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Molecular analysis of peptides from the GH loop of foot-and-mouth disease virus C-S30 using surface plasmon resonance: a role for kinetic rate constants

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Abstract

A foot-and-mouth disease virus (FMDV) field variant, isolate C-S30 (also named C_1-Barcelona), is known to contain four changes within the main antigenic site A (GH loop of capsid protein VP1, residues 136–150), at least one of which (Leu147 → Val) involves a highly conserved position, critical for antibody recognition in the reference strain C-S8c1. However, immunoenzymatic analysis of FMDV C-S30 showed it was recognised by 4C4, a monoclonal antibody that specifically targets site A. This remarkable behaviour has led us to analyse the individual and combined contributions of the four mutations to the antigenicity of C-S30, by surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) studies of pentadecapeptides displaying all possible combinations of the four replacements. Analysis of this family of C-S30-derived analogues shows a certain level of antibody recognition by SPR. In addition, SPR data suggest that kinetic rate constants provide an indirect measure, on the one hand, of paratope accessibility (association rate constant) and, on the other hand, of peptide fitness to the same paratope (dissociation rate constant).

Keywords: Foot-and-mouth disease virus; Surface plasmon resonance; Synthetic peptide antigens; Field variant isolates; BIAcore

1. Introduction

Foot-and-mouth disease virus (FMDV), an aphthovirus from the *Picornaviridae* family, causes the economically most important disease of farm animals. The very high antigenic and genetic variability of the virus is a major obstacle to the eradication of the disease. Conventional inactivated virus-based vaccines protect only against homologous viruses and have been related to several disease outbreaks in the field (Pereira, 1981; Mateu et al., 1988; Gebauer et al., 1988; Kitching et al., 1988; Domingo et al., 1990).

Most anti-FMDV neutralizing antibodies are directed towards the GH loop of capsid protein VP1,

Abbreviations: AA, amino acid residue; AAA, amino acid analysis; AM, 2-[4-aminomethyl-(2,4-dimethoxyphenyl)]phenoxyacetic acid (linker); C_A, analyte concentration; DMF, *N*,*N*′-dimethylformamide; EDC, *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide; EID, enzyme-linked immunodot; EITB, enzyme-linked immunoelectrotransfer blot. ELISA, enzyme-linked immunosorbent assay; ESMS, electrospray mass spectrometry; Et_3SiH, triethylsilane; FMDV, foot-and-mouth disease virus; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibition concentration; k_a, association rate constant ($M^{-1}\ s^{-1}$); K_A, thermodynamic association constant (M^{-1}); k_d, dissociation rate constant (s^{-1}); K_D, thermodynamic dissociation constant (M); KLH, keyhole limpet hemocyanin; k_s, apparent rate constant (s^{-1}); MALDI-TOF MS, matrix-assisted laser desorption time-of-flight mass spectrometry; MBHA, *p*-methylbenzhydrylamine resin; MBS, *m*-maleimidobenzoyl-*N*-hydroxisuccinimide ester; MeCN, acetonitrile; MPLC, medium pressure liquid chromatography; NHS, *N*-hydroxysuccinimide; mAb, neutralising monoclonal antibody; NMM, *N*-methylmorpholine; PBS, phosphate buffer saline; R_{eq}, equilibrium response; RI, bulk refractive index; R_{immob}, immobilisation response; R_{max}, maximum response; RU, resonance units; SPPS, solid phase peptide synthesis; SPR, surface plasmon resonance; ^{t}Bu, *tert*-butyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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Table 1
Synthetic peptides reproducing mutations in FMDV C-S30

NAME	SEQUENCE[a]	MUTANTS
A15[b]	YTASARGDLAHLTTT	reference
A15(138T)	--T----------	
A15(140T)	----T----------	one
A15(147V)	----------V---	point
A15(149A)	----------A-	
A15(138T,140T)	--T-T----------	
A15(138T,147V)	--T--------V---	
A15(138T,149A)	--T----------A-	two
A15(140T,147V)	----T------V---	point
A15Brescia[c]	----T--------A-	
A15(147V,149A)	----------V-A-	
A15(138T,140T,147V)	--T-T------V---	
A15(138T,140T,149A)	--T-T--------A-	three
A15(138T,147V,149A)	--T--------V-A-	point
A15(140T,147V,149A)	----T------V-A-	
A15S30[d]	--T-T------V-A-	four point
A15Scr[e]	RAGTATTLADLHYST	control

[a] all sequences were C-terminal carboxamides.
[b] corresponds to site A of FMDV C-S8c1 isolate;
[c] corresponds to site A of FMDV C_1-Brescia isolate;
[d] corresponds to site A of FMDV C-S30;
[e] peptide used as a negative control.

corresponding approximately to residues 136–150. This immunodominant site, termed site A, consists of several overlapping continuous epitopes which have been faithfully reproduced by synthetic peptides. Antigenic site A includes highly conserved residues such as the integrin-binding Arg–Gly–Asp (RGD) motif or the Leu residues at positions 144 and 147, all of them critically involved in cell and antibody recognition events (Strohmaier et al., 1982; Cheung et al., 1983; Fox et al., 1989; Mateu et al., 1989, 1990; Martínez et al., 1991; Mateu et al., 1996; Verdaguer et al., 1998).

