4. Materials & Methods

4.1 General procedures

4.1.1 Solvents and chemicals

	Supplier
Amino acids and resins for SPPS	Advanced Chemtech, Propeptide, Bachem Feinchemikalien AG and Calbiochem-Novabiochem
Coupling reagents for SPPS	
DIP, HOBt	Fluka
TBTU	Propeptide/Neosystem
Solvents and other reagents for SPPS	
DCM, *Normasolv p. a.*	Scharlau
DMF, *peptide synthesis*	Scharlau/Panreac
NMP, *peptide synthesis*	Applied Biosystems
NMM, *peptide synthesis*	Merck
DIEA, *p. s.*	Merck, Acros
Piperidine, *p. a.*	Aldrich
TFA, *p. s.*	KalieChemie
Tert-butylmethylether* *>99%*	Fluka
Water	De-ionised and filtered with a MilliQ Plus system (Millipore) to a resistivity superior to 18 MΩ cm^{-1}
MeOH, MeCN, *hplc grade*	Merck, Panreac, Scharlau
Glacial AcOH, *p. a.*	Merck
Anisole, *p. s.*	Merck
1,2-ethanedithiol, *90%+*	Aldrich
Thioanisole, *>99%*	Fluka
Triethylsilane, *> 99%*	Aldrich
Hydrochloric acid 37%, *p. a.*	Merck
Phenol, *p. s.*	Aldrich
Ninhydrin, *p. a.*	Merck

*Stabilised with BTH and stored with sodium

Materials and reagents for ELISA

PBS, tablets for buffer preparation	Sigma
BSA, fraction V	Boehringer Manheim
Goat-anti-mouse IgG antibody - peroxidase conjugate, *blotting grade*	Bio Rad
orto-phenylenediamine	Sigma
Hydrogen peroxide 35%, *stabilised p. a.*	Acros
Tween 20, *Plusone*	Pharmacia Biotech
PVC 96-well plates, *highly activated*	Titertek, 77-172-05

Materials and reagents for SPR analysis

EDC (*amine coupling kit*), *BIAcertified*	
NHS (*amine coupling kit*), *BIAcertified*	
Ethanolamine hydrochloride (*amine coupling kit*), *BIAcertified*	Biosensor AB
HBS buffer, *BIAcertified*	
Sensor chip CM5, *BIAcertified*	
Oxalic acid, *p. s.*	Merck
orto-phosphoric acid, *p. s.*	Scharlau
Formic acid, *p. a.*	Merck
Malonic acid, *p. a.*	Merck
Sodium phosphate, *p. a.*	Merck
Ethanolamine, *p. a.*	Aldrich
Piperazine, *p. a.*	Aldrich
Glycine, *p. a.*	Fluka
Potassium thiocianate, *p. s.*	Merck
Magnesium chloride, *p. a.*	Merck

General reagents and materials for biochemistry

KLH, 18 mg/ml in 65% aqueous ammonium sulphate	Calbiochem
Glutaraldehyde, *p. a.*	Sigma
HCA I (E. C. 4.2.1.1)	Sigma, C-4396
Guanidine hydrochloride, *purum >98%*	Fluka
Citric acid, *p. a.*	Merck
Sodium citrate, *p. a.*	Merck
E.D.T.A. (Titriplex III), *p. a.*	Merck
Sodium chloride, *p. a.*	Merck
Tris, *p. a.*	Merck
Urea, *p. a.*	Merck
Iodoacetic acid, *p. a.*	Merck
Cysteine, *for molecular biology*	Sigma
β-mercaptoethanol, *purum >99%*	Fluka
Ammonium sulphate, *for biochemistry*	Merck
Sodium azide, *purum p. a. >99%*	Fluka
Papain (E. C. 3.4.22.2)	Sigma, P-4762
Dialysis membrane (MW cut-off=15-20 kDa), ∅=16 mm	Servapor, 4415
Centriprep-3 concentrators	Amicon

Reagents for SDS-PAGE

Acrylamide/Bis 37.5:1, *ultrapure grade*	Amresco
APS, *p. a.*	Serva
TEMED, *p. a.*	Merck
Glycine, *for molecular biology*	Sigma
SDS	Boehringer
Glycerol, *p. a.*	Sigma
Bromophenol blue	Bio Rad
Coomassie brilliant blue R-250	Bio Rad

Solvents and materials for NMR spectroscopy

Deuterium oxide, *99.95% Uvasol®*	Merck
2,2,2-Trifluoroethanol - d_3	SDS
1,4 – dioxane, *for spectroscopy Uvasol®*	Merck
NMR quartz tubes, 5 mm OD (high magnetic field)	SDS
	SDS

4.1.2 Instrumentation

*Amino acid analysis**	Beckman System 6300 - elution with sodium salts - 250 × 4 mm column containing a polysulphonate resin for cationic exchange - *post*-column detection by the ninhydrin reaction
*Mass spectrometry***	
- ES-MS	Fisons Instruments VG Quatro
- MALDI-TOF (matrices: ACH, SA)	Finnigan MAT Lasermat 2000, Bruker II Biflex
UV-Vis spectrometry	Perkin-Elmer Lambda 5
pH-meter	Crison MicropH 2002
Centrifuge	Beckman GS-15R
Lyophiliser	Virtis Freezemobile 12EL
ELISA spectrometer	Labsystem Multiskan MS
SPR instrument	BIAcore 1000, IFC4 with recovery
*NMR spectrometry****	Varian VXR500
*X-ray diffraction*****	MarResearch image plate detector (180 × 0.10 mm, 1800 pixels); Rigaku RU-200B rotating anode
SDS-PAGE	Bio Rad Mini-PROTEAN II electrophoresis cell; Bio Rad gel dryer, model 583.

* amino acid analyses were performed by Dr. C. Carreño, Dr. M. L.Valero and Ms. M. E. Méndez, at the *Servei de Sintesi de Pèptids de la Universitat de Barcelona*.
** mass spectra were acquired by Drs. I. Fernández, M. Vilaseca, M. L. Valero and E. de Oliveira, at the *Servei de Espectrometria de Masses de la Divisió de Ciències de la UB*.
*** NMR spectra were recorded by Dr. M. A. Molins at the *Servei de RMN, SCT, Universitat de Barcelona.*
**** X-ray diffraction data were acquired by Ms. W. F. Ochoa at the *European Synchrotron Facility in Grenoble.*

4.1.3 Analytical methods

4.1.3.1. Qualitative ninhydrin assay

This assay serves to detect free amine groups on polymeric supports (resins) for SPPS and is performed as described by Kaiser *et al.*[1]

4.1.3.2 Qualitative Ellman assay

This assay allows the detection of free thiol groups either in solution or on polymeric supports compatible with aqueous media, according to Ellman *et al.*[2]

4.1.3.3. Amino acid analysis

The content and proportion of amino acid residues present in a free or resin-bound peptide are determined by amino acid analysis (AAA), following a previous hydrolysis step. To hydrolyse a peptide-resin[3], 1 – 10 mg of dried resin are placed into a Pyrex glass tube and 250 μl of a 1:1 (v/v) mixture of 12 M hydrochloric and propionic acids are added. The tube is sealed and hydrolysis is carried out at 155 °C for 90 minutes. The procedure for a free peptide[4] is similar, hydrolysing with 6 M HCl for 45 minutes. The hydrolysed mixture is then evaporated to dryness and the residue dissolved in a known volume of a 0.06 M citrate buffer, pH 2.0. After filtration through a nylon filter ($\varnothing_{pore}$=0.45 μm), the sample is ready for AAA.

