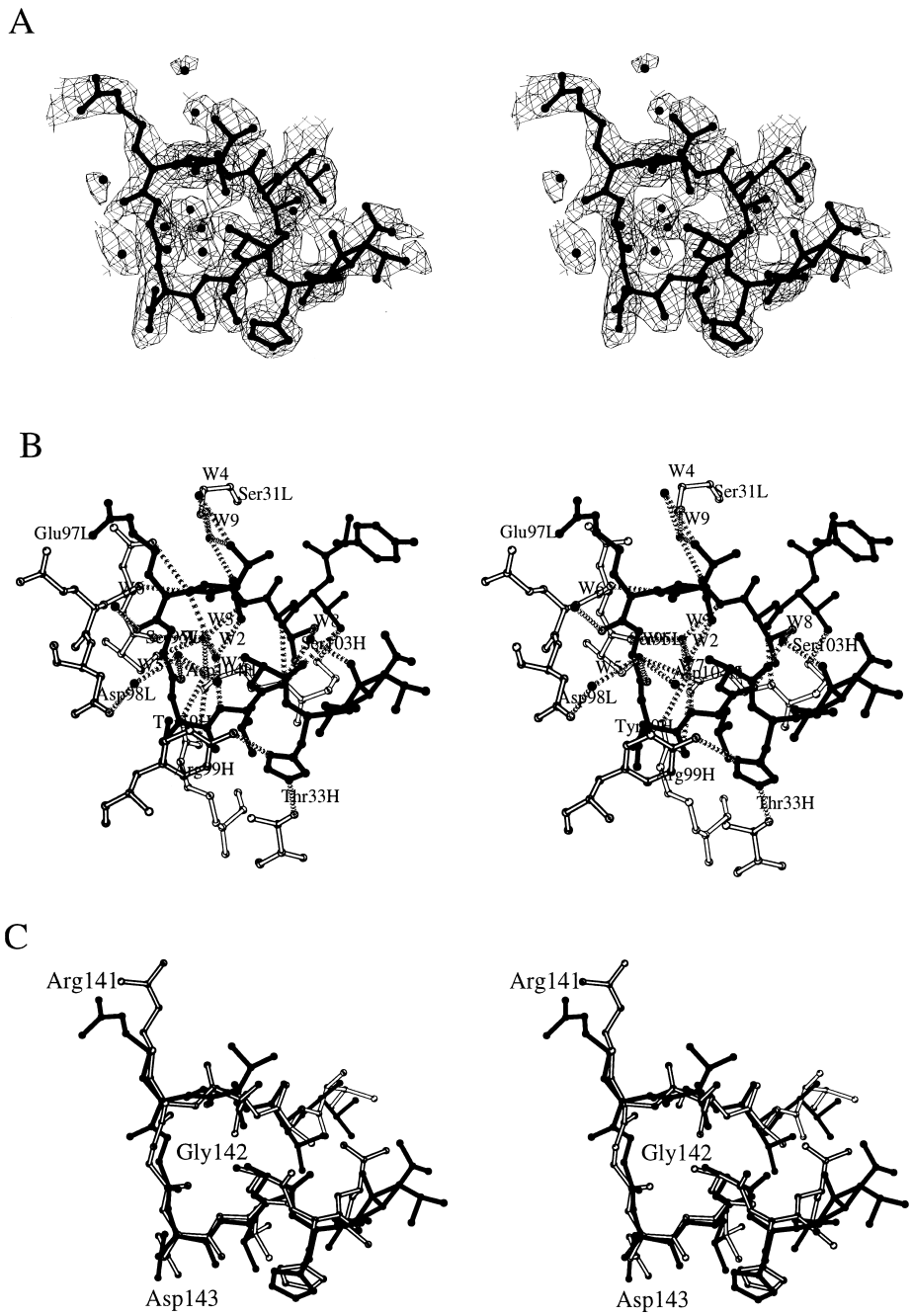

Fig. 1. (A) Stereo view of the (2Fo-Fc) electron density map corresponding to the antigenic peptide A15-C-S30. The peptide model and nearby solvent molecules (isolated dots) are also shown. (B) Stereo view of the Fab–peptide interactions. Only the Fab residues in direct contact with the peptide are depicted (open rods). Water molecules (W) in direct contact with the peptide or mediating the antibody recognition are also shown. Hydrogen bonds are indicated by broken lines. (C) Superposition of the A15 peptide conformations found in the 4C4 Fab complexes with the A15-C-S30 (filled) and the A15-C-S8c1 (open) peptides.