Site A of FMDV C-S8c1 is mimicked by peptide A15 (Table 1) and the interactions of this peptide with neutralizing mAbs such as SD6 [raised against C-S8c1 (Mateu et al., 1988)] and 4C4 [raised against C_1-Brescia (Mateu et al., 1988)] have been fully characterized by means of ELISA, SPR and X-ray crystallographic studies (Verdaguer et al., 1998; Gomes et al., 2000a,b). Interactions of A15 with each of these mAbs share many common features. Indeed, in the crystal state, the peptide adopts a quasi-cyclic conformation when complexed with the Fab fragment of either mAb, with substantial induced-fit in both molecules and the RGD triplet playing a key role in the interaction (Verdaguer et al., 1998).

On previous genomic studies of FMDV field isolates, a natural variant, C-S30, was characterized as containing four mutations in the GH loop (Ala138→Thr, Ala140→Thr, Leu147→Val and Thr149→Ala) relative to the reference strain C-S8c1 (Mateu et al., 1987, 1988). This viral isolate was neutralized by the site A-directed mAb 4C4 (Mateu, personal communication) and its capsid protein VP1 was recognized by the same mAb in EITB assays (Mateu et al., 1990). This behavior was confirmed on peptide-KLH conjugates reproducing the four mutations of site A (Mateu et al., 1992). Parallel studies had established that at least one of the mutations (Leu147→Val) was clearly detrimental for recognition by 4C4 (Carreño et al., 1992). At the time, these findings (one mutation abolishing antigenicity, three additional ones apparently restoring it) were interpreted in the sense that multiple replacements on the GH loop of C-S30 were not strictly additive (Mateu et al., 1992; Carreño et al., 1992).

Additional evidence on the antigenicity of C-S30 has come from recent crystallographic studies showing that

a site A pentadecapeptide with the four mutations is capable of forming a complex with 4C4 with a similar array of interactions than the reference peptide A15 (Ochoa et al., 2000).

To further clarify these issues, we have decided to quantitate the effects of single and combined mutations at the four positions of interest within site A. We have analyzed sixteen peptides with the relevant substitutions (Table 1) and studied their interactions with site A-specific mAbs SD6, 4C4 and 3E5 by ELISA and SPR.

Results are totally self-consistent, showing a modest level of mAb recognition for C-S30 peptides. Additionally, SPR data obtained with this family seems to show that, while the dissociation rate constant gives a measure of peptide antigenicity, the association rate constant appears to be related to the particular features of the antibody paratope.

2. Experimental

2.1. Materials and methods

2.1.1. Peptide synthesis and purification

Peptides (Table 1) were synthesized by SPPS in an ABIMED AMS 422 multiple peptide synthesizer using Fmoc/^{t}Bu chemistry on an AM-MBHA resin (0.51 mmol/g). The synthesis scale was 0.025 mmol and couplings were done with four equivalents (eq) of Fmoc-AA-OH, 4 eq of TBTU and 8 eq of NMM. A 20% piperidine solution in DMF was used for the deprotection cycles. Peptides were fully deprotected and cleaved from the resins by treatment with 1 ml of a 95:2.5:2.5 (v/v) TFA/Et_3SiH/H_2O cocktail for 2 h at room temperature. The crude peptides were precipitated from the TFA solution by treatment with cold *tert*-butyl methyl ether, lyophilized and purified by MPLC, employing a gradient of 5→25% MeCN in water with 0.05% TFA. Purified peptides were satisfactorily characterised by AAA, HPLC and ESMS or MALDI-TOF MS.

2.1.2. Peptide and mAb solutions for SPR analysis

Peptide stock solutions, ~2.5 mM in 0.1 M acetic acid, were prepared and quantitated by AAA. Solutions for BIAcore analysis were obtained by 1000-fold and subsequent serial dilutions in HBS. Stock solutions of mAbs SD6 and 4C4 in PBS with 0.02% sodium azide, pH 7.3 (supplied by Drs Núria Verdaguer and Wendy Ochoa), were quantitated by the Pierce BCA Assay. mAb 3E5 was purified from ascitic fluid (supplied by Dr Emiliana Brocchi) using a HiTrap Protein A affinity column (Pharmacia Biotech) and quantitated by UV–Vis spectroscopy [1 OD_{280} unit = 0.75 mg/ml]. The solutions used for mAb immobilization (in 10 mM acetate buffer, pH 5.0) had a 5 μg/ml final concentration.