4.1.4 Chromatographic methods

4.1.4.1 High performance liquid chromatography

Analytical HPLC is performed in either of the following systems:

Waters – composed by a controller and a quaternary pump 600E with a low pressure mixer, an automatic injection system Waters 712, a variable wavelength UV-Vis detector 490E and a integrator/recorder either D-2000 (Merck-Hitachi) or Chromatopac C-R5A.

Shimadzu – composed by two LC-6A pumps with a high pressure mixer, an SCL-6B controller, an SIL-6B auto-injection system, a variable wavelength UV-Vis detector SPD-6A and a integrator/recorder Chromatopac C-R6A.

The HPLC column is a 250 × 4 mm Nucleosil C_{18}, with a reverse solid phase of octadecylsyloxane ($\varnothing_{beads}$=5 μm; $\varnothing_{pore}$=120 Å). The mobile phases are gradients of H_2O (0.045% v/v TFA) and MeCN (0.036% v/v TFA) at a 1 ml/min flow.

4.1.4.2 Medium pressure preparative liquid chromatography

Peptides are purified by MPLC in systems composed by LCD/Milton or Duramat ProMinent piston pumps, variable wavelength Applied Biosystems or single wavelength Uvicord 2158 SD (LKB) detectors at 220 nm, Ultrorac 2070II (LKB) or Gilson FC205 fraction collectors and Servoscribe (Phillips) or Pharmacia-LKB REC101 recorders. Glass columns (ℓ=200-300 mm, $\varnothing_{internal}$=25 mm) with Vydac C_{18} reverse phase ($\varnothing_{beads}$=15-20μm, $\varnothing_{pore}$=300 Å) are used and the mobile phase consisted on a binary linear gradient of H_2O and MeCN with 0.05% TFA at a constant flow of 120-150 ml/h.

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4.2 Solid-phase peptide synthesis

4.2.1 Solid-phase peptide synthesis protocols

4.2.1.1 Preparation of resins for peptide synthesis

The dry resin (MBHA[1] or PEG-PS[2]) is placed in a polypropylene syringe containing a polyethylene filter. The resin is allowed to swell in 40% TFA in DCM (1 × 1 min + 1 × 20 min), filtered and neutralised with 5% DIEA in DCM (3 × 1 min), then washed with DCM (5 × 30 s) and DMF (3 × 1 min).

A mixture of the chosen two-functional spacer (or *handle*) and HOBt (3 eq each) is dissolved in a minimum volume of DMF and added to the resin in the syringe. The coupling agent DIP (3 eq) is then added and coupling is allowed to proceed overnight or until a negative ninhydrin test is obtained. If the ninhydrin test remains positive after 18 hours of reaction, the resin is washed and a re-coupling step (1 eq of all reactants in similar conditions) is performed. When coupling is complete, the resin is filtered, thoroughly rinsed with DMF and dried.

4.2.1.2 Fmoc/ ^{t}Bu chemistry

4.2.1.2.1 Manual synthesis[3]

Manual syntheses are performed in polypropylene syringes with a polyethylene porous filter. The volumes of solvents and reagent solutions added should cover the entire resin to allow optimal solvating and swelling of the beads. Stirring is done with a teflon rod and, at the end of each cycle, excess reagents, by-products and solvents are eliminated by filtration and washing with DMF and DCM.

Peptide chain elongation is performed according to the following steps:

Step	*Reagent*[a]	*Treatment*	*Time/min*
1	DMF	Wash	3 × 1
2	20 % piperidine in DMF	Pre-equilibrate	1
3	20 % piperidine in DMF	Deblock (Fmoc removal)	10
4	DMF	Wash	3 × 1
5	Fmoc-AA-OH/coupling agent/DIEA[b]	Coupling	45 – 60
6	DMF	Wash	3 × 1
7	DMF	Wash	3 × 1
8	Ac_2O/DIEA 1:1 in DMF	Acetylation (block non-reacted amino groups)	15
9	DCM	Wash	3 × 1

[a] volumetric reagent/solvent proportions
[b] coupling agents used: DIP, TBTU; Base (DIEA) is required with TBTU only (2 eq DIEA/ 1 eq TBTU)

The coupling agents employed in this work were DIP and TBTU[4], the latter requiring the addition of base (DIEA) in a 2:1 molar proportion between base and reagent. The Fmoc-AA-OH were dissolved in the minimum volume of DMF and, once coupling time was over and washing steps performed (step 6), a ninhydrin assay was done. When the assay was negative, chain elongation proceeded to the incorporation of the following amino acid residue, starting with the removal of the Fmoc group (step 2 and the following). When the ninhydrin test was positive, a recoupling cycle was performed (step 5 and the following). If the addition of recoupling steps could not improve coupling efficiency, then acetylation (steps 7 – 9) could be used to block the non-reacted amino groups.

4.2.1.2.2 Machine-assisted synthesis

Peptides can also be synthesised in a MilliGen 9050 Plus PepSynthesiser, which dissolves amino acids and coupling reagents and works with a continuous flow Fmoc/^{t}Bu chemistry (instead of filtration steps after each cycle). The inlet/outlet detectors allow a constant monitoring of the synthesis at each step and it is possible to choose synthesis scale and coupling reagents.
The general protocol consists on Fmoc-AA-OH/coupling agent dissolution in DMF, followed by addition of 0.6 M DIEA in DMF. The coupling mixture is activated through a 5 min bubbling step with nitrogen and subsequent transfer of the solution to the column reactor, which contains the previously de-blocked resin. Chain elongation proceeds as follows:

Step	*Reagent*[a]	*Treatment*	*Flow /ml.min^{-1}*	*Time*
1	DMF	Wash	3	15 s
2	20 % piperidine in DMF	Pre-equilibrate	3	1 min
3	20 % piperidine in DMF	Deblock (Fmoc removal)	3	5 min
4	DMF	Wash	3	7 min
5	Activated Fmoc-AA-OH[b]	Coupling	3	5 s
6	Activated Fmoc-AA-OH	Coupling	3	60 min
7	DMF	Wash	3	4 min

[a] volumetric reagent/solvent proportions
[b] coupling agents used: TBTU

Once the synthesis is completed, the peptide-resin is transferred to a polypropylene syringe to be washed and dried as described in 4.2.1.2.1.