AMoRe package with the 4C4 Fab coordinates as searching model (Navaza, 1994). The initial solution was then optimized by allowing to move as four separated rigid bodies the variable heavy, variable light, constant heavy and constant light domains. The resulting R-factor was 0·36% in the resolution range of 15 to 4 Å. Examination of the electron density maps, calculated at this stage, clearly showed extra density corresponding to the oligopeptide occupying the antigen-binding site. The final model for the structure of the complex was obtained by iterative cycles of model rebuilding by using the program O (Jones *et al.*, 1991) and positional refinement with XPLOR (Brünger, 1992), including bulk

Table 3. Hydrogen bonds between the A15-CS30 and A15-CS8 peptides and the 4C4 Fab

The participants in the various hydrogen bonds are shown. Crystallographic data are described in Table 2 and the three-dimensional structures of the two complexes are shown in Fig. 2. The CDR loops are given to which the 4C4 Fab atoms listed belong.

Peptide atom	Water molecule	4C4 Fab atom	CDR loop	Distance (Å)
A15-CS30				
Thr-137 O	–	Asp-104 N	H3	3·2
Thr-137 Oγ1	–	Ser-103 Oγ	H3	3·1
Thr-138 Oγ1	w8	–	–	2·7
Ser-139 N	w3	Ser-95 O	L3	3·0/2·7
Ser-139 N	w3	Asp-104 Oδ	H3	3·0/2·8
Ser-139 Oγ	–	Asn-96 Oδ1	L3	2·7
Ser-139 O	w2	–	–	2·7
Thr-140 N	w9	Ser-31 Oγ	L1	2·7/2·6
Thr-140 Oγ1	w9	–	–	3·2
Thr-140 Oγ1	w4	–	–	3·3
Arg-141 O	–	Asp-98 N	L3	2·9
Arg-141 O	w6	–	–	3·0
Arg-141 NH1	–	Glu-97 Oε1	L3	2·7
Gly-142 N	w1/w5	Asp-98 Oδ1	L3	2·9/3·1/2·8
Gly-142 N	w2	–	–	3·4
Gly-142 O	w1	–	–	2·9
Asp-143 N	w2	–	–	3·3
Asp-143 Oδ2	–	Arg-99 Nη2	H3	3·0
Asp-143 Oδ1	–	Arg-99 Nε	H3	2·8
Asp-143 Oδ1	–	Thr-50 Oγ1	H2	2·9
Leu-144 N	w2	–	–	2·8
Leu-144 O	w8	–	–	2·9
His-146 N	–	Tyr-59 Oη	H2	3·4
His-146 Nδ1	–	Tyr-59 Oη	H2	3·1
His-146 Nε2	–	Thr-33 Oγ1	H1	2·8
Val-147 O	w8	–	–	2·5
–	w7/w1	–	–	3·0
A15-CS8c1				
Thr-137 N	–	Arg-54 Nη2	L2	2·6
Thr-137 O	–	Asp-104 N	H3	3·2
Thr-137 Oδ1	–	Asp-34 Nδ2	L1	2·9
Ser-139 Oδ	–	Asn-96 Oδ1	L3	3·4
Asp-143 O	–	Tyr-59 Oη	H2	3·4
Asp-143 Oδ1	–	Arg-9 Nη2	H3	2·7
Asp-143 Oδ2	–	Arg-99 Nη1	H3	2·8
Asp-143 Oδ2	–	Thr-50 Oγ1	H2	3·0
His-146 Nδ1	–	Tyr-59 Oη	H2	2·6
His-146 Nδ2	–	Thr-33 Oδ	H1	3·2
His-146 Nδ2	–	Ser-52 Oδ	H2	2·8

solvent correction. The refined model converged to crystallographic agreement factors R and R_{free} of 24% and 26%, respectively, for 17943 reflections in the resolution shell 15·0–2·3 Å (Table 2).