2.1.3. Surface plasmon resonance analysis

BIAcore 1000 instrument, sensor chips CM5, HBS buffer (10 mM Hepes with 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20 at pH 7.4), amine coupling kit (NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide) and ethanolamine were all from Biosensor AB. Immobilization of mAbs was performed according to the manufacturer's instructions: a 5 μl/min HBS continuous flow was maintained and the carboxymethyl surface was activated by a 7-min injection of a solution containing 0.2 M EDC and 0.05 M NHS; biospecific surfaces were obtained by injecting 35 μl of the mAb solutions; unreacted activated groups were blocked by a 6-min injection of ethanolamine and remaining non-covalently bound molecules were washed off with a 3-min pulse of 100 mM HCl for SD6 or 10 mM NaOH for 4C4 and 3E5 surfaces. Final surface densities were typically around 1500 RU.

All kinetic SPR analyses were run at a 60 μl/min HBS flow and each peptide was injected at six different concentrations, ranging from ~80 to 2500 nM for SD6, ~40 to 1250 nM for 4C4 and ~20 to 625 nM for 3E5 surfaces. Sensorgrams were generated by injections of peptide solutions with 90 s association steps followed by 240 s dissociation in running buffer (kinjection mode). A 90 s pulse of 100 mM HCl or 10 mM NaOH (SD6 and 4C4/3E5 surfaces, respectively) was applied to regenerate the surfaces at the end of each cycle and washing steps were added to avoid carry-over. The scrambled pentadecapeptide A15scr was injected under the same conditions as a control for non-specific binding and instrumental drift effects.

Biosensor data were prepared, modeled and fitted by means of BIAevaluation 3.0.2 software, where calculations are carried out by numerical integration (Morton et al., 1995) and global curve fitting is done by non-linear least-squares analysis (O'Shannessy et al., 1993) applied simultaneously to the entire data set. The quality of the fitted data was evaluated by visual comparison between calculated and experimental curves as well as by the magnitudes of the Chi squared parameter and of the standard errors associated to the experimental constants.

Some SPR experiments were also run in reverse order, immobilizing peptide and using mAb as the soluble reactant. These assays were carried out merely from a qualitative and comparative point of view and were not fully optimized. Peptide immobilization was identical to the one described above for mAbs: 25 μl of peptide solutions (~200 μg/ml in 10 mM acetate buffer, pH 5.0) were injected over the activated surfaces and final immobilization responses (R_{immob}) were ~170 RU. mAb solutions (in HBS buffer) ranged from 25 to 800 nM (SD6) and from 16 to 500 nM (4C4 and 3E5) and sensorgrams were run, modeled and fitted as above.

Table 2
Kinetic data for the interactions between mAb SD6 and site A peptides, representing all combinations of the four mutations found in FMDV C-S30 (138T, 140T, 147V, 149A)

PEPTIDE	$k_a/M^{-1}s^{-1}$	k_d/s^{-1}	K_A/M^{-1}	ELISA[a]
A15	7.3×10^{4}	1.4×10^{-3}	5.4×10^{7}	
A15(138T)	1.0×10^{5}	1.5×10^{-2}	6.5×10^{6}	
A15(140T)	1.4×10^{5}	3.0×10^{-3}	$4.7\times10^{7-}$	
A15(147V)	1.1×10^{5}	1.0×10^{-2}	1.0×10^{7}	
A15(149A)	1.2×10^{5}	2.2×10^{-3}	5.5×10^{7}	
A15(138T,140T)	1.6×10^{5}	1.5×10^{-2}	1.1×10^{7}	
A15(138T,147V)	3.8×10^{4}	4.2×10^{-2}	9.1×10^{5}	
A15(138T,149A)	1.2×10^{5}	1.7×10^{-2}	7.0×10^{6}	
A15(140T,147V)	7.8×10^{4}	1.3×10^{-2}	6.1×10^{6}	
A15Brescia	9.0×10^{4}	8.0×10^{-3}	1.2×10^{7}	
A15(147V,149A)	1.0×10^{5}	1.7×10^{-2}	6.0×10^{6}	
A15(138T,140T,147V)		ni[b]		
A15(138T,140T,149A)	2.2×10^{5}	2.2×10^{-2}	9.7×10^{6}	
A15(138T,147V,149A)		ni		
A15(140T,147V,149A)	1.7×10^{5}	1.8×10^{-2}	9.1×10^{6}	
A15S30	3.8×10^{4}	8.8×10^{-2}	4.3×10^{5}	

[a] Qualitative data from ELISA competition assays are represented, with a black box corresponding to $IC_{50} > 100$, a dark gray box to $30<IC_{50}\leq100$ a light gray box to $5<IC_{50}\leq30$ and a white box to $IC_{50}\leq5$;
[b] "ni" stands for "no interaction".

2.1.4. Competition ELISA

A fast qualitative competition ELISA screening of the peptides was performed with the sole purpose of confirming the peptide ranking withdrawn from SPR studies.

