

Cyclic disulfide model of the major antigenic site of serotype-C foot-and-mouth disease virus

Synthetic, conformational and immunochemical studies

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A cyclic disulfide peptide representing antigenic site A of foot-and-mouth-disease virus (FMDV) strain C-S8c1 (residues 134 to 155 of viral protein 1 (VP1) with Tyr¹³⁶ and Arg¹⁵³ replaced by cystine; TTCTASARGDLAHLTTTHACHL) was synthesized by solid phase methods. Formation of the cyclic disulfide was carried out by air oxidation of the fully deprotected and reduced bis-cysteine precursor, under high dilution conditions. The identity of the cyclic peptide was confirmed by both physical and enzymatic methods. A conformational study of the cyclic peptide and of its linear parent structure (YTASARGDLAHLTTTHARHLP, residues 136–156 of VP1 of FMDV C-S8c1) by circular dichroism in the presence of a structure-inducing solvent showed the cyclic disulfide analog to adopt lower levels of α -helix than its linear counterpart. In competitive ELISA assays both peptides reacted with similar affinity against a representative panel of neutralizing monoclonal antibodies directed towards antigenic site A. Thus, a high inherent flexibility of this loop may preclude a conformational restriction strong enough to alter recognition by anti-virus antibodies.

Antigenic peptide; Conformational restriction; Circular dichroism; Monoclonal antibody; Solid phase peptide synthesis

1. INTRODUCTION

Foot-and-mouth disease virus (FMDV) includes a major antigenic site (termed site A) involved in neutralization of infectivity, and located within amino acids 135 to 160 of capsid protein VP1 [1–3]. The three-dimensional structure of FMDV of serotype O (strain O₁BFS) [4] showed that this region is a protein loop highly exposed on the capsid surface, with a diffuse density suggestive of high molecular disorder and flexibility. Only upon reduction of a disulfide bridge at the base of the loop a structure became apparent, but this segment remained one of the most mobile regions of the virus surface [5]. It has been proposed that, as with a peptide in solution, this loop may not have a unique conformation [6]. This could explain the relative success as synthetic vaccines of peptides mimicking this segment [3,8]. The loop could adopt, with little cost in free energy, the conformations required to interact with many of the antibodies elicited against the homologous synthetic peptide. It has been also suggested that in FMDV O₁BFS this loop exists at least in two spatial arrangements oriented towards different locations on the capsid

surface [9]. Such a change in orientation may also involve conformational alterations within the loop.

For FMDV type C, linear peptides reproducing site A have been shown to be recognized almost as efficiently as the entire VP1 or complete virions by neutralizing monoclonal antibodies (nMAbs) elicited against intact FMDV [10,11]. In addition, the effect of amino acid substitutions within site A on the recognition of intact virus by nMAbs directed to this site has been repeatedly reproduced with the corresponding substituted peptides ([10–12], Mateu et al., unpublished). These and other results [7] suggest that the antigenic site A of FMDV of serotype C is composed of continuous epitopes defined mainly by local conformation(s) within the VP1 G-H loop. However, some anti-FMDV type C MAbs recognized site A in the virus somewhat better than linear peptides representing the relevant sequence (Mateu et al., unpublished). Also, the immunogenicity of peptides reproducing site A is lower than that of the intact virus. Through many factors can account for this latter observation, an adequate conformational restriction may improve both the antigenicity and the immunogenicity of FMDV site A peptides.

In many viral [13–15] and non-viral [16–18] proteins which depict well defined, structured epitopes, the spatial features of those sites have been mimicked by cyclic peptide structures with varying degrees of conforma-

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tional restriction more effectively than by linear peptides. For FMDV, the limited structural definition of site A is a serious drawback in designing constrained peptide models which could react better than linear site A peptides with anti-virus antibodies and thus become candidate synthetic vaccines. In this paper we describe the synthesis of a cyclic disulfide peptide defining a 18-residue (56-atom) loop corresponding to antigenic site A of FMDV C-S8c1. We compare the reactivity of antibodies with this cyclic peptide and its linear counterpart. The similar reactivity of the two peptides suggests a considerable degree of structural flexibility of this segment, regardless of the disulfide bridge linking its ends.