4.2.1.2.3 Machine-assisted parallel synthesis

Multiple peptide synthesis can be performed on an Abimed MAS 422 synthesiser, which allows the simultaneous synthesis of up to 48 peptide-resins, using Fmoc/^{t}Bu chemistry with *in situ* activation. The synthesis programmes are quite flexible in what concerns synthesis scale, number of coupling steps *per* amino acid residue and duration of each step. This synthesiser operates as follows: the Fmoc-AA-OH (0.6 M in DMF, except for Fmoc-His(Trt)-OH and Fmoc-Phe-OH, which are dissolved in NMP), the coupling reagent (0.5 M TBTU) and the base (4 M NMM), which are previously dissolved and placed in appropriate racks and sealed with septa, are added to 2 ml syringes containing previously deblocked and washed resin, and fitted to a 48-port manifold system. The addition is done in a pre-defined sequence, according to the reactivity of each amino acid residue which is added to each syringe. Chain elongation proceeds according to steps 1-6:

Step	***Reagent***[a]	***Treatment***	***Number of repeats***	***Time/min***
1	20 % piperidine in DMF	Deblock	2 × 1 ml	5[b]
2	DMF	Wash	2 × 1 ml	0.5
3	DMF	Wash	3 × 0.3 ml	0.5
4	Fmoc-AA-OH/TBTU/NMM	Coupling	1	30[c]
5	DMF	Wash	12 × 1 ml	0.5
6	DMF	Wash	2 × 0.3 ml	0.5
7[d]	DMF	Wash	2 × 1 ml	0.5
8	DMF	Wash	3 × 0.3 ml	0.5
9	20 % piperidine in DMF	Deblock	2 × 1 ml	5[b]
10	DMF	Wash	2 × 1 ml	0.5
11	DMF	Wash	3 × 0.3 ml	0.5
12	DCM	Wash	3 × 0.3 ml	0.5

[a] volumetric reagent/solvent proportions;
[b] reaction time is increased along chain elongation;
[c] 100 µl of DCM are added at 80% of the coupling total time ;
[d] steps 7-12 correspond to the final cycles for resin deblocking, washing and drying.

4.2.1.2.4 Peptide cleavage from the resin and removal of side-chain protecting groups

Up to 500 mg of dry resin (N^{α} - Fmoc previously removed) are placed in a Falcon centrifuge tube. The cleavage reagent (cocktail R^5) is prepared: 90% TFA, 2% anisole, 5% thioanisole and 3% 1,2 – ethanedithiol, and added to the resin at the proportion of 1 ml cocktail : 100 mg resin. The reaction is carried out at room temperature for 2 hours with constant shaking. Anhydrous *tert*-butylmethylether (40 ml) is then added and the mixture cooled at -78 °C for peptide precipitation. The suspension is stirred, then centrifuged at 4 °C and 4000 r. p. m. for 15 minutes, after which the supernatant is decanted. The procedure is repeated 5 times from the ether addition step. The final peptide precipitate is dried with nitrogen, resuspended in AcOH 10% and filtered through a polypropylene syringe containing a polyethylene filter. The peptide solution is then lyophilised.

4.2.2 Synthesis of peptides from the GH loop of FMDV

4.2.2.1 Peptides for SPR and ELISA

Peptide sequences and their characterisation are compiled in chapters 2 and 3.

4.2.2.1.1 Linear 15-mer peptides from the FMDV strain C_1-Barcelona (C-S30)

These peptides were prepared by machine-assisted parallel synthesis at a 25 μmol scale (section 4.2.1.2.3) on an Fmoc-AM-MBHA resin (0.51 mmol/g), where the handle AM[6] was incorporated as described in 4.2.1.1. The usual side-chain protecting groups in Fmoc/^{t}Bu synthesis were employed: Asp(O^{t}Bu), Arg(Pmc), His(Trt), Ser(^{t}Bu), Thr(^{t}Bu) and Tyr (^{t}Bu).
The peptides, which were obtained as C-terminal carboxamides, were cleaved and deprotected as described in 4.2.1.2.4, with some modifications: a polystyrene pipette tip was adapted to each resin-containing syringe and the latter was introduced in a 10 ml Sarsted polypropylene tube, previously containing the cleavage cocktail (1 ml). The cocktail was then sucked up into the syringe and, after air bubbles were carefully expelled, the reaction was carried out as previously described. Once the first 2-hour period was over, the peptide crudes were expelled from the syringes to the Sarsted tubes and additional 0.5 ml of fresh cleavage cocktail were sucked up into the syringes and reaction proceeded for further 30 minutes. The second filtrates were mixed with the first ones and processed as described in chapter 4.2.1.2.4.
Crude products were analysed by HPLC (5→95% B and 10→45% B), AAA and ES MS(+) or MALDI-TOF MS (section 4.1.4.1). Peptides with more than 15% of byproducts were purified by reverse phase MPLC (5→25% B, section 4.1.4.2).

4.2.2.1.2 Larger versions of the FMDV GH loop: peptides A21 and A21S30

The 21-residue peptides A21 and A21S30 were synthesised either by manual Fmoc/ ^{t}Bu chemistry (section 4.2.1.2.1) or by machine-assisted synthesis on a MilliGen 9050 Plus PepSynthesiser (section 4.2.1.2.2) at a 50 μmol scale. In either case, an Fmoc-AM-PEG-PS resin (0.20 mmol/g) was employed and procedures were as already described in previous chapters. Peptide cleavage from the deblocked resin was done as described in 4.2.1.2.4. Both peptides were purified by reverse phase MPLC (10→30% B) and characterised as usual.