Each peptide (0.1–242 pmol/well) was made to compete with an A21-KLH conjugate (5 pmol peptide/well; conjugate prepared by coupling KLH to A21-Cys using *N*-hydroxysuccinimide 3-maleimidobenzoyl ester) for the same mAb, as previously described (Mateu et al., 1995). Absorbance at 492 nm was determined on a Labsystems Multiskan RC instrument and corrected by subtraction of the mean absorbance of three negative controls (no A21-KLH conjugate, no mAb, and neither conjugate nor mAb). Average from duplicate series of readings was expressed as percentage of the maximal absorbance of positive controls (conjugate + mAb and no peptide competitor). These data were converted to IC_{50}s, defined as the concentrations giving 50% of maximal absorbance. Final antigenicities are expressed as relative IC_{50}s, normalized to the IC_{50} of the reference peptide, A15.

3. Results

3.1. Peptides

Sixteen 15-residue peptides (Table 1) were synthesized, one representing site A of FMDV C-S8c1 (A15), another representing the same site of four-point mutant strain C-S30 (A15S30) and 14 others corresponding to all possible intermediate (one-, two- and three-point) combinations of the mutations found in FMDV C-S30. A scrambled sequence pentadecapeptide, A15Scr, was also prepared as a negative control. All peptides were synthesized by Fmoc-based solid phase methods (Fields and Noble, 1990; Mateu et al., 1996) and satisfactorily identified (AAA, ESMS, MALDI-TOF MS) as the target sequences, with purities always higher than 90% (HPLC).

Table 3
Kinetic data for the interactions between mAb 4C4 and the peptides under study

PEPTIDE	$k_a/M^{-1}s^{-1}$	k_d/s^{-1}	K_A/M^{-1}	ELISA[a]
A15	3.8×10^{5}	1.9×10^{-3}	1.9×10^{8}	
A15(138T)	2.5×10^{5}	5.9×10^{-3}	4.2×10^{7}	
A15(140T)	6.0×10^{5}	2.6×10^{-3}	2.3×10^{8}	
A15(147V)	9.5×10^{4}	4.4×10^{-2}	2.2×10^{6}	
A15(149A)	6.4×10^{5}	3.1×10^{-3}	2.1×10^{8}	
A15(138T,140T)	2.6×10^{5}	8.2×10^{-3}	3.1×10^{7}	
A15(138T,147V)	2.4×10^{5}	4.2×10^{-2}	5.7×10^{6}	
A15(138T,149A)	2.5×10^{5}	3.2×10^{-3}	7.6×10^{7}	
A15(140T,147V)	2.4×10^{5}	6.5×10^{-2}	3.7×10^{6}	
A15Brescia	2.6×10^{5}	1.6×10^{-3}	1.6×10^{8}	
A15(147V,149A)	2.1×10^{5}	4.4×10^{-2}	4.7×10^{6}	
A15(138T,140T,147V)	3.4×10^{5}	1.6×10^{-1}	2.1×10^{6}	
A15(138T,140T,149A)	2.1×10^{5}	9.6×10^{-3}	2.2×10^{7}	
A15(138T,147V,149A)	3.1×10^{5}	5.2×10^{-2}	6.0×10^{6}	
A15(140T,147V,149A)	4.4×10^{5}	7.2×10^{-2}	6.1×10^{6}	
A15S30	2.2×10^{5}	1.2×10^{-1}	2.0×10^{6}	

[a] Qualitative data from ELISA competition assays are represented as before.

3.2. Competition ELISA studies

Competition between plate-bound A21 (KLH conjugate) and each peptide in Table 1 in soluble form for mAbs SD6, 4C4 and 3E5 was qualitatively measured by ELISA. Single point mutants Ala140→Thr and Thr149→Ala were shown to bind effectively to all three mAbs (Tables 2–4). Mutant Ala138→Thr was not a good competitor ($IC_{50} > 30$) for mAb SD6, but its binding behaviour towards mAbs 4C4 and 3E5 was rated as good ($IC_{50} \leq 5$) and reasonable ($5 < IC_{50} \leq 30$), respectively. Mutant Leu147→Val was a reasonable ligand to mAb SD6 but a bad competitor when mAbs 4C4 and 3E5 were employed. The remaining mutants, corresponding to all possible combinations of the four mutations, were generally modest competitors. Results were consistent with an overall additive effect, where the worse competitors usually included mutations Ala138→Thr and Leu147→Val, while the best corresponded to A15Brescia. Peptide A15S30 followed this same trend (Fig. 1).