2. MATERIALS AND METHODS

2.1. Peptide synthesis

Solvents and reagents for solid phase peptide synthesis were as previously described [10]. Automated peptide synthesis was performed in an ABI 430A instrument using Boc chemistry (manufacturer SSRC and std1 protocols for 0.1 and 0.5 mmol, respectively), with minor modifications. Manual couplings by the standard DCC procedure were performed as in [10]. Couplings mediated by benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) were performed by preincubation of Boc-amino acid (0.4 M in dimethylformamide (DMF), 5 ml), BOP (1 M in DMF, 2 ml) and diisopropylethylamine (4 mmol, 680 μ l) for 10 min. After assembly of the complete sequence, removal of the 2,4-dinitrophenyl (Dnp) His protection and full deprotection and cleavage of the peptide resin by HF were performed as previously described [10].

2.2. Purification, oxidation and characterization

The crude linear peptide was purified in a medium-pressure C₁₈-silica column (Michel-Miller, 20 \times 300 mm) using a 15–20% acetonitrile gradient in water (both solvents with 0.05% trifluoroacetic acid (TFA)). The eluate was monitored at 220 nm and collected into ca. 6-ml fractions which were analyzed by HPLC (Shimadzu) on a Nucleosil C₁₈ column eluted with a linear 15–30% acetonitrile (+ 0.036% TFA) gradient into 0.045% TFA in water, and by HPCE (ABI 270A; fused silica 72 cm capillary, 50 cm to detector; 50 μ m i.d.) at 210 V/cm and 30°C in 20 mM citrate, pH 2.5 buffer. Fractions of the highest purity were pooled and re-run as above to give material of satisfactory quality (> 97% by HPLC and HPCE). The purified linear peptide was diluted to 50 μ M with 0.02 M Tris-HCl buffer, pH 8. The solution was vigorously stirred for 3–5 days at room temperature (r.t.) until oxidation was shown to be complete by HPLC. The reaction was then quenched by addition of glacial HOAc to pH 3–4, lyophilized and desalted on Sephadex G-10 (2 \times 100 cm) in 0.1 M HOAc. All peptide fractions were of satisfactory purity (> 95%) by HPLC and HPCE and were pooled and lyophilized. The purified cyclic disulfide peptide was characterized by amino acid analysis (6 M HCl, 110°C, 24 h hydrolysis; Beckman 6300 analyzer) and FAB-MS (VG Quattro, Fisons Instruments). Tryptic digests were performed on a 0.5 mg sample in 400 μ l of 50 mM NH₄HCO₃, pH 8.4, treated with 20 μ l of agarose-bound trypsin (80 U/ml, Sigma) for 120 min at 37°C. The reaction mixture was stopped by addition of glacial HOAc and centrifuged. The supernatant was analyzed by HPLC using a linear 5–65% acetonitrile gradient in water over 30 min. Each peak was collected, analyzed for homogeneity by HPCE and submitted to amino acid analysis. For DTT reductin, cyclic peptide (0.5 mg in 400 μ l of 50 mM NH₄HCO₃, pH 8.4) was treated with 50 μ l of 25 mM DTT (110 \times molar excess) in water for 2 h at 37°C. The reaction was quenched by HOAc and analyzed by HPLC and HPCE as above.

2.3. Competitive ELISA assay

Neutralizing MAbs raised against FMDV serotype C and which recognize site A have been previously described and their epitopes characterized [7,11,20]. Linear peptide (5 pmol, 100 μ l) conjugated to KLH [21] was incubated overnight at 4°C as coating antigen in microtiter plates. These were saturated for 2 h at r.t. with 5% BSA in PBS, and washed. Then, 100 μ l of a solution containing a non-saturating fixed amount of MAb preincubated for 1.5 h at r.t. with different amounts of the competitor antigens (1.6, 5, 15, 45, 135 and 405 pmol) in 1% BSA in PBS were added to the wells and further incubated for 1 h at r.t. After washing, 100 μ l of peroxidase-conjugated goat-anti mouse IgG (Bio-Rad, 1:3000 dilution) were added and incubated for 1 h at r.t., followed by extensive washing. Bound antibody was detected using H₂O₂ and 4-chloro-1-naphthol as substrate. The reaction was stopped with 100 μ l of 4 N H₂SO₄ and the absorbance at 492 nm read in a Titertek multiscan photometer.