4.2.2.1.3 Cyclic versions of the FMDV GH loop: peptides cyc16S30 and cyc16^{147}Val

The cyclic peptides, cyc16S30 and cyc16^{147}Val, were synthesised by intra-molecular disulphide bridge formation[7] of the corresponding linear bis-thiol precursors (see chapter 2). The linear bis-thiol

peptides were synthesised by similar methods as those described in 4.2.2.1.2, using Fmoc-Cys(Trt)-OH and Fmoc-*Ahx*-OH in addition to the other protected amino acids usually employed (section 4.2.2.1.1). Peptide cleavage, characterisation and purification by MPLC (10→25% B) were performed as already described, having the extra care that peptides were always kept under acidic conditions[A] to avoid intermolecular disulphide bridge cross-linking. Once the linear bis-thiol precursors were purified and lyophilised, cyclization proceeded by air oxidation at high peptide dilution and pH 8. The peptide was added stepwise to 100 mM ammonium bicarbonate buffer, pH 8, to a final concentration of 50 μM, under vigorous stirring, and left to react at open air.
The extent of cyclization was monitored by HPLC (10→45% B) and by the Ellman qualitative assay, and usually reached completion within 1 hour. The reaction was stopped upon dropwise addition of glacial acetic acid until pH 3. Cyclic peptides were characterised by HPLC, AAA and MALDI-TOF MS and repeatedly lyophilised from water to eliminate the ammonium salts.

4.2.2.1.4 Linear 15-mer peptides bearing the mutations $^{137}T\rightarrow I$, $^{138}A\rightarrow F$, $^{140}A\rightarrow P$, $^{142}G\rightarrow S$ and $^{148}T\rightarrow I$

These peptides were prepared by machine-assisted parallel synthesis at a 25 μmol scale on an Fmoc-AM-MBHA resin (0.51 mmol/g), by similar methods as those described in section 4.2.2.1.1. Peptide cleavage was performed according to the same section and, again, crude peptides having more than 15% of impurities were purified by reverse phase MPLC (15→45% B). Peptide characterisation and quantification was as previously described.

4.2.2.2 Single syntheses of peptides for structural studies

The set of FMDV peptides for NMR and X-ray diffraction studies were prepared in individual syntheses and exhaustively purified to meet the purity requirements of both techniques. Syntheses were performed at a 100 μmol scale on an Fmoc-AM-MBHA resin (0.20 mmol/g) in the MilliGen 9050 Plus PepSynthesiser by methods similar to those described. Peptide purification by MPLC was carried out as usual, regarding that at least 5 mg of 99% pure peptide should be obtained.

[A] MPLC peptide purification fractions were collected on tubes containing 100 μl of 0.1 M AcOH.

References

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4.3 Antigenic evaluation of the FMDV peptides

4.3.1 SPR analysis of peptide-antibody interactions

The technical and scientific bases for real-time surface plasmon resonance biospecific interaction analysis are exposed in sections 0.1 to 0.3 of the present work. A BIAcore SPR biosensor was used, and standard amide immobilisation chemistry on a CM5 sensor chip were employed. Both immobilisation chemistry and sensor chip features are described in section 0.2. Standard procedures, following manufacturer's instructions, were employed as far as possible. Equipments and reagents for biosensor analysis are specified in section 4.1.

4.3.1.1 Peptide and mAb solutions for biosensor analysis

Peptide stock solutions *ca.* 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) were desalted and buffer-exchanged on an NAP-5 Sephadex G-25 column (Pharmacia Biotech) and final mAb concentrations were determined by measurement of optical density at 280 nm, considering that 1 $OD_{280} \approx 0.75$ mg (protein)/ml.

4.3.1.2 Optimisation of the direct kinetic analysis of immobilised mAb – peptide interactions

SD6 solutions (100 and 50 μg/ml, in either 10 mM sodium acetate, pH 5.5, or 5 mM sodium maleate, pH 6.5) were separately injected (30 μl) at 5 μl/min over a non-activated sensor surface, to determine which gave the most efficient mAb pre-concentration into the dextran matrix.
Three SD6 surfaces were prepared using the standard amine coupling procedure as described by the manufacturer[1]: each carboxymethyl surface was activated with a 35 μl injection (at 5 μl/min) of a solution containing 0.2 M EDC and 0.05 M NHS, and SD6 was then coupled at three different densities by injecting over each surface 35 μl of 50, 5 and 3 μg/ml SD6 in 10 mM sodium acetate

buffer, pH 5.5, respectively. Non-reacted activated groups were then blocked by a 30 µl injection of ethanolamine hydrochloride and remaining non-covalently bound material was washed off in a regeneration step with a 3-min pulse of 100 mM HCl. Surface densities obtained were of 8000, 1700 and 800 RU, respectively, where 1 RU (resonance unit) corresponds to 1 ng (protein)/mm^2 (surface).

A few sets of experiments, using A15 as analyte, were run on the three SD6 surfaces at different peptide concentrations (ranging from 1 to 2500 nM) and flow rates (5 and 60 µl/min). All experiments were done with HBS as running buffer at 25 °C, using the *kinjection* mode. Sensorgrams were generated with 7-min peptide injections in the HBS flow, followed by 6-min dissociation in running buffer and then by a 2-min regeneration step with 100 mM HCl.

Biosensor data were prepared, modelled and fitted by means of the BIAEvaluation 3.0.2 software[2] (Biosensor AB, 1994-97, run on Windows '95). The quality of the fits was assessed by visual comparison between experimental and modelled sensorgrams, as well as by statistical parameters such as χ^2 and standard errors associated to the calculated constants, or by further inspection of residual distribution.

4.3.1.3 Systematic screening of FMDV peptide antigens: validation of the SPR methodology

Immobilisation of mAbs SD6 and 4C4 was performed as described in the previous section. Biospecific surfaces were obtained by injecting 35 and 16 µl of the 5 µg/ml SD6 and 4C4 solutions in 10 mM acetate buffer pH 5.5, respectively. Following the capping step with ethanolamine hydrochloride, remaining non-covalently bound molecules were washed off with a 3-min pulse of 100 mM HCl or 10 mM NaOH for SD6 or 4C4 surfaces, respectively. The final immobilisation responses were of about 1600 RU.

All kinetic SPR analyses were run at a 60 µl/min HBS flow and each peptide was analysed at 6 different concentrations, ranging from *ca.* 80 to 2500 nM for SD6 and *ca*. 40 to 1250 nM for 4C4. Sensorgrams were generated by *kinjections* of peptide solutions with 90 s association steps followed by 240 s dissociation in running buffer. A 90 s pulse of 100 mM HCl or 10 mM NaOH (SD6 and 4C4 surfaces, respectively) was applied to regenerate the surfaces at the end of each cycle and wash steps (needle, IFC, system flush) were added to avoid carry-over. The pentadecapeptide A15scr, containing the constituent amino acids of A15 in scrambled form, was injected under the same conditions as a control for non-specific binding to the sensor chip surfaces.

After subtracting the response of peptide A15scr to the responses of the relevant peptides, data were prepared, modelled and fitted by means of BIAevaluation software as already described.

4.3.1.4 Antigenic evaluation of FMDV C-S30 peptides by direct SPR kinetic analysis

Peptides from the FMDV C-S8c1 and C-S30 GH loops (syntheses described in section 4.2.2) were screened by SPR as described in section 4.3.1.3. This screening included an additional anti-FMDV monoclonal antibody, mAb 3E5. This mAb was purified from ascitic fluid as follows.