3.3. SPR analysis

Although illustrative, data from competition ELISA did not provide a very good description of the mAb–peptide interactions, since a number of peptides did not compete with plate-bound A21 within the concentration range chosen (1–2420 nM) and thus no IC_{50}s could be calculated. Therefore, in order to achieve a fast, direct screening of a fairly large number of FMDV peptides, we immobilized the three anti-FMDV mAbs on BIAcore CM5 chips and used the peptides as soluble analytes. Whenever interactions could not be reliably measured (denoted as 'ni' on Tables 1–4), sensorgrams had square-wave-like shapes, either due to a mere refractive index jump or to extremely low-affinity. All the measurable interactions fitted to a pseudo-first-order 1:1 kinetic model (with baseline drift correction when required) and provided self-consistent results (Schuck and Minton, 1996). Real-time kinetic data on these peptide–mAb interactions (Tables 2–4) generally showed good correlation with ELISA.

Table 4
Kinetic data for the interactions between mAb 4C4 and the peptides under study

PEPTIDE	$k_a/M^{-1}s^{-1}$	k_d/s^{-1}	K_A/M^{-1}	ELISA[a]
A15	1.6×10^{5}	1.6×10^{-3}	9.4×10^{7}	
A15(138T)	1.1×10^{5}	8.5×10^{-3}	1.3×10^{7}	
A15(140T)	2.6×10^{5}	1.5×10^{-3}	1.8×10^{8}	
A15(147V)	3.3×10^{5}	5.0×10^{-2}	6.6×10^{6}	
A15(149A)	4.8×10^{5}	1.5×10^{-3}	3.2×10^{8}	
A15(138T,140T)	2.4×10^{5}	8.4×10^{-3}	2.9×10^{7}	
A15(138T,147V)	2.6×10^{5}	4.2×10^{-2}	6.3×10^{6}	
A15(138T,149A)	3.2×10^{5}	8.3×10^{-3}	3.9×10^{7}	
A15(140T,147V)	3.8×10^{5}	4.8×10^{-2}	8.0×10^{6}	
A15Brescia	4.4×10^{5}	4.2×10^{-3}	1.0×10^{8}	
A15(147V,149A)	4.8×10^{5}	3.7×10^{-2}	1.3×10^{7}	
A15(138T,140T,147V)		ni[b]		
A15(138T,140T,149A)	3.4×10^{5}	1.4×10^{-2}	2.5×10^{7}	
A15(138T,147V,149A)	3.6×10^{5}	4.1×10^{-2}	8.9×10^{6}	
A15(140T,147V,149A)	5.9×10^{5}	5.2×10^{-2}	1.1×10^{7}	
A15S30	3.2×10^{5}	7.2×10^{-2}	4.5×10^{6}	

[a] Qualitative data from ELISA competition assays are represented as before.
[b] "ni" stands for "no interaction".

For single-point mutants, similarly to what was observed with competition ELISA, SPR analysis showed substitutions Ala140→Thr and Thr149→Ala to be well tolerated by the three mAbs. This was to be expected, since both replacements are present in field isolate C_1-Brescia, previously shown to be recognized by these mAbs. Mutations Ala138→Thr and Leu147→Val affected antibody recognition to different extents: mAb SD6 was more sensitive to Ala138→Thr than to Leu147→Val, quite the opposite to mAb 4C4, which tolerated the first replacement much better than the second one. Similar effects were observed for mAb 3E5. These results are consistent with recent crystallographic studies, where it was found that Ala138 has a higher percentage of residue contact with mAb SD6 than with mAb 4C4 (Verdaguer et al., 1998).

Peptides with two or three replacements displayed antigenicities in agreement with the above pattern (Tables 2–4). While A15Brescia is the most antigenic peptide of the set, decreases in mAb recognition are seen whenever mutations Ala138→Thr and Leu147→Val are simultaneously introduced. Once again, SPR-derived affinities correlated well with data from ELISA.

Finally, the four-point mutant pentadecapeptide reproducing C-S30 site A followed the same trend already described for the partial mutants. Simultaneous insertion of the Ala138→Thr, Ala140→Thr, Leu147→Val and Thr149→Ala replacements resulted in modest antigenic responses towards the three mAbs (Tables 2–4), with important differences in the dissociation rate constants of the corresponding mAb-peptide complexes when compared to the reference peptide A15 (Fig. 2(A–B)). A biosensor assay in reverse order (immobilized peptide and mAb as soluble analyte) was performed to further confirm the antigenicity ranking of A15S30. Although less accurate than the previous configuration,[2] this assay format furnished data of good quality that clearly supported the above results. Complexes of the three mAbs with A15S30 dissociated

[2] Unlike the previously described SPR configuration, these assays were not fully optimized. Ideally, to ensure a 1:1 interaction, Fab fragments instead of whole mAbs should have been used. Analytes such as mAbs are prone to bind under diffusion-controlled kinetics and to cause steric hindrance effects. Also, differences in peptide immobilization levels can make comparisons among them unreliable.