2.4. Circular dichroism studies

CD measurements were performed on a Jasco J720 spectropolarimeter flushed with N₂ (3 l/min). Spectra were recorded at 5°C in a 1.0 mm-pathlength cell containing 20 μ M peptide in 1 mM citrate, 1 mM phosphate, 15 mM NaCl buffer, pH 5.3 in the presence of varying amounts of hexafluoroisopropanol (HFIP). Data were typically collected using a 0.2 nm spectral bandwidth, a 4 s time constant (10 nm/min) and averaging the data from three scans.

2.5. Structural analysis of cyclic peptide

Molecular modelling was performed on a Silicon Graphics workstation using the INSIGHT II and DISCOVER programs. The initial conformation was generated from the previously calculated lowest energy conformation of the nonapeptide Ac-TASARGDLA-NHMe [22], and appending to this fragment the remaining residues of the sequence as a predetermined α -helix. This starting conformation was minimized using first the steepest descent method (500 iterations) and then the conjugate gradient method (500 iterations). The resulting conformation was subjected to 10 ps molecular dynamics (MD) trajectory calculation at 750K. An integration step of 0.5 fs was used in all the calculations. During the trajectory, 20 structures were stored at regular times (0.5 ps) and minimized using the conjugate gradient algorithm until the norm of the gradient was less than 0.01 kcal/mol. The last structure stored and minimized was newly subjected to the same MD trajectory calculation as above for a more efficient search of the conformational space. Solvent was not explicitly included in the calculation but, in order to simulate its effect, a distance-dependent dielectric constant of 4r was used.

3. RESULTS

The cyclic disulfide model of the FMDV loop was designed on the basis of the reported X-ray diffraction structure of FMDV of serotype O₁BFS [4] and on appropriate alignment of site A sequences from serotypes O₁ and C₁ (Fig. 1). The region between residues 134 and 155 of VP1 was chosen, and since it has been suggested that the distance between the two C α of residues Tyr¹³⁶ and Arg¹⁵⁷ in serotype O₁ is ca. 10 Å [23], the corresponding amino acids in the C₁ sequence (Tyr¹³⁶ and Arg¹⁵³) were replaced by two cysteine residues to allow cyclization through an internal disulfide bridge.

The linear precursor of the cyclic disulfide peptide (Fig. 1) was synthesized by solid phase methods [24] using Boc chemistry on *p*-methylbenzhydrylamine resin and the following side chain protections: Ser(Bzl), Thr(Bzl), Arg(Tos), His(Dnp), Asp(OcHex) and Cys(Meb). A first synthesis was done using rapid (30

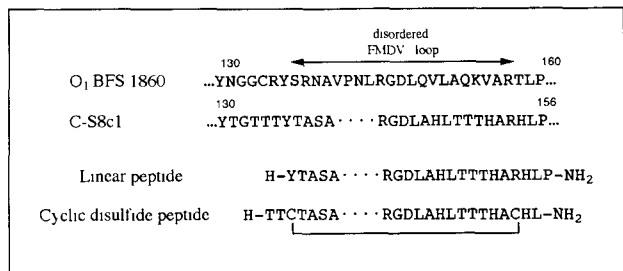


Fig. 1. Amino acid sequence of the major antigenic site of FMDV O₁BFS [4], of C-S8c1 [35], and of synthetic peptide models (linear and cyclic disulfide) of the latter virus. A dot in the sequence indicates deletion of one amino acid. The double arrow above the sequence of FMDV O₁BFS spans the disordered region of the VP1 G-H loop of this virus [4], resolved upon reduction of the virion [5].