4.3.1.5 Purification of mAb 3E5 from ascitic fluid

Ascitic fluid was unfrozen and divided into 1 ml aliquots, which were then centrifuged for 5 min at 10000 r.p.m. (4 °C). The supernatants were pooled and an equivalent volume of buffer A was added (*buffer A*: 112.4 g/l glycine, 175.4.g/l NaCl, pH 8.9). This mixture was again divided into 1 ml aliquots and centrifuged. The aqueous fraction was separated from lipids and pellets.
A HiTrap protein A – Sepharose affinity column (Pharmacia Biotech), coupled to a 2132 Microperpex (LKB) peristaltic pump, was prepared by extensive rinsing, with 100 mM sodium citrate buffer, pH 3, then with buffer A, at a constant flow of 20 ml/h.
The sample was applied to the column and eluted with buffer A until $OD_{280} \leq 0.01$; elution then proceeded with 100 mM sodium citrate buffer, pH 5.0, and fractions were collected on glass tubes containing 100 µl of 100 mM Tris-HCl buffer, pH 8.5. Fractions were monitored at 280 nm and, once an absorbance peak was observed, the elution buffer was changed to 100 mM sodium citrate, pH 3, until a second smaller peak was observed. Fractions collected at pH 5 with $OD_{280} \geq 0.5$ were pooled and dialysed against PBS overnight (*PBS, phosphate buffered saline*: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3; 3 × 1 l) and final concentration was determined by optical density at 280 nm. For the present purpose, further steps for mAb concentration (upon precipitation with ammonium sulphate and subsequent dialysis against PBS and gel filtration on Sephadex G-100) were not required.

4.3.1.6 SPR interaction analysis of free FMDV C-S30 peptides with immobilised mAbs SD6, 4C4 and 3E5

Immobilisation of mAbs SD6 and 4C4 were as described in section 4.3.1.3. MAb 3E5 was immobilised similarly to the described for mAb 4C4. Kinetic analyses were run as described in section 4.3.1.3 and peptide concentrations injected over the 3E5 surface ranged from *ca*. 35 to *ca.* 625 nM. Data evaluation procedures were as already described, using the *1:1 langmuirian binding* (either with or without baseline drift) kinetic model[2].

4.3.1.7 SPR interaction analysis of immobilised FMDV C-S30 peptides with free mAbs SD6, 4C4 and 3E5

Peptides A15, A15S30, A21, A21S30, A15Brescia, cyc16S30, cyc16^{147}Val and A15scr were immobilised by methods identical to those described for mAb immobilisation. Following a surface-activation step similar to that previously described, 25 µl of the peptide solutions (200 µg/ml in 10 mM acetate buffer, pH 5.0) were injected over the surface and final immobilisation responses of *ca.* 170 RU were obtained. Surfaces were regenerated by 2-min pulses of 100 mM HCl.
mAb solutions, 25 to 800 nM in HBS, were injected as previously described for peptides and sensorgrams were run, modelled and fitted as before.

4.3.1.8 Antigenic evaluation of multiply substituted FMDV peptides by direct SPR kinetic analysis

Direct SPR kinetic analysis of peptides reproducing combinations of the substitutions ^{137}T→I, ^{138}A→F, ^{140}A→P, ^{142}G→S and ^{148}T→I could not be accomplished due to experimental problems related either to extremely slow complex dissociation or to insufficient surface regeneration (see chapter 3).

4.3.1.9 Alternative strategies for surface regeneration

A screening of alternative regeneration conditions, based on the multi-cocktail approach of Andersson and co-workers[5], was performed (see chapter 3, Tables 3.5 and 3.6).
The evaluation of the screening cocktails is performed by a 10-min analyte injection at a flow rate of 2 µl/min, followed by 30 s injections (at 20 µl/min) of a given cocktail until analyte level decreases to 30% or less of the original value. At this point, a new analyte injection is applied, followed by injections of another regeneration cocktail. After a regeneration effectiveness is attributed to each cocktail, a more extensive optimisation can be performed, relying on combinatorial mixing of the different stock cocktails.
Cocktails including stock solutions A, B, C, U or I' (see chapter 3) were screened as described, but none led to satisfactory results.

4.3.1.10 Indirect SPR kinetic analysis by competition assays using an FMDV peptide – carrier protein conjugate

The preparation of a 1:1 peptide – protein conjugate made use of the chemistry of the Npys thiol protecting group[7]. An Fmoc-peptide A15-AM-MBHA resin was prepared by solid phase peptide Fmoc/^{t}Bu chemistry (scale 50 µmol) on an Fmoc-AM-MBHA resin (0.20 mmol/g) in the MilliGen 9050 Plus PepSynthesiser. After removal of the N^{α} - Fmoc protecting group and subsequent

washing cycles, Boc-Cys(Npys)-OH was manually coupled (using 4 eq. of DIP in DCM) to the peptide-resin. Once coupling was completed and resin conveniently washed and dried, peptide cleavage and side-chain deprotection (except for the Npys group, stable to strong acids) was performed with a 90% TFA, 5% H_2O and 5% Et_3Si cleavage mixture The Cys(Npys)A15 peptide was characterised by AAA, HPLC and MALDI-TOF MS and lyophilised prior to heterodimerisation with protein HCA I (human carboxy anhydrase I, a 28 kDa monomer with a single Cys residue)[8].

The reaction of disulphide heterodimerisation between Cys(Npys)A15 and HCA I was performed under denaturating acidic conditions. HCA I (5 mg, 0.17 μmol) was dissolved in the minimum amount of 6 M guanidine hydrochloride in water (pH 4.2) and left under magnetic stirring for 30 min, until a positive Ellman test was obtained. Peptide Cys(Npys)A15 (3.13 mg, 1.7 μmol) was added to the protein solution and reaction was allowed to proceed overnight, monitored by HPLC (15→65% B) and by the qualitative Ellman assay. When reaction reached equilibrium, the mixture was diluted to 2 ml with 6 M guanidine hydrochloride and dialysed during 48 h against decreasing concentrations of guanidine hydrochloride (4 M, 1 × 1 l → 2 M, 1 × 1 l → 1 M, 1 × 1 l → water, 3 × 1 l). The dialysed solution was characterised by HPLC, MALDI-TOF MS and AAA, and the total protein content was determined by optical density at 280 nm. After adding sodium azide (0.03%), the protein solution was divided into 500 μl aliquots that were stored at –20 °C prior to SPR assays.