■ **Molecular dynamics (MD) simulation.** Three sets of MD simulations, corresponding to the variable module (Fv) of the 4C4 Fab interacting with peptides A15-C-S8c1, A15-C-S8c1/LV and A15-C-S30 (Table 1), were performed by using the GROMOS96 package with its standard protein and water force fields (van Gunsteren *et al.*, 1996). Crystallographic coordinates of peptides A15-C-S8c1 and A15-C-S30 (residues 137–148) were used as starting models in the corresponding simulations. Thr-148, not visible in the electron density maps of the A15-C-S8c1 complex, was added to avoid end effects in the critical Leu-147 residue, by using the information available in the A15-C-S30 structure. Starting coordinates for the A15-C-S8c1/LV peptide were then derived, with the graphic program TURBO (Rousel & Cambillau, 1989), by

replacing Leu-147 with valine. Crystallographic water molecules determined in the present work for the complex of 4C4 Fab with the A15-C-S30 peptide were not included in the simulations.

In every simulation, the structure of the corresponding complex was placed initially at the centre of a truncated octahedron, the dimensions of which were chosen such that the minimum distance of any protein atom from the closest wall was 7 Å. The edge lengths of the corresponding cubic boxes were about 73 Å. Systems were treated as immersed into an equilibrium configuration of bulk simple point charge (SPC) water (Berendsen *et al.*, 1986). Water molecules outside the box or with a distance to a solute atom of less than 2·3 Å were removed. The numbers of water molecules considered in the three simulations were 5271, 5272 and 5264, respectively. To relax strong water–water and water–protein non-bonded interactions, steepest-descent energy minimization was performed until stabilization was reached. After that, counter-ions were added to neutralize charged protein sites, with a subsequent energy minimization. Simulations were performed at constant volume and temperature (300 K) with periodic boundary conditions and an integration step of 2 fs. Temperature was kept constant by weak coupling to an external bath (Berendsen *et al.*, 1984, 1986). Bond lengths were constrained to equilibrium values by using the SHAKE algorithm (Rickaert *et al.*, 1977). Equilibrations were achieved within 50 ps and, afterwards, the three systems were simulated for a total time of 300 ps with the total potential energy remaining essentially constant. Only amino acids from the peptides and all water molecules were allowed to move during calculations. Analysis were performed, mainly with programs contained in the GROMOS96 package, using the structures generated every 0·1 ps during the interval spanning from equilibration until the end (from about 50 to 350 ps).

■ **Coordinates.** Coordinates have been deposited in the Brookhaven protein database under accession number 1EJO.

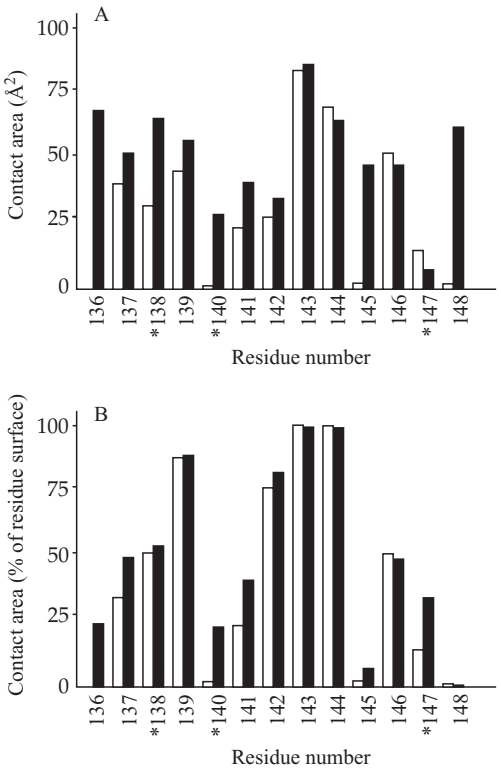

Fig. 2. Contact areas for the A15 peptides in the 4C4 Fab complexes with the A15-C-S30 (filled bars) and the A15-C-S8c1 (open bars) peptides expressed as absolute values (A) or as percentages of the residue surface (B). Asterisks indicate A15-C-S30 residues that differ in A15-C-S8c1. Peptide sequences are shown in Table 1.

