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Synthesis, solution structure analysis and antibody binding of cyclic epitope peptides from glycoprotein D of Herpes simplex virus type I

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Abstract

Two cyclic peptides with a thioether bond have been synthesised corresponding to the 9–22 (^{9}LKMADPNRFRGKDL^{22}) sequence of glycoprotein D (gD-1) of Herpes simplex virus. The role of the secondary structure in protein-specific monoclonal antibody recognition was investigated. The sequence selected for this study comprises a strongly antigenic site adopting a β-turn at residues ^{14}Pro–^{15}Asn. Thioether bond was formed between the free thiol group of cysteine or homocysteine inserted in position 11 and the chloroacetylated side chain of lysine in position 18. We report here the preparation of cyclic peptides containing Cys or Hcy in position 11, differing only in one methylene group. The linear precursor peptides were synthesised by Boc/Bzl strategy on MBHA resin, and the cyclisation was carried out in alkaline solution. The secondary structure of the peptides was studied by CD, FT-IR and NMR spectroscopy. The CD and FT-IR data have revealed fundamental changes in the solution conformation of the two compounds. The CH_2 group difference significantly resulted in the altered turn structure at the ^{12}Ala and ^{13}Asp as identified by NMR spectroscopy. The antibody binding properties of the cyclopeptides studied by gD-specific monoclonal antibody (A16) in direct and competition enzyme-linked immunosorbent assay (ELISA) were also not the same. We found that peptide LK[HcyADPNRFK]GKDL exhibited higher affinity to Mab A16 than peptide LK[CADPNRFK]GKDL, however, their reactivity was significantly lower compared to the linear ones. Our results clearly show the importance of secondary structure in an antibody binding and demonstrate that even a slight modification of the primary structure dramatically could influence the immune recognition of the synthetic antigens.
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Keywords: HSV gD-1 epitope; Cyclic peptides; Thioether bond; Solution conformation; NMR; Antibody binding

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1. Introduction

The use of cyclic peptides based on the bioactive proteins offers an approach to construct an antigen with optimal binding and pharmacological properties. Epitope sequences are mainly present in the hydrophilic surface regions of immunogenic proteins, often adopting β-turn structures [1,2]. However, short peptides are flexible molecules, and they might not adopt the biologically relevant conformation for B-cell recognition. Cycle formation is a common method to reduce the number of conformers of linear peptides. Peptide stabilised by cyclisation, can often better mimic the native conformation influencing the biological response [3–7]. In addition, cyclopeptides are more stable and resistant against the enzymatic degradation, which makes them suitable for the development of potential synthetic antigen. Strong correlation between the solution conformation and the biological activity was recently documented in several cases, for linear [8–11] and also for cyclic peptides [12–15].

The application of amide (lactam) [16,17] or disulfide bridges [18] for cycle formation are among the most common methods. However, amide bond formation requires the protection of functional groups with orthogonal cleavable protecting groups and needs mainly organic solvent. It is believed that the disulfide bond is not stable enough in vivo. In order to avoid oligomerisation, reaction for disulfide strategy based cyclisation is usually performed at high dilution. Reports have also described the application of oxime [19], thiazolidine [20] or thioether [21] bond formation for cyclopeptides.

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) are among the most common infectious agents in human. Glycoprotein D (gD) is a structural component of the HSV envelope, and appears to be the major target for the immune response. Immunisations with gD can protect the animals from HSV infection [22,23]. The N-terminal 1–23 region of HSV gD-1 is a strongly antigenic site, and peptides from this domain are able to induce both B and T-cell responses [24]. Peptide 9–21 (LKMADPNRFRGKD) was determined as the optimal epitope of the N-terminal part, in which three amino acids, the ^{13}Asp, ^{16}Arg and ^{17}Phe residues are essential for antibody recognition [25]. Previous experiments have shown that the replacement of methionine in position 11 by norleucine (Nle) is a permitted and practical modification, since by this way, oxidation of the methionine residue can be avoided [26]. The structure of peptide 1–23 and its fragments were studied earlier by prediction algorithms, NMR and CD spectroscopy. It was demonstrated that the peptides could adopt a β-turn-like structure at residues ^{14}Pro–^{15}Asn under appropriate conditions [27–29].