The protein heterodimer HCA I – CysA15 was injected on sensor chip flow cells where mAbs SD6, 4C4 and 3E5 had previously been immobilised by standard procedures (*ca.* 600 RU of each mAb were immobilised). A total protein concentration of 300 nM was injected over the three mAb surfaces and also over a fourth mock surface (EDC/NHS activation plus ethanolamine hydrochloride capping, without protein injection) for non-specific binding evaluation. Injections were performed as already described for peptide injection. Protein response was studied at three different pH values, upon protein dilution in either HBS (pH 7.3), 10 mM Tris – HCl (pH 8.5) or 10 mM sodium acetate (pH 5.5) buffers. In neither case was a specific response observed. A non-specific response was observed at pH 5.5, due to the electrostatic attraction between protonated protein – pI ≈ 6 – and the negatively charged carboxymethyl dextran matrix.

4.3.1.11 Indirect SPR kinetic analysis by competition assays using an engineered recombinant protein expressing the GH loop of FMDV C-S8c1

Protein JX249A is a recombinant β-galactosidase from *Escherichia coli*[9], with solvent exposed loops where a 24-residue peptide from the GH loop of FMDV C-S8c1 (TT^{136}YTASARGDLAHLTT^{150}THARHLP)[10] has been inserted. The protein is a 472 kDa homotetramer with one GH loop *per* monomer and is highly antigenic towards a panel of anti-GH loop antibodies.

Protein samples (305 μg/ml in *buffer Z*: 0.06 M sodium hydrogen phosphate, 0.04 M sodium di-hydrogen phosphate, 0.01 M potassium chloride and 1 mM magnesium sulphate) were diluted in HBS to a total FMDV peptide concentration of 624 nM (and subsequent serial dilutions) and tested

for SPR analysis. Protein was injected over SD6, 4C4 and 3E5 surfaces (*ca.* 600 RU of mAb immobilisation level), as previously described. Insufficient mAb surface regeneration was observed, which could not be overcome by alternative regeneration procedures based on the multi-cocktail approach described in section 4.3.1.9. The regeneration strategy that yielded better results consisted on three successive 1-min injections of 40 mM NaOH, but even so surface life-time was significantly reduced due to the inefficient removal of bound protein and consequent decreasing availability of mAb binding sites.

In spite of the surface regeneration problems observed, a set of preliminary tests for SPR surface competition analysis was performed using peptides A15 and A15scr as competitors. A constant amount of protein JX249A (total final concentration in FMDV peptide = 160 nM) was added to six peptide solutions with concentrations ranging from 0 to 300 nM. The peptide – protein mixtures were then injected as previously described for peptide injections and three 1-min pulses of 40 mM NaOH for partial surface regeneration were added at the end of each injection. Data was processed with the BIAEvaluation 3.0.2 software, using the *heterogeneous analyte* kinetic model[2] (competition between two different analytes, section 0.3).

4.3.1.12 Indirect SPR kinetic analysis by competition assays using cysteine-capped protein JX249A

Since one of the possible causes for JX249A irreversible binding to mAb surfaces could be the fact that all cysteine thiol groups in native bacterial β-galactosidases are reduced, capping of the thiol groups was performed using iodoacetic acid[11]. A denaturating solution (2 ml, 7.5 M urea, 4 mM EDTA, 0.25 Tris-HCl, pH 8.5) was added to a 170 μg/ml JX240A solution in buffer Z (2 ml), corresponding to 46 nmol of total cysteine. β-mercaptoethanol was added (2 μl, 26 μmol) to cleave any disulphide bonds in the protein and the mixture was left to stand at 60 °C for 1 h in the dark, followed by another hour at room temperature. Then, iodoacetic acid (5 μl, 39 mM in 0.1 M NaOH) was added to the mixture and reaction was allowed to proceed for further 30 min in the dark at room temperature. Reaction was quenched by excess β-mercaptoethanol (100 μl) and the mixture was then dialysed for 48 hours against decreasing concentrations of urea (5 M, 1 × 1 l → 2 M, 1 × 1 l → 1 M, 1 × 1 l → water, 3 × 1 l).

The dialysed solution was analysed by AAA to determine the degree of cysteine carboxymethylation (sample and two carboxymethylcysteine standards were submitted to the same AAA protocol) and by SDS-PAGE to check for protein integrity.

SDS-PAGE analysis[12] of the protein JX249A before and after cysteine carboxymethylation was performed on a BIO RAD Mini-PROTEAN II electrophoretic cell. An 8% acrylamide gel (7 × 8 cm) was prepared by mixing a 40% acrylamide solution (2 ml of an acrylamide/bis-acrylamide mixture, 37.5:1) with H_2O (5.5 ml), "lower" buffer (2.5 ml, 1.5 M Tris-HCl and 0.4% SDS, pH < 8.7), 15 % APS (40 μl) and TEMED (5 μl); the mixture was poured in the aligned clamp assembly, covered with a water layer and left to polimerise at room temperature for 40 min. The upper gel layer for sample loading was prepared by mixing the 40% acrylamide solution (150 μl) with H_2O (1.3 ml),

"upper" buffer (500 µl, 0.5 M Tris-HCl and 0.4% SDS, pH < 8.7), 15% APS (20 µl) and TEMED (2 µl); this mixture was poured over the lower solidified gel and left to polimerise at room temperature for 50 min (a teflon comb was used to mould the sample loading wells). Protein samples (JX249A and carboxymethylated JX249A, 200 µg/ml) were diluted in "sample" buffer (1:1 v/v dilution in 20% glycerol, 4% SDS, 0.125 M Tris-HCl, 0.04% bromophenol blue, pH 6.8) and, after adding β-mercaptoethanol (2 µl), were heated at 110 °C for 2 min. A mixture of protein molecular weight standards including carbonic anhydrase (28 kDa), ovalbumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa) was prepared by similar methods. The gel assembly was introduced in the inner cooling core and completely covered with "running" buffer (500 ml, 1.92 M glycine, 0.25 M Tris-HCl, 1 % SDS, pH<8.7). Both samples and standards were loaded (20 µl) in the corresponding wells. The gel was then run at a constant voltage of 150 V for approximately 1 hour. After cutting off the upper layer, the gel was submerged into a Coomassie blue staining bath (0.1% Coomassie blue R-250 in fixative medium: 40% MeOH/10% AcOH) and left under mechanic shaking for 30 min. Destaining of background colour was done with several changes of 40% MeOH/10% AcOH (3 changes, overnight). Colour-developed gel was dried under vacuum and heat (2 h) on a BIO RAD 583 gel dryer, using a slowly increasing temperature gradient, followed by constant heating at 80 °C and a final fast cooling step.
The carboxymethylated JX249A fraction was analysed by SPR under conditions identical to those described for the original protein. Similar results were obtained.