Results

Structure of the 4C4 Fab in complex with the A15-CS30 peptide

The final electron density maps of the complex between the 4C4 Fab and the A15-C-S30 peptide, at 2·3 Å resolution, allowed the positioning of the main and side chains of 431 of the total of 436 4C4 Fab residues (216 from the light chain and 220 from the heavy chain) and of 12 of the 15 peptide residues (Fig. 1A). Heavy-chain Fab residues 136–143, in the constant domain, and terminal peptide residues Ala-149, Thr-150 and the side chain of Tyr-136 were disordered, and their coordinates have not been introduced in the present model. The quality of the final maps is also reflected in the 166 well-defined solvent molecules found, nine of which participate in the interactions between the peptide and the Fab CDRs (Table 3), and three water molecules establish bridges among peptide residues (Fig. 1B).

The A15-C-S30 peptide shows an overall conformation closely related to the one found in other site A complexes determined previously (Verdaguer *et al.*, 1995, 1998) (Fig. 1C). The RGD motif, residues 141–143, is located in an open turn conformation preceded by an extended region, residues 136–140, and followed by a short helix, positions 144–148 (Fig. 1). All of the peptide residues incorporated into the model interact directly with the hypervariable regions of the Fab (Table 3 and Fig. 2). In particular, the entire molecular surfaces of Asp-143, from the RGD motif, and Leu-144 are in contact with the antibody, similar to what had been observed in complexes involving Fab A15-C-S8c1 (Fig. 2) (Verdaguer *et al.*, 1995, 1998). However, the substituted residues Thr-138 and, particularly, Thr-140 and Val-147 do not present extensive interactions with the antibody (Fig. 2). The fourth substitution, Ala-149, not visible in the electron density and not included in the present model, must also remain exposed to the solvent and far from the antibody.

Comparisons of the site A variant peptides A15-CS30 and A15-CS8c1

The root-mean-square deviation (RMSD) between the main-chain peptide atoms in the A15-C-S30 and A15-C-S8c1 Fab 4C4 complexes is only 0·4 Å for the common residues 137–147. The Arg-141 side chain has different dispositions in the two peptides (Fig. 1C), although the electron density corresponding to this residue was weak in both complexes (Fig. 1A; see also Verdaguer *et al.*, 1995, 1998). The replaced residues, Thr-138 and Val-147, show the largest main-chain