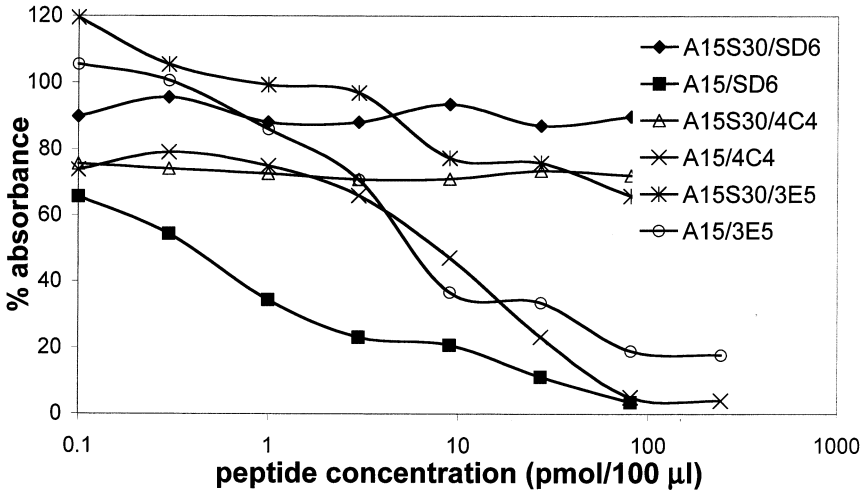

Fig. 1. Absorbance vs. concentration plots obtained in the competition ELISA analysis of peptides A15 and A15S30.

much faster than the corresponding complexes with A15 (Table 5, Fig. 2(C–D)).

4. Discussion

SPR biosensors allow fast and reliable screening of antigens and provide kinetic data on antigen–antibody interactions (Altschuh et al., 1992; Garland, 1996; Schuck, 1997). However, artifacts such as ligand site heterogeneity, ligand steric hindrance, mass-transport limitations, conformational change and non-specific binding have been identified and caution must be taken when considering the kinetic constants as absolute values (Schuck and Minton, 1996; O'Shannessy and Winzor, 1996; Hall et al., 1996). Another limitation is the size of the analyte, which should ideally be over 5 kDa for BIAcore 1000 instruments (Karlsson, 1994). When large numbers of small peptides are to be tested, the conventional assay format, involving peptide immobilization and injection of mAb as soluble analyte, has several drawbacks: (1) it involves substantial costs on mAbs, sensor chips and immobilization reagents; (2) comparison between different peptides is not reliable, since immobilization conditions are difficult to reproduce; (3) the high amounts of peptide required to obtain measurable immobilization responses cause problems such as ligand site heterogeneity and diffusion-controlled kinetics; (4) the large size of the mAb also favors steric hindrance and diffusion-controlled kinetics; (5) the process is quite time-consuming.

In previous studies, we have shown that reliable SPR screening of low molecular weight peptide analytes ($\sim$ 1.6 kDa) (Gomes et al., 2000a,b) can be achieved by the 'reverse' assay format, where soluble peptide is injected over a mAb surface, with standard deviations not higher than 9% observed for the determined kinetic constants. The use of non-specific amine coupling in the mAb immobilization step did not seem to be a source of ligand heterogeneity, and thus allowed to avoid problems typical of the oriented affinity capture immobilization (e.g. via the Fc using a rabbit-anti-mouse immunoglobulin), such as surface decay due to ligand dissociation. In the present study, the choice of mAb immobilization has again provided self-consistent results, further confirmed by an excellent agreement with data from parallel ELISA experiments. Moreover, since all peptides were always injected over the same mAb surface (for each one of the three mAbs), peptide ranking in terms of antigenic recognition is totally meaningful.

Deconvolution of the four-point mutation sequence found for site A of FMDV C-S30 into single-, double- and triple-point mutant peptides revealed a coherent pattern. We were able to confirm earlier studies in which replacements Ala138 $\rightarrow$ Thr and Leu147 $\rightarrow$ Val were clearly detrimental for peptide recognition by anti-site A mAbs (Mateu et al., 1992; Verdaguer et al., 1998; Gomes et al., 2000a,b). Peptides containing the Leu147 $\rightarrow$ Val mutation were generally weak antigens towards all three mAbs assayed and became even worse upon addition of the Ala138 $\rightarrow$ Thr mutation. The present study clearly shows that further insertion of the well-tolerated mutations Ala140 $\rightarrow$ Thr and Thr149 $\rightarrow$ Ala in the 15-mer sequences is not enough to restore antigenicity levels to those of peptides A15 (reference strain C-S8c1) or A15Brescia (C_1-Brescia strain). Possibly, the tight interactions between 4C4 and FMDV C-S30 (or peptides representing its GH loop), as seen in previous immunological and crystallographic experiments, involve an important conformational change in the loop, not measurable in the time-scale of SPR analysis. The kinetic rate constants for the A15S30-mAb interactions show that although the peptide