min/cycle) single couplings with symmetrical anhydrides in DMF for all residues except Boc-Arg(Tos) and Boc-His(Dnp), which were coupled as HOBT esters. Thiolytic Dnp removal, HF cleavage and purification led to a major product (fairly homogeneous by HPLC but later found heterogeneous by HPCE) the mass spectra of which showed two peaks (3:2 ratio) corresponding to the lack of one (M-101) and two (M-202) Thr residues, respectively. These results prompted a new synthesis of the linear precursor using longer coupling times (60 min/average residue) and systematic recoupling of Thr residues, which were monitored by the Kaiser test. The problematic Thr couplings were found to be those of residues 134, 135 or 137, whereas the 148–150 Thr triplet was trouble-free. The ninhydrin test after recoupling of Thr¹³⁷ was still clearly positive and it was decided to carry out the rest of the synthesis manually. Standard DCC and DCC/HOBT couplings were again ineffective for this residue, which was only successfully coupled by

the BOP method [25]. Similar coupling difficulties at Cys¹³⁶, Thr¹³⁵ and Thr¹³⁴ were solved likewise. Thiolytic Dnp removal [26] and HF acidolysis of the protected peptide resin gave a crude peptide that could be purified in two MPLC steps (global HF and purification yield 27%) to an HPLC- and HPCE-homogeneous material (> 97%) with amino acid analysis and mass spectra consistent with theory. This linear peptide (5×10^{-4} M) was air-oxidized at pH 8. HPLC monitoring of the reaction (Fig. 2) showed the oxidation to be complete in 3–5 days for 7–10 μ mol batches; for smaller samples, shorter times (1–2 days) sufficed. The oxidized material was remarkably clean and required only desalting to furnish the cyclic disulfide in high purity (95–97% by HPCE and HPLC, respectively). MS analysis gave a molecular peak two units less than the linear precursor. The internal disulfide identity of this material was further confirmed by a negative Ellman test [27] and by reversal to the reduced form upon treatment with DTT at pH 8.4 (data not shown). Trypsin hydrolysis at the single Arg residue of the sequence afforded additional evidence in favor of the cyclic structure. As shown in Scheme 1, the cyclic disulfide yields a single tryptic product with the amino acid composition of the parent peptide, whereas other disulfide arrangements will produce more complex tryptic maps. Both HPCE (Fig. 3) and HPLC (not shown) tryptic maps gave a major component that did not coelute with either the starting cyclic disulfide or its reduced form and with an amino acid composition identical to the parent sequence (Table I). MS analysis of this peak gave a molecular peak 18 units higher than the cyclic peptide. On the basis of this and the above evidence, it can be concluded that the oxidized material is indeed the desired cyclic monomeric form of the target peptide. Minor components of the tryptic digest (Table I) were attributed to lability of the disulfide under the alkaline proteolysis conditions.

The conformational features of the cyclic disulfide were examined by CD and compared to those of a linear peptide. In HFIP titration experiments, the cyclic ana-

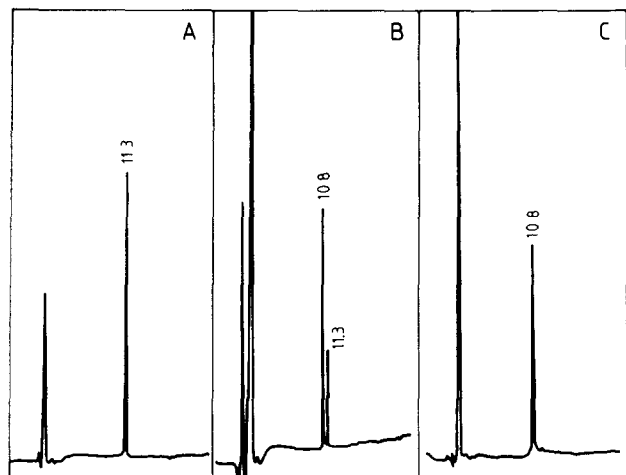
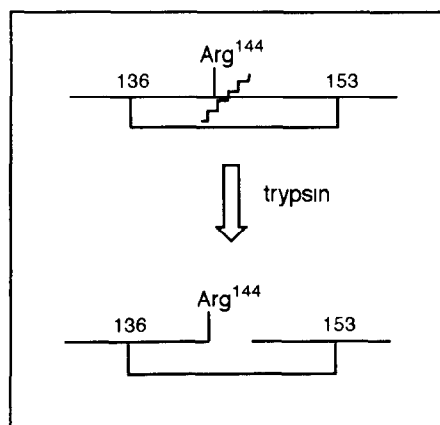


Fig. 2. Reverse-phase HPLC analysis of different stages of cyclic disulfide formation. (A) reduced, purified linear precursor, (B) after 24-h oxidation; and (C) fully oxidized, purified by gel permeation chromatography. HPLC conditions, see text.