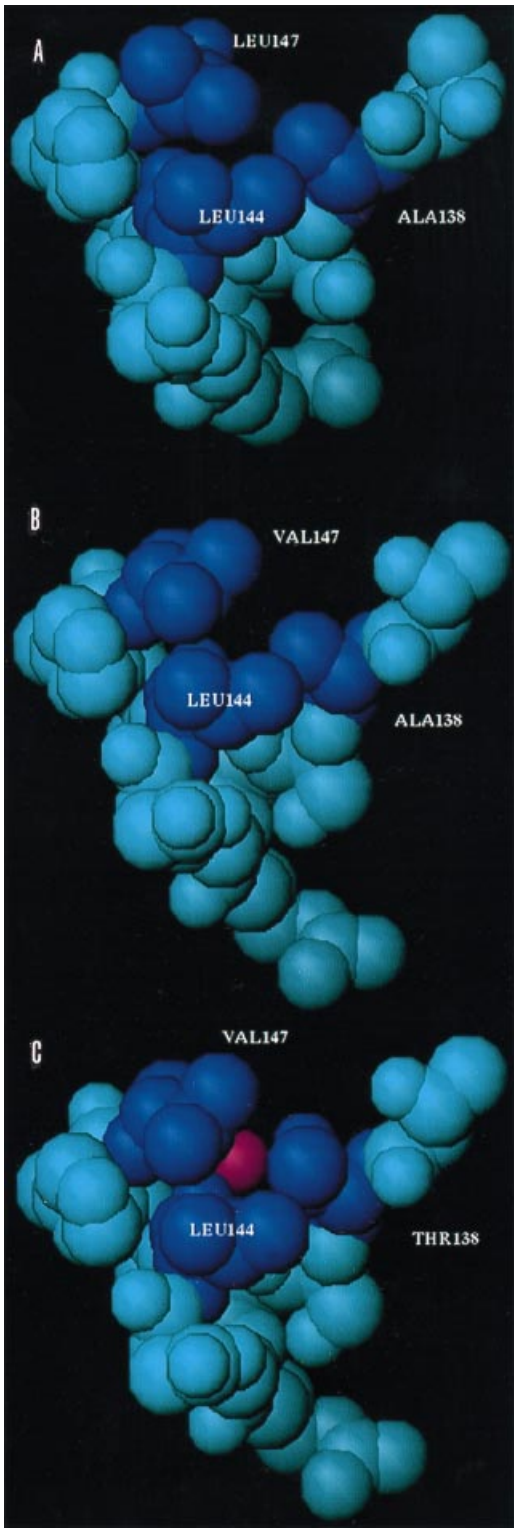

Fig. 3. Space-filling representation of the three peptide models A15-C-S8c1 (A), A15-C-S8/LV (B) and A15-C-S30 (C). Peptide residues that contribute to intrapeptide hydrophobic interactions are highlighted in dark-blue. The water molecule found in contact with the substituted Thr-138 in the A15-C-S30 peptide is displayed in red (C).

deviations of 0·7 and 1·0 Å, respectively. Despite the high structural similarity between the two peptides A15-C-S30 and A15-C-S8c1, differences in the main-chain conformational angles are important, particularly around the RGD motif (Fig. 2). Thus, in the A15-C-S30 peptide, residues Thr-140 and Arg-141 are situated in the Ramachandran region corresponding to α-helices while, in the A15-C-S8c1 peptide, Ala-140 and Arg-141 are in the regions of β-strands and left-handed helices, respectively. A third situation is found in the structure reported for the G–H loop of the reduced FMDV serotype O_1 (Lea *et al.*, 1993, 1995), where the corresponding residues Leu-144 and Arg-145 are both found in the Ramachandran region corresponding to β-strands. The flexibility of Gly-142 allows compensatory main-chain torsional angles that result in the overall structural similarity of the three peptides. It is important to notice the presence, in the A15-CS30 structure, of one water molecule (named w8 in Fig. 1) that is hydrogen-bonded with the side chain of Thr-138 and the main-chain oxygen atoms of residues Leu-144 and Leu-147. This water molecule cannot be present, for steric reasons, in the A15-C-S8c1 structure, where the bulkier Leu-147 side chain fills the available space (Fig. 3).

MD analysis

Three MD simulations corresponding to complexes of the Fv 4C4 with peptides A15-C-S8c1, A15-C-S8c1/LV and A15-C-S30 were carried out as described in Methods. The main difference between the averaged conformations of the three peptides is the opening of the C-terminal region in the simulation of the single-substituted A15-C-S8c1/LV peptide (Fig. 4A). Other significant differences can also be seen at the amino termini and for the Arg-141 side chain. The stability of the averaged conformations was analysed by evaluating the RMSD values of the peptide fluctuations in every simulation. The lowest values correspond to the A15-C-S30 peptide, while the more constant main-chain region includes the RGD motif and Leu-144 in the three peptides, with Asp-143 having the smallest variation. The side chains of Asp-143 and Leu-144 also appear extremely invariant, probably reflecting the extent and the stability of the interactions of these residues with the antibody. The stability of the tetra-substituted peptide A15-C-S30 observed during the simulation is contributed by a hydrogen-bonded water molecule located in a site very close to the position of the bridging crystallographic water (w8) (Figs 1B and 4B). This water site presents a high occupation and a low RMSD of about 0·1 Å. Hydrogen-bond interactions take place between the water molecule that occupies this site and polar atoms from the substituted residues Thr-138, Leu-144 and Val-147 (Table 3). The increased hydrophobic surface exposed to the solvent appears to explain the departure from the 'correct' site A-like conformation observed by MD with the A15-CS8/LV peptide (Fig. 3).