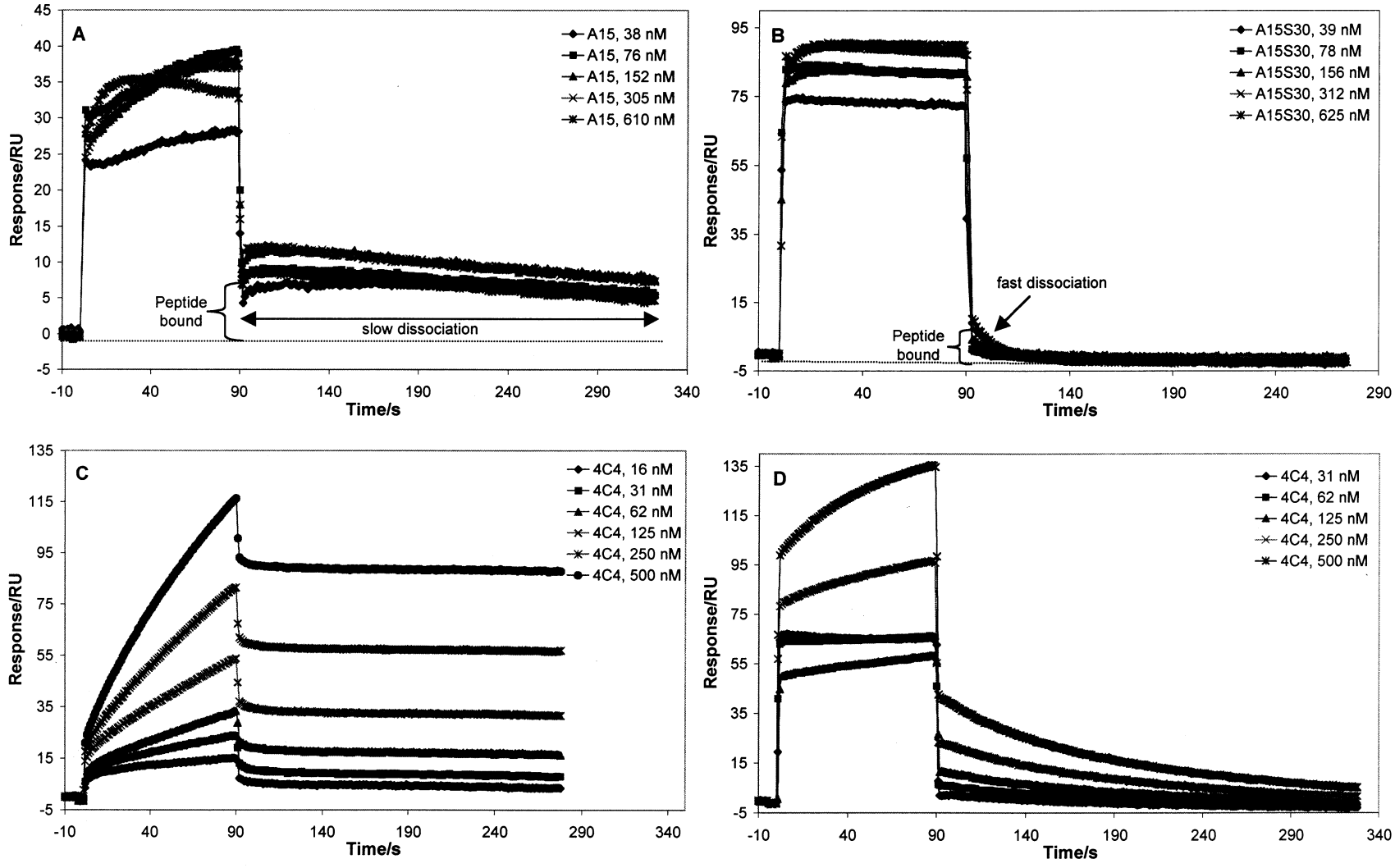

Fig. 2. Sensorgrams obtained in the analysis of the interactions between site A15 and A15S30 peptides and mAb 4C4: (A) A15 vs. immobilized mAb; (B) A15S30 vs. immobilized mAb; (C) 4C4 vs. immobilized A15; and (D) 4C4 vs. immobilized A15S30.

Table 5
Kinetic data for the interactions between mAbs SD6, 4C4, 3E5 and immobilized peptides

Mab	Peptide	$k_a/M^{-1}s^{-1}$	k_d/s^{-1}	K_A/M^{-1}
SD6	A15	1.2×10^4	2.5×10^{-4}	5.0×10^7
	A15Brescia[a]	1.8×10^4	–	
	A15S30	2.9×10^3	1.8×10^{-2}	1.6×10^5
4C4	A15	2.3×10^4	2.2×10^{-4}	1.1×10^8
	A15Brescia[a]	1.8×10^4	–	–
	A15S30	2.5×10^4	1.1×10^{-2}	2.3×10^6
3E5	A15	1.2×10^5	7.3×10^{-4}	1.7×10^8
	A15Brescia[b]	—	–	–
	A15S30	3.5×10^4	1.1×10^{-2}	3.2×10^6

[a] Under the conditions of analysis, values of k_d were too small to provide a reliable measure and values of k_a were calculated from the slope of k_s versus analyte concentration (locally fitted $k_s = k_a \times C + k_d$).