The aim of our study was to design and prepare synthetic antigens with HSV-1 specificity. For this, cyclic peptides with thioether bond corresponding to the 9–22 sequence of the HSV gD-1 were synthesised and the role of the secondary structure in antibody recognition was investigated.

Cyclisation via thioether bond is a simple and selective method, which can be carried out with unprotected peptides. The thioether bond forms rapidly under mild alkaline solution, between a thiol group and—for example—a chloroacetylated amino group [21]. In most of the cases, this reaction is faster than the competing oxidation, which can lead to the disulfide formation; therefore, this side reaction is not considerable. The chloroacetyl group is stable under the normal HF deprotection conditions, even when free thiols are used as scavengers [30]. The thioether bond is especially used for the conjugation of peptide epitopes to proteins [31], and it was also successfully applied for chemical ligation, the synthesis of cyclic peptides and peptidomimetics [32–35]. Beside the simplicity of the synthesis, an important advantage of the thioether bond, is the increased metabolic stability in vivo compared to the disulfide bridge, which could be a fundamental feature in the design of a synthetic antigen.

In this article, we report on the synthesis, CD, FT-IR and NMR based conformational analysis and monoclonal antibody binding of two cyclic peptides, which differ only in a single methylene group. The data show that this small difference leads to considerable changes both in the solution conformation and in the recognition of protein-specific monoclonal antibody (Mab).

Table 1
Characterisation of the cyclic and linear peptides

Peptide	Yield (%)	R_t (min)[a]	Elemental composition	Rel. Mol. Mass	
				Calc.	Found[b]
[9]LKNleADPNRFRGKDL[22]	60	27.4	$C_{73}H_{124}O_{19}N_{24}$	1641.9	1642.5
[9]LKMADPNRFKGKDL[22]	51	27.0	$C_{72}H_{122}O_{19}N_{22}S$	1632.0	1633.0
[9]LKNleADPNRFKGKDL[22]	68	28.1	$C_{73}H_{124}O_{19}N_{22}$	1613.9	1614.5
[9]LK[HcyADPNRFK]GKDL[22]	25	25.8	$C_{73}H_{120}O_{20}N_{22}S$	1658.0	1658.8
[9]LK[CADPNRFK]GKDL[22]	27	25.7	$C_{72}H_{118}O_{20}N_{22}S$	1643.9	1644.4

[a] Column: Phenomenex Jupiter C_{18} (250×4.6 mm, 5 μ, 300 Å); eluent A: 0.1% TFA/H_2O, B: 0.1% TFA/Acetonitrile-H_2O (80:20 v/v); gradient: 0 min 0% B; 5 min 0% B; 50 min 90% B; flow rate: 1 ml/min.
[b] ESI-MS, $[MH]^+$.

2. Materials and methods

2.1. Materials

All amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland), while *m*- and *p*-cresol, *p*-thiocresol, *N*,*N′*-dicyclohexylcarbodiimide (DCC), *N*,*N′*-diisopropyl-ethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA) and hydrogen fluoride (HF) were Fluka (Buchs, Switzerland) products. Solvents for synthesis and purification were obtained from Reanal (Budapest, Hungary). *o*-Phthalaldehyde (OPA) and all other reagents for amino acid analysis were from Reanal (Budapest, Hungary) and from Merck (Darmstadt, Germany).