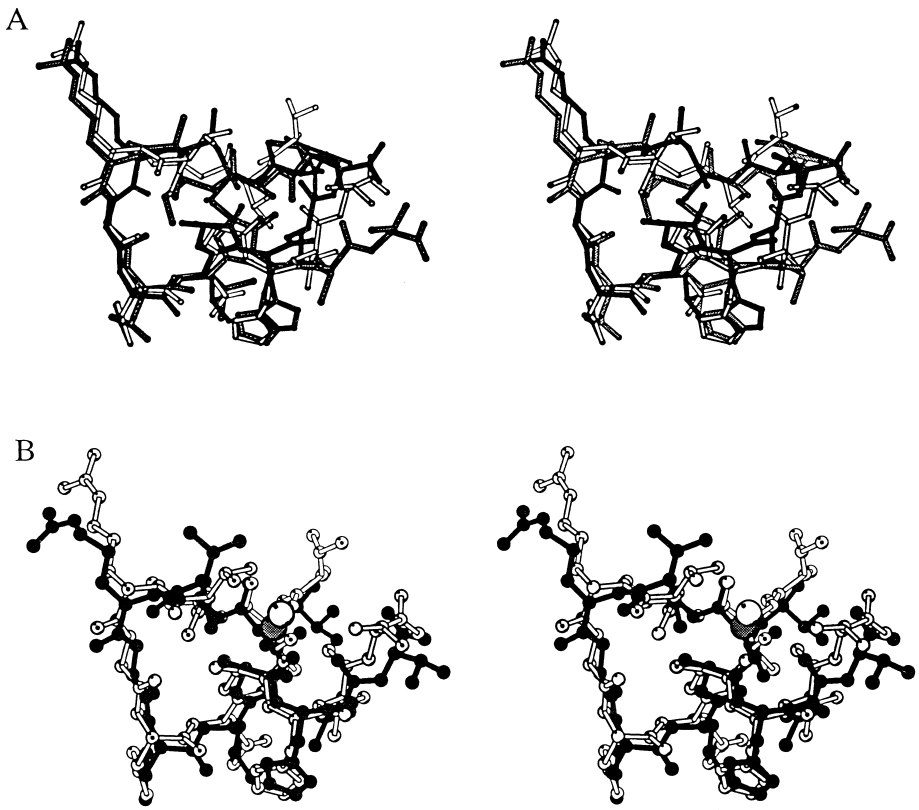

Fig. 4. (A) Stereo view of the superposition of the A15-C-S8c1 (filled), A15-C-S8/LV (shaded) and A15-C-S30 (open) peptides corresponding to the averaged conformations calculated in 300 ps of MD simulations of the corresponding Fv–peptide complexes. (B) Stereo view of the superposition of the A15-C-S30 coordinates from the crystal (filled) and the MD simulation (open). The water molecule found in the crystal structure (shaded) is situated close to the water site determined in the MD simulation (open).

Discussion

The effects of single or multiple amino acid substitutions at antigenic site A of FMDV of serotype C on the reactivity of site A-specific neutralizing MAbs have been studied extensively by immunochemical assays employing variant viruses and substituted synthetic peptides (Mateu *et al.*, 1989, 1990, 1992; Martínez *et al.*, 1997; Novella *et al.*, 1993; Verdaguer *et al.*, 1998). Replacements at position 147 altered the binding of most antibodies substantially, including 4C4, even though residue 147 had only marginal interactions with that antibody. In particular, substitution of Leu-147 by valine diminished the binding of most site A-specific MAbs analysed (Mateu *et al.*, 1992). However, the effect of this single substitution Leu-147 → Val was apparently compensated for, at least in part, by the replacement Ala-138 → Thr (Mateu *et al.*, 1992). These observations and the fact that natural FMDV isolate C-S30 (Martínez *et al.*, 1991; Villaverde *et al.*, 1991) contains these critical replacements in its VP1 G–H loop relative to clone C-S8c1 (Table 1) encouraged the present study.