[b] k_a could not be determined due to non linearity (mass-transport effects) of k_s versus C.

promptly binds to the paratope, the complex dissociates quite fast. Structural constraints present in the virion may have, in this particular case, an important role in the recognition of the loop. Therefore, presentation of peptide A15S30 would be a key feature for peptide antigenicity. Results using conformationally restricted (cyclic) versions of A15S30 seem to confirm this hypothesis (Gomes et al., 2001). Further investigations are presently in progress.

A remarkable conclusion of the present work is that SPR-derived kinetic constants seem to have a specific significance in ligand–receptor interactions. Indeed, affinities of the different peptide analogues towards a same mAb, whether it was SD6, 4C4 or 3E5, were mainly determined by the dissociation rate constant, k_d (Figs. 3 and 4). This result had been previously reported in the literature (VanCott et al., 1994; England et al., 1997). A plot of affinity (K_D) versus dissociation rate constant (k_d) would be a straight line with slope

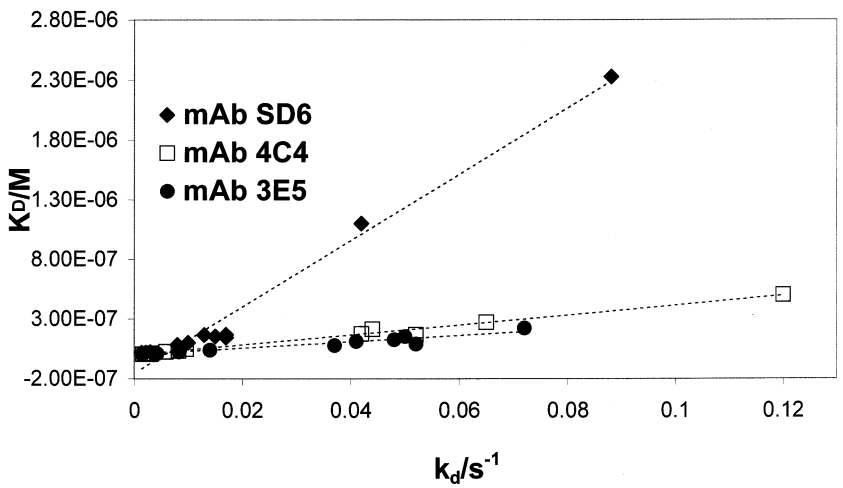

Fig. 3. Correlation between thermodynamic affinity constant K_D and rate dissociation constant k_d for the interactions between peptides containing combinations of the mutations Ala138→Thr, Ala140→Thr, Leu147→Val, T149→Ala and mAbs SD6 (slope $= 3\times10^{-5}$, $r^2 = 0.98$), 4C4 (slope $= 4\times10^{-6}$, $r^2 = 0.99$) and 3E5 (slope $= 3\times10^{-6}$, $r^2 = 0.92$).

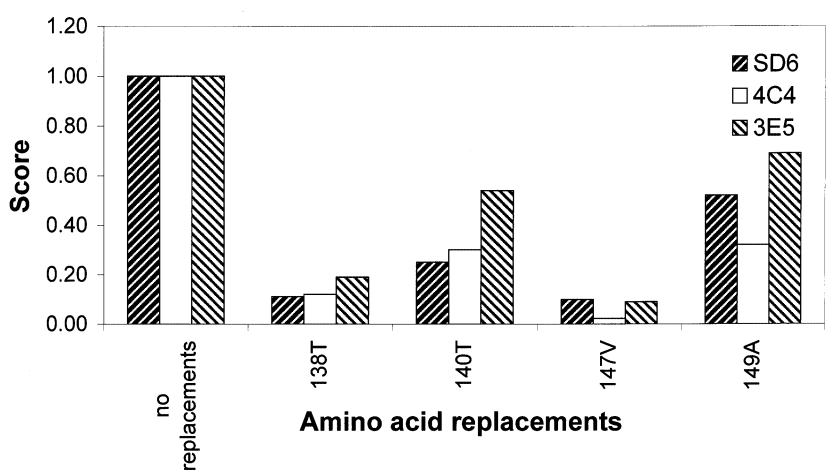

Fig. 4. Global effects of the amino acid substitutions Ala138→Thr, Ala140→Thr, Leu147→Val, T149→Ala on peptide antigenicity towards mAbs SD6, 4C4 and 3E5 (average of the relative affinities corresponding to each peptide containing each one of the four replacements).

equal to reciprocal the association rate constant, k_a. We did, in fact, observe this behaviour and, furthermore, were able to see that this slope, i.e. $1/k_a$, depended on the antibody assayed. This suggests a role for k_a, as a measure for the global affinity of a particular antibody to its specific epitopes.

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