Scheme 1

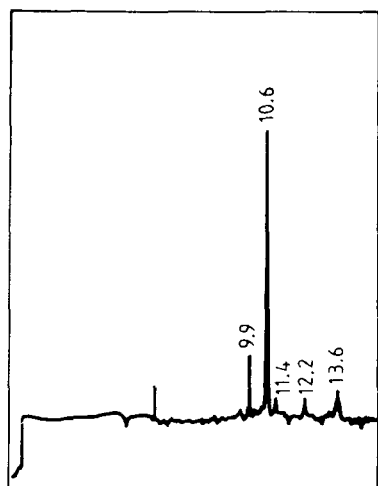


Fig. 3. Capillary electrophoresis tryptic map of the cyclic disulfide peptide (1 s vacuum injection, ca. 4 nl from a 1 $\mu\text{g}/\mu\text{l}$ digestion mixture). HPCE conditions, see text

log showed $-\left[\theta_{\text{MR}}\right]_{222}$ (negative molar ellipticity per residue at 222 nm) values consistently larger than its linear counterpart at any given HFIP concentration (Table II). Maximal helicity was reached at 14% HFIP for the linear peptide, whereas twice that amount was required by the cyclic analog to attain comparable, though still lower, α -helix levels.

To evaluate the relevance of partial conformational restriction on the antigenic recognition by antibodies, both the linear peptide and its cyclic disulfide analog were tested in a competitive ELISA assay against a representative panel of eight nMAbs elicited against intact virus and directed towards distinguishable epitopes within the VP1 G-H loop. The fully reduced linear precursor of the cyclic disulfide was used as a control. Results obtained with two MAbs (SD6 and 4C4) are presented in Fig. 4, and are representative of those obtained with the other six MAbs (5A2, 6D11, 7FC12, 7AH1 and 7CA11, [7]). These results showed that there were no significant differences in antigenic recognition between the linear and the cyclic forms.

4. DISCUSSION

The representation of B-cell continuous epitopes in linear peptides has as a main drawback the fact that such peptides exist in solution in a variety of random conformations, only a fraction of which may be recognized by protein-specific antibodies [28,29]. This is illustrated by the fact that a large excess of peptide is often required to equal the antigenic reactivity of the native protein. Drawing from experience in the hormone-receptor field [30], it has been suggested that appropriate conformational constraints may induce improved molecular recognition between peptides and their cognate antibodies. Several examples of conformational restric-

Table I
Tryptic fragments of disulfide cyclic peptide

HPCE retention time ¹	Abundance	Amino acid composition ²
9.9	15%	Asp ₁ Thr ₃ Gly ₁ Ala ₂ Leu ₃ His ₃ (C-terminal fragment)
10.6	72%	Asp ₁ Thr ₆ Ser ₁ Gly ₁ Ala ₂ Leu ₃ His ₃ Arg ₁ (open sequence)
11.4	3%	n.d.
12.2	5%	Thr ₃ Ser ₁ Ala ₂ Leu ₁ Arg ₁ (N-terminal fragment)
13.6	5%	n.d.

¹See Fig. 3

²From the corresponding HPLC-isolated fractions. Cys not determined

tion by cyclization have been reported for influenza [13,14], hepatitis B [15], lysozyme [31] and FMDV [32] immunopeptides. Applying similar principles, we have sought to impose a conformational constraint on the G-H loop of serotype C of FMDV by means of an internal cyclic disulfide. To assess the severity of the conformational restriction, the cyclic structure was subjected to molecular dynamics to evaluate the distance between the two C α of the cystine residue and compare it with that reported for serotype O₁. This distance was measured in the three lowest energy conformers within 3 kcal/mol and averaged according to Maxwell-Boltzman distribution at 278K to give a final value of 5.6 Å (vs. ca. 10 Å for serotype O₁BFS).