2.2. Synthesis

2.2.1. Synthesis of linear 9–22 peptides from HSV gD-1

Synthesis was carried out manually by solid phase methodology on a 4-methyl-benzhydrylamine (MBHA) resin (0.1 g, 1.04 mmol/g) by Boc/Bzl strategy. The amino acid side chain protecting groups were cyclohexyl ester for Asp, 2-chlorobenzyloxycarbonyl for Lys and mesitylene-sulfonyl for Arg. Activation and coupling were carried out using the DCC/HOBt method [36] with three equivalents of amino acid derivatives and coupling agents calculated to the resin capacity in DCM/DMF (3:1, V/V) solvent mixture. The efficacy of coupling was monitored by ninhydrin [37] and bromophenol blue [38] assays. Boc-group was removed by 33% TFA/DCM, while neutralisation was performed with 10% DIEA in DCM. The peptides were cleaved from the resin by 10 ml HF using 700 mg *p*-cresol as scavenger. Characteristic data are presented in Table 1.

2.2.2. Synthesis of cyclic 9–22 peptides from HSV gD-1

The synthesis of the linear pre-cursor peptides for cyclisation was carried out manually on an MBHA resin (0.5 g, 1.1 mmol/g) by Boc/Bzl strategy. The amino acid side-chain protecting groups were cyclohexyl ester for Asp, 2-chlorobenzyloxycarbonyl for Lys, except [18]Lys, which was blocked by Fmoc group, tosyl for Arg and 4-methyl-benzyl for Cys and Hcy. The synthesis, including activation, coupling and monitoring was the same as above mentioned. The N-terminal amino acid was coupled with DIC/HOBt, to avoid the presence of DCU in the chloroacetylation step. After completion of the synthesis, the Fmoc protecting group of the [18]Lys was removed by 50% piperidine/DMF, and the side chain was chloroacetylated on the resin by 5 equiv chloroacetic acid pentachlorophenyl ester in DCM/DMF (1:4, V/V) solvent mixture for 1.5 h. Afterwards, the N-terminal Boc protecting group was removed by 33% TFA/DCM, and the peptide was cleaved from the resins by 10 ml HF using 0.5 ml *m*-cresol and 0.5 g *p*-thiocresol scavenger mixture [30] for 1.5 h at 0 °C. Crude peptides were precipitated by dry ether, dissolved in 10% acetic acid and freeze-dried.

Table 2
Amino acid composition of peptide LK[CADPNRFK]GKDL

Amino acid (calculated)	Found			
	24 h[a]		48 h[a]	
	OPA	Ninhydrin	OPA	Ninhydrin
Asp (3.00)	2.89	2.80	2.95	2.90
CMC[b] (1.00)	0.30	0.33	0.65	0.60
Pro (1.00)	nd	1.00	nd	1.05
Gly (1.00)	0.95	1.00	1.05	1.05
Ala (1.00)	0.90	0.95	0.98	1.05
Leu (2.00)	1.90	2.20	2.10	2.15
Phe (1.00)	0.90	0.97	0.89	0.95
Lys (3.00)	2.50	2.70	2.95	3.07
Arg (1.00)	0.67	0.75	0.95	0.87

nd: not determined.
[a] Acid hydrolysis (6 M HCl, 110 °C, N_2 atmosphere).
[b] S-carboxymethyl-cysteine.

Prior to cyclisation, the crude linear peptides were purified by HPLC as described below. Cyclisation was carried out in solution. The purified solid chloroacetylated peptide was added to ammonia/water solution (pH 8) in small portions for 0.5 h at room temperature. The final concentration was 0.5 mg/ml. The mixture was stirred further for 3 h, followed by freeze-drying. Peptides were purified on RP-HPLC and characterised by HPLC, ESI-MS (Table 1) and amino acid analysis, using either the ninhydrine or OPA method (Table 2).

2.3. Analysis

2.3.1. HPLC

Analytical RP-HPLC was performed using Phenomenex Jupiter C_{18} (250×4.6 mm, 5 μm, 300 Å) reverse phase column and Waters system composed of No. 600 pump, No. 600 controller and No. 490 programmable multiwave length detector (Nihon Waters Ltd., Tokyo, Japan). Eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile–water (80:20 V/V). Linear gradient of eluents (0 min 0% B; 5 min 0% B; 50 min 90% B) with 1 ml/min flow rate was used as mobile phase. Three different wavelengths were applied for detection of the peaks ($\lambda = 214$, 254 and 280 nm).