4.3.1.13 Indirect SPR affinity analysis by solution competition experiments

A solution competition SPR approach was employed for the determination of peptide – antibody affinities[13] (section 0.2). In this approach, similar to a competition ELISA experiment, a known constant Fab concentration is incubated with known increasing competitor antigen (peptide) concentrations. When equilibrium is reached, the peptide – antibody mixtures are put in contact with a surface covered with specific antigen (for instance, the C-S8c1 GH loop peptide A15) and the relationship between free Fab in competitor concentration provides a measure for competitor peptide – antibody affinity.

Fab fragments of both SD6 and 4C4 monoclonal antibodies were kindly supplied by Ms. Wendy F. Ochoa and Dr. Nuria Verdaguer (IIQAB – CSIC, Barcelona). Isolation and purification of Fab 3E5 were performed at 4 °C (except where mentioned otherwise) as follows: mAb was purified from ascitic fluid as described in section 4.3.1.5 and then concentrated by precipitation with 45% ammonium sulphate. The suspension was centrifuged (10000 r.p.m.) for 20 min and pellet was resuspended in the minimum volume of PBS buffer. This suspension was then dialysed against PBS overnight (3 × 1 l). To a Falcon centrifuge tube containing the antibody solution (3 mg in 2 ml of PBS) were added the following reagents: 24 µl of 0.1 M EDTA, 126 µl of 100 mM cysteine and 30 µg of papain. The volume was completed to 3 ml with PBS buffer and the tube was sealed and left

at 37 ºC for 5 hours. The digestion was then quenched by addition of iodoacetamide (180 µl). The digestion mixture was analysed by SDS-PAGE on a 12% acrylamide gel as described in section 4.3.1.12, except for pre-treatment of samples, which were not submitted to heating neither to β-mercaptoethanol addition prior to loading in the gel. mAb and Fab 4C4 samples were used as standards. Proteins in the digestion mixture were precipitated with 85% ammonium sulphate and the suspension was centrifuged (10000 r.p.m.) for 20 min. Pellet was resuspended in the minimum volume of 1:1 PBS/buffer A and the suspension dialysed overnight against buffer A (3 × 1 l). After centrifuging at 12000 r.p.m. to remove remaining solid particles, the protein solution was eluted in a protein A – Sepharose column as previously described for mAb purification. Fractions with $OD_{280} \geq 0.5$ (first elution peak) were pooled and concentrated to a final volume of 2 ml, using a Centriprep-3 concentrator* at 2000 r. p. m.

Fab was purified by gel filtration on a Sephadex G-100 support previously conditioned and equilibrated (overnight) at a constant PBS flow of 20 ml/h. Sample elution was performed at the same buffer flow and monitored at 280 nm. Three peaks were collected and their composition was analysed by SDS-PAGE as previously described in this section. Fab-containing fractions were pooled, concentrated with a Centriprep-3 concentrator and quantitated by optical density at 280 nm.

A biospecific surface was prepared upon peptide A15 immobilisation on a CM5 sensor chip as previously described (section 4.3.1.7), injecting 50 µl of the peptide solution (200 µg/ml in 10 mM acetate buffer, pH 5.5) in order to obtain high surface peptide density (*ca.* 300 RU) and therefore favour mass transport limitations (see chapter 3).

Fab SD6, 4C4 and 3E5 stock solutions were diluted in HBS to a final concentration of 320 nM (and subsequent serial dilutions). Series of 7 different Fab concentrations (ranging from 0 to 320 nM) were injected over the sensor chip surface with immobilised A15: 5-min injections at 5 µl/min were applied, and 1-min pulses of 100 mM HCl were used for regeneration. Under mass transport limitations, initial binding rate is related to analyte concentration[14], therefore a calibration curve for *initial rate = ƒ (Fab concentration)* could be built from the dependence of curve initial slope (measured at the 100th second of injection time with a 10-second time window) on Fab concentration (see chapter 3).

* this system is used for concentration and desalting of 5 – 15 ml samples, having a 3 kDa molecular weight cut-off. Sample is placed in a container where a filtrate collector is immersed and twist-locked. Immersion creates a slight hydrostatic pressure differential which is increased upon centrifugation of the assembly. Therefore, solvent and materials below the molecular weight cut-off are forced through the membrane into the filtrate collector until equilibrium is reached (hydrostatic pressure differential = 0). Upon removal of filtrate solution, the differential is restablished and successive concentration cycles can be carried out.

Peptide solutions (5 to 1250 nM in HBS) were incubated with Fab (80 nM) overnight at 4 °C. The solutions were then allowed to stand at room temperature for 1 hour for re-equilibration prior to injection in the SPR system. Each peptide – Fab mixture was then injected over the sensor chip surface (5-min injections at 5 µl/min) and 100 mM HCl 1-min pulses were used to regenerate the peptide surface after each injection. Remaining free Fab in each injected mixture was measured from curve initial slope and subsequent intrapolation in the corresponding calibration curve. The dependence of remaining free Fab on competitor peptide concentration was plotted and processed by the following two methods:

Data points from the titration series where free Fab concentration was measured from the binding rate were fitted to the equation (Fab total concentration is constant, peptide concentration is the independent variable and K_D is the fitted parameter):

$$\frac{[Fab]-[peptide]-K_D}{2}+\sqrt{\frac{([peptide]+[Fab]+K_D)^2}{4}-[peptide]\times[Fab]}$$

that is included in the BIAEvaluation *solution affinity* model[2] (chapter 3). This evaluation of K_D ($K_D=1/K_A$) does not take into account the effects of the immobilised peptide antigen.

Another method, that takes into account the influence of the immobilised peptide, is based on the *Cheng and Prusoff's* formula[15] (chapter 3):

$$K_i = 1+\frac{K_A[Fab]}{IC_{50}}$$

where K_A is the immobilised peptide – Fab affinity (determined independently, for instance, by SPR kinetic analysis), [Fab] is the Fab total concentration and IC_{50} is the 50% inhibitory concentration for the competitor peptide in solution (determined from the *free Fab = ƒ (peptide concentration)* plot).