Examination of the conformational properties of the cyclic peptide by CD in HFIP-containing buffers revealed considerably lower levels of α -helix than its linear counterpart. Maximal ellipticity values were almost 50% larger for the linear than for the cyclic peptide, and were reached at lower concentrations of organic solvent. NMR studies (Roig et al., manuscript in preparation) confirm the α -helical character of the linear peptide in the presence of moderate amounts of HFIP. The VP1 G-H loops of FMDV serotypes O and A have also been described as helical on the basis of CD and NMR work on synthetic peptide models [32,33]. Assuming our

Table II
 $[\theta_{\text{MR}}]_{222}$ at different HFIP concentrations for linear and cyclic disulfide peptides

HFIP%	Linear	Cyclic disulfide
0	-1.15×10^3	-1.36×10^3
4	-1.09×10^3	-2.06×10^3
8	-3.27×10^3	-2.67×10^3
12	-1.70×10^4	-3.82×10^3
14	-1.92×10^4	-7.49×10^3
16	-1.90×10^4	-1.13×10^4
20	-1.93×10^4	-1.27×10^4
28	n.d.	-1.43×10^4

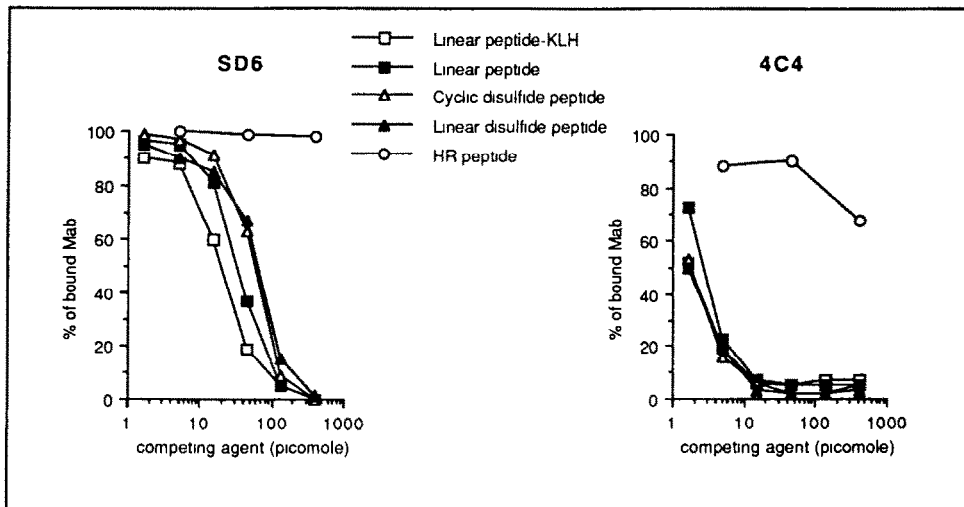
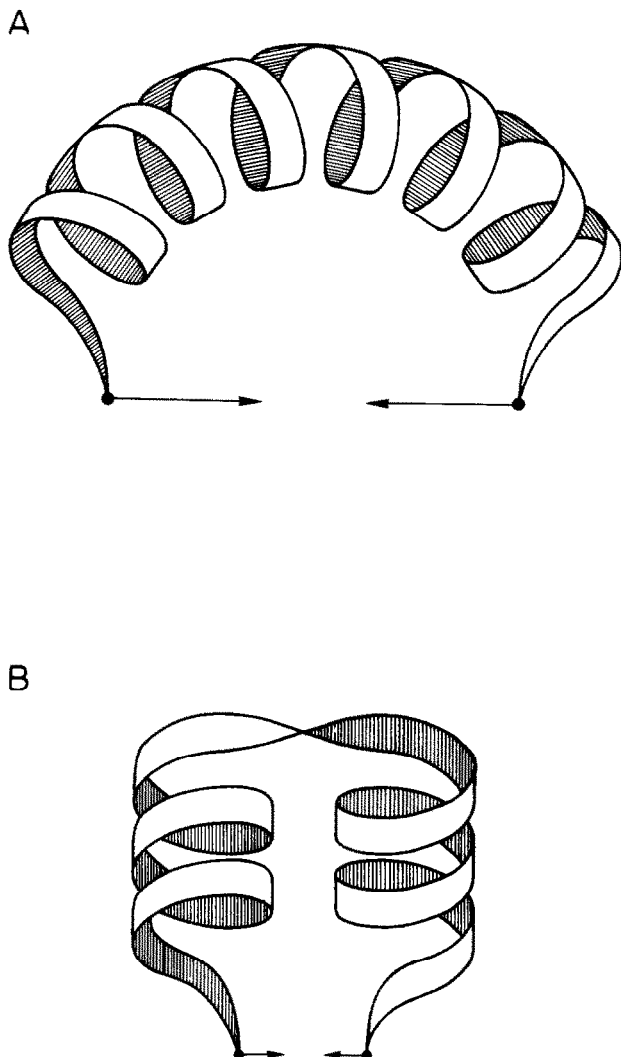