Purification of linear and cyclopeptides was carried out on a Phenomenex Jupiter C_{18} (250×10 mm, 10 μm, 300 Å) semipreparative column using the same eluents with 5 ml/min flow rate. The gradient was 0 min 10% B; 5 min 10% B; 50 min 50% B. Detection was carried out at $\lambda = 214$, 220 and 254 nm.

2.3.2. Ninhydrin based amino acid analysis

The amino acid composition of peptides was determined by using a Beckman Model 6300 (Fullerton, CA, USA) analyzer. Prior to analysis, the samples were hydrolysed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

2.3.3. OPA based amino acid analysis with pre-column derivatisation

The amino acid analyses were performed on the laboratory assembled KNAUER HPLC system using precolumn derivatisation with OPA (*ortho*-phthalaldehyde) reagent. Hydrolysis of the cyclopeptides was carried out using 6 M HCl in N_2 atmosphere at 110 °C for 8, 24 or 48 h.

The OPA solution ($c \approx 10$ mg/ml) was freshly prepared from 10 mg OPA, 900 μl methanol, 100 μl sodium borate buffer (0.5 M, pH 9.5) and 10 μl 2-mercaptoethanol. Amino acid standards and samples were diluted with methanol:water:sodium borate buffer (0.5 M, pH 9.5) = 1:1:1 V/V.

The reaction was performed at an ambient temperature for 120 s, and the reagent–sample ratio was 1:4 (V/V) (250 μl). After reaction, 25 μl 0.75 M HCl was added. The amino acid derivatives were separated in linear gradient (0 min 10% B, 10 min 24% B, 25 min 35% B, 40 min 60% B, flow rate: 1 ml/min) on KNAUER-Eurospher C_{18} column (4 μm silica, 100 Å pore size) using eluent A: sodium acetate buffer (0.05 M, pH 7.0) and eluent B: methanol. Detection was performed using a fluorescent detector (Shimadzu, Japan) at λ(excitation)/λ(emission) = 330/445 nm.

2.3.4. Mass spectrometry

Positive ion electrospray ionisation mass spectrometric (ESI-MS) analyses were performed on a PE API 2000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). Spray voltage was set to 4.8 kV, and 30 V orifice voltage was applied.

Samples were dissolved in methanol–water (1:1 V/V) mixture containing 0.1% acetic acid, and 5 μl of sample was injected with a flow rate of 100 μl/min. The instrument was used in Q_1 scan mode in the range of m/z 400–1700, with a step size of 0.3 amu and a dwell time of 0.5 ms.

2.3.5. Circular dichroism spectroscopy

Circular dichroism was measured on a Jobin Yvon (Longjumeau, France) Mark VI Dichrographe. CD spectra were recorded in quartz cell with path length 0.02 cm from 185–280 nm at room temperature. Trifluoroethanol (TFE) (Aldrich, NMR grade), distilled water and 50% mixture (V/V) of TFE and water were used as solvents. Concentration was in the range of 0.5–0.1 mg/ml. CD band intensities are expressed in molar ellipticity ($[\Theta]_{MR}$ in deg cm^2/dmol).

2.3.6. Fourier transform infrared spectroscopy

FT-IR spectra were obtained in TFE solution with a Bruker IFS-55 FT-IR spectrometer (Germany) at a resolution 2 cm^{-1}, using a 0.02 cm cell with CaF_2 windows. The peptide concentration was 2 mg/ml. The amide I region of the spectra was decomposed into component bands by the Levenberg–Marcquardt non-linear curve-fitting method using weighted sums of Lorentz and Gauss functions. The choice of starting parameters was assisted by Fourier self-deconvolution (FSD). Both the curve-fitting and FSD procedures were part of the instrument's software package (OPUS, version 2.0).