4.3.2 Enzyme-linked immunosorbent assays – ELISA

The antigenicity of the FMDV synthetic peptides towards mAbs SD6, 4C4 and 3E5 was also determined by immuno-enzymatic assays[16], namely, competition ELISA[17]. Procedures were as follows:

Peptide A21 conjugated to KLH[#] (5 pmol of peptide in 100 µl PBS per well) was incubated overnight at 4 ºC as coating antigen in micro-titer ELISA 96-well plates. The latter were saturated for 3 h with 5% BSA in PBS and then liquid was removed upon suction under reduced pressure with a Pasteur pipette. This and all subsequent steps were carried out at room temperature. Then, 100 µl of a solution containing a non-saturating, constant amount of mAb – pre-incubated for 1.5 h with different concentrations of the competitor peptide antigens (serial dilutions from 243 to 0.1 pmol/100 µl) in 1% BSA in PBS – was added to the wells and further incubated for 1 h. After washing with 0.1% BSA, 0.1% Tween 20 in PBS, 100 µl of peroxidase-conjugated goat anti-mouse IgG (1:3000 dilution in PBS) were added to each well. Incubation was for 1 h, followed by thorough rinsing with 0.1% BSA, 0.1% Tween 20 in PBS. Bound antibody was detected using H_2O_2 and *orto*-phenylenediamine as substrate. Colour was allowed to develop in the dark for 10 minutes and reaction was quenched upon addition of 2 M H_2SO_4 (100 µl/well). The absorbance at 492 nm was immediately read.

The assay included a series of positive and negative controls: a positive control A21-KLH + mAb (without competitor peptide) in triplicate and five negative controls, respectively, A21-KLH + PBS (× 2), PBS + mAb (× 2) and PBS + PBS (× 1). Absorbances were corrected upon subtraction of the average absorbance measured for negative controls and expressed as percentages of the maximum absorbance (average of positive controls). Competitor peptide antigenicity was expressed as IC_{50}, that is, 50% of inhibitory concentration (competitor concentration leading to a 50% decrease in maximum absorbance) and normalised to the IC_{50} obtained for the standard peptide A15 (IC_{50} rel = IC_{50} competitor/IC_{50} A15, sections 2 and 3).

[#] this conjugate had been already prepared by Dr. M. L. Valero and M. E. Méndez.

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4.4 Structural studies of the FMDV peptides

4.4.1 Two-dimensional proton nuclear magnetic resonance[1]

NMR spectra were acquired both in aqueous solution (85% H_2O + 15% D_2O) and in the presence of the structuring agent TFE (30% TFE + 60% H_2O + 10% D_2O) at a peptide concentration of 2 mM. Experiments were run at 25 °C and 1,4-dioxane was added to all samples as an internal reference standard.
All experiments were carried out with a Varian VXR-500 S NMR spectrometer and further processed with the VNMR[3] software programs. The 2D ^{1}H-NMR experiments performed were:

- TOCSY[3], with 70 millisecond mixing time;
- NOESY[4], with mixing time of either 200 or 400 milliseconds;
- ROESY[5], with 200 millisecond mixing time.

Water signal elimination was carried out either upon pre-saturation or using the WATERGATE[6] method. Prior to the Fourier transform, both FIDs and interferograms were multiplied by an exponential function.

4.4.2 Protein X-ray diffraction crystallography[7,8]

4.4.2.1 Protein crystallisation

Crystals of the complex between the Fab of 4C4 and peptide A15(138F,140P,142S) were obtained by the *hanging drop vapour diffusion*[9] technique and subsequent micro and macro-seeding steps.
Fab (40 µl, 18 mg/ml in PBS) and peptide (8 µl, 10 mg/ml in H_2O) were incubated at 4 °C for 2 hours. A simple search for crystallisation conditions was performed in the vicinity of the conditions found by W. F. Ochoa for the crystallisation of similar Fab 4C4 – FMDV peptide complexes at 20 °C: 1 µl droplets of the peptide-Fab mixture were mixed with equivalent volumes of the precipitating agents; these agents were based on different dilutions of PEG 4K in water, 100 mM Tris-HCl buffer at variable pH and 400 mM LiCl. Each precipitating solution (1 ml) was poured on a well of 24-well cell culture plates, which acted as solution reservoirs. Protein droplets were put on pre-treated[&] glass covers that were then inverted and stuck, using silicone grease, to the top of the corresponding solution reservoir.
Small twined needles were formed at 18% polyethyleneglycol (PEG) 4K, pH 8.5 and then used for micro-seeding: a cat whisker was soaked in a needle-containing drop and then in a fresh protein drop that was equilibrated against a solution reservoir as previously described. This micro-seeding produced larger needles at 16% PEG 4K, which were harvested (upon suction with a capillary quartz tube, ∅=0.2 mm) and washed in crystallising solution. These needles were used for macro-seeding in 2 µl droplets containing 7 mg/ml of Fab, 1.8 mg/ml of peptide, 6.5% PEG 4K, 0.2 M LiCl with 50 mM Tris HCl (pH=8.5), equilibrated against a reservoir containing 13% PEG 4K equally buffered at room temperature. Small needle-shaped crystals (0.6 × 0.05 × 0.03 mm) were reproducibly formed under these conditions and, occasionally, unstable hexagonal crystals were also observed.

4.4.2.2 Data collection

Crystals for cryogenic data collection were soaked in harvesting solutions with 20% of glycerol and flash-frozen under a stream of boiled-off nitrogen at 100 K. X-ray data sets were collected by W. F. Ochoa on a MarResearch image plate detector (180 × 0.10 mm, 1800 pixels) system using a Rigaku RU-200B rotating anode, on the European Synchrotron Radiation Facility at Grenoble. A 2.2 Å resolution data set was collected with 1° rotations (a total of 91 rotations) at a crystal-detector distance of 180 mm. Crystals were orthorhombic, space group $P2_12_12_1$, and unit cell parameters as presented in chapter 3, containing one molecule of the complex per asymmetric unit. Diffraction data were auto-indexed and integrated and merged using programs DENZO and SCALEPACK[10].

[&] glass covers were treated as follows: 30 min in a dichloromethylsilane bath (hood) → 30 min in a water bath → 30 min in a fresh water bath → 30 min in an ethanol bath; the covers were then allowed to dry prior to their utilisation.

4.4.2.3 Structure solution and refinement

Crystals of the complex seemed related to crystals formed with the same Fab and the wild-type peptide A15, whose structure had been previously solved. However, the unit cell parameters differed and the structure was newly determined by molecular replacement[11] using the AmoRe package[12], employing the 4C4 Fab coordinates as searching model. The initial solutions were then optimised by allowing to move as four separated rigid bodies the variable heavy, variable light, constant heavy and constant light domains. Examination of the electron density maps, calculated at this stage, clearly showed extra densities corresponding to peptide occupying the antigen binding site. The final model for the structure of the complex was obtained by iterative cycles of manual modelling of water molecules and rebuilding of protein/peptide chains using the program O[13], alternating with positional refinement using standard protocols in the CNS package[14]. Bulk solvent correction was applied, allowing the use of all reflections in the resolution shell 15.0 – 2.3 Å. The refined models converged to satisfactory crystallographic agreement factors, as presented in chapter 3. Structural refinement analysis was done with PROCHECK[15] and graphic representation of the structure was processed with program SETOR[16].

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