In the structure of the 4C4 Fab complexed with the variant peptide A15-C-S30 reported here, the two amino acids Thr-138 and Val-147 that were replaced in the peptide show only minor interactions with the Fab (Table 2 and Fig. 2). Differences in binding affinities for peptides with substitutions in those two residues should therefore be due mainly to free-energy differences when adopting a 'correct' site A-like loop structure, the one recognized by antibodies or by the receptor (Fig. 5). The reduced stability of that conformation in the peptide with the single substitution Leu-147 → Val would then explain the reduced affinity observed for the antibody. Instead, the double-substituted peptide could recover binding affinity by re-stabilizing the 'correct' conformation with the additional water molecule (w8) that bridges the chain hydroxyl group of the replaced Thr-138 with the main-chain oxygen atoms of residues Leu-144 and Leu-147 (Table 3 and Fig. 3C). In the MD simulation of the A15-C-S30 variant peptide, a water molecule with a low RMSD is placed in almost exactly the same location as the one found in the crystal structure (Fig. 4B). These structural results provide a rationale for how a general change in specificity to a large diversity of antibodies can be achieved by a destabilization of the original antigen structure. In those cases, further substitutions can restore the affinity for the antibodies by increasing the stability of the original antigen conformation, even when none of the residues substituted

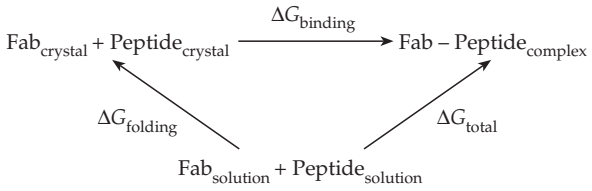

Fig. 5. A simplified thermodynamic cycle of a Fab interacting with an antigenic peptide can be described as a two-step process. In the first step, the peptide adopts individually the structure seen in the complex that is formed in the second step, so that $\Delta G_{total} = \Delta G_{folding} + \Delta G_{binding}$, where $\Delta G_{folding}$ refers to the free-energy difference between the peptide structure in solution and that found in the complex (the Fab reorganization, if required, can also be included in this term) and $\Delta G_{binding}$ refers to the free-energy difference due to the formation of the interactions between the peptide and the Fab. A 'lock-and-key' recognition mechanism, in which structures remain unchanged on complex formation, corresponds to $\Delta G_{folding} = 0$. Instead, $\Delta G_{folding} < 0$ describes an 'induced-fit' mechanism, where the complex would only be stabilized by a favourable $\Delta G_{binding}$ contribution. Analysis of Fab 4C4 complexes with a number of A15 peptides, both from the available crystal structures and with MD simulations, suggest that $\Delta G_{binding}$(A15-C-S30) $\approx \Delta G_{binding}$(A15-C-S8/LV), while the experimental data on the relative stabilities of these complexes (Mateu *et al.*, 1992) imply that ΔG_{total}(A15-C-S30) $< \Delta G_{total}$(A15-C-S8/LV), which would allow us to conclude that $\Delta G_{folding}$(A15-C-S30) $< \Delta G_{folding}$(A15-C-S8/LV). The reduced stability of the 'correct' site A-like conformation for the A15-C-S8/LV peptide would explain the lack of affinity of most site A MAbs for this variant peptide (see text).

participates in direct antigen–antibody interactions (Freire, 1999).

Recognition of continuous epitopes, like the VP1 G–H loop of FMDV studied in this work, depends not only on the amino acid sequence but also on the spatial conformation of the epitope, where solvent molecules often play a critical role. Interpretation of mechanisms of virus escape from antibody recognition, essential in RNA virus evolution and vaccine design, must also take into consideration the effects of amino acid replacements in connection with the solvent rearrangements imposed. In spite of difficulties in predicting the effects of amino acid replacements in antigen–antibody recognition, the present results for FMDV encourage the design of quasi-cyclic structures reproducing antigenic site A. Data for the G–H loop from a number of different antibodies, including the multiply substituted peptide reported here, suggest a robust and strongly immunogenic structure despite the mobility of this antigenic site on the virion surface (Parry *et al.*, 1990; Logan *et al.*, 1993). Cocktails of quasi-circular peptides representing the most frequent escape mutants found at site A should be candidates for incorporation into vaccine formulations. Such formulations should include additional B-cell and T-cell epitopes to ensure a broad immune response to minimize the selection of escape mutants and vaccine failures (Domingo & Holland, 1992; Taboga *et al.*, 1997).

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