Fig. 4. Inhibition of monoclonal antibody (SD6 and 4C4) binding to linear peptide-KLH by different competing antigens in ELISA. Peptide HR, a 21-residue peptide with a replacement of His¹⁴⁰ by Arg that reproduces site A of a virus mutant which does not bind any Mab directed to this site [7], and linear peptide conjugated to KLH were used as negative and positive controls, respectively.



linear peptide model of the VP1 loop to be helical under appropriate structure-inducing conditions, a possible interpretation for the observed loss in helical potential upon cyclization is given in Fig. 5, where the different effects of an internal constraint on two possible types of α -helical loop are compared. If the loop consists of an uninterrupted stretch of α -helix (A), it is not possible to draw together its two ends by cyclization without seriously disrupting its hydrogen bonding pattern, with a resulting decrease in the helical character. Alternatively, one can envisage a loop formed by two antiparallel stretches of α -helix (B), with lower amount of α -helix but where a covalent bond can be formed between the two ends without affecting the net helical content. The decrease in helicity observed by CD in going from the linear to the cyclic model of FMDV loop suggests that, while the former peptide is likely to exist as a continuously α -helical structure in the presence of HFIP (model A), the cyclic analog is more prone to adopt an arrangement such as model B, with a lower intrinsic helical content. This interpretation of CD data awaits further confirmation by ongoing NMR experiments. A previous CD study on FMDV serotype A₁₂ peptides [33] showed also some decrease in α -helix content upon cyclization. The VP1 G-H loop of FMDV O₁BFS, as recently visualized by X-ray crystallography of DTT-treated virions, is partly helical [5]. However, since there is very low sequence similarity between the VP1 G-H

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Fig. 5. Two possible arrangements of a helical loop. In model A, considerable helix disruption is predicted when the two ends of the loop are brought together by internal cyclization. The helical content of B, lower than A, is however less affected by the internal constraint.

loop of O₁BFS and C-S8c1 -including a four amino acid deletion in the latter- it is not clear whether the structure of O₁BFS may apply to C-S8c1.

The antigenic behavior of the conformationally restricted peptide towards a representative panel of nMAbs elicited against intact virus and directed to site A was essentially identical to that of its linear counterpart. In attempting to relate this finding with the CD results discussed above, lack of data on the effects of antibody binding on peptide conformation is a serious limitation. A relatively straightforward interpretation would be to assume that the disruption caused by cyclization, while substantial enough to be detectable by CD, does not reduce the flexibility of the peptide in the area of contact with antibodies to the extent that significant recognition features are abolished. Cyclic disulfide peptides of small (6-residue) size have been shown to be rather flexible structures [34], and one should therefore expect considerably more flexibility for our 18-residue disulfide cycle. If confirmed by future results, such flexibility may in part defeat the purpose and eventual application of our approach. It should be noted, however, that in designing the present cycle an extrapolation of structural data from the O₁BFS to the C-S8c1 serotype was introduced (see section 3). Since the VP1 loops of the two serotypes are not of the same length (C-S8c1 is four residues shorter), the possibility still remains that cyclic peptides (disulfide or otherwise) of ring size slightly smaller than the present one are better recognized by MAbs than the linear peptides used so far and thus become improved models of the main antigenic site of FMDV. On the other hand, once it has been shown that a cyclic peptide model of this antigenic site is well recognized by nMAbs generated from FMDV, further work will be directed to assess the immunogenic potential of such cyclic structures.

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