2.3.7. Nuclear magnetic resonance spectroscopy

Parameters of spectra: One-dimensional and two-dimensional NMR spectra were recorded at 298 K and pH 3, in H_2O:D_2O (9:1, V/V) on BRUKER DRX 500 MHz instrument (Germany). Sample concentration was 6.4 mg/ml. All spectra were referenced to water (4.706 ppm). Spin systems identification and sequential assignments were achieved by 2D-NOESY, 2D-COSY and 2D-TOCSY experiments. In the case of peptide LK[HcyADPNRFK]GKDL, three mixing times (140, 175, 230 ms) were used for NOESY, and two different spin-locks (60 and 75 ms) for TOCSY spectra. For peptide LK[CADPNRFK]GKDL, two mixing times were used for NOESY (175 and 250 ms), and two for TOCSY (40 and 60 ms) spectra.

Methods: All resonance assignments were achieved using the NMR triad module of program SYBYL 6.0. Distance restraints were collected by using the 2D-NOESY (140 ms) for peptide LK[HcyADPNRFK]GKDL and 2D-NOESY (250 ms) for LK[CADPNRFK]GKDL. Structure calculation was performed with the program X-PLOR 3.851 based on collected distance restraints, using simulated annealing protocol. Thirty structures were calculated for each different molecule. Following the structure analysis 15–15 conformers (structures) were considered, and used to calculate mean average structures.

2.3.8. Enzyme-linked immunosorbent assay

For the direct ELISA, 96-well ELISA plates (Greiner Labortechnik, Germany) were coated with serial dilutions (starting at 10 μg/well) of the peptides. The dilutions were performed in coating buffer (0.05 M sodium carbonate–bicarbonate buffer, pH 9.6). Plates were incubated overnight at 4 °C. Plates were washed three times with washing buffer (0.3% Tween-20, 1 M NaCl, in phosphate buffered saline, pH 7.2 (PBS)). Monoclonal antibody A16 was diluted (250 times) in dilution buffer (0.3% Tween-20, 0.2 M NaCl, in PBS) and 100 μl of the diluted Mab was added to each well. Thereafter the plates were incubated at room temperature for 1 h, and subsequently washed three times with washing buffer. Then 100 μl of peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark) diluted 1000 times in dilution buffer was added to each well and the plates were incubated at 37 °C for 1 h. After incubation, the plates were washed three times with washing buffer and 100 μl of *o*-phenylenediamine dihydrochloride (0.5 mg/ml) in substrate buffer (0.05 M sodium phosphate-citrate buffer, pH 5.0) with 100 μl of 30% hydrogen peroxide per 100 ml of substrate buffer was added. After 30 min, the reaction was stopped by adding 50 μl of 2 M H_2SO_4 and the absorbance at $\lambda = 490$ nm was measured. As a standard, peptide 9–22 with Nle in position 11 was used.

The competition ELISA was performed as described earlier [25]. Briefly, dilution series of gD peptide 9–22 and the cyclic analogous of peptide 9–22 were pre-incubated for 2 h at room temperature with optimal concentrations (dilution 800×) of Mab A16. After pre-incubation the residual binding capacity of Mab A16 in the pre-incubation mixture was determined by ELISA in microtiter wells plates coated with linear or cyclopeptide (0.5 μg/well). As standard, a dilution series of peptide 9–22 with a fixed dilution of Mab A16 was always included: approximately 3 pmol peptide 9–22 per 100 μl resulted in an OD_{490} of 1.0. The relative amount of the peptides in the pre-incubation mixture required to give an OD_{490} of 1.0 was estimated graphically from plots of OD vs. peptide concentrations. Data represent at least three independent experiments for each compound.

3. Results and discussion

3.1. Synthesis of linear and cyclic 9–22 peptides of HSV gD-1

Two cyclopeptides corresponding to the 9–22 sequence of the HSV-1 gD protein with thioether bond were synthesised, as outlined in Fig. 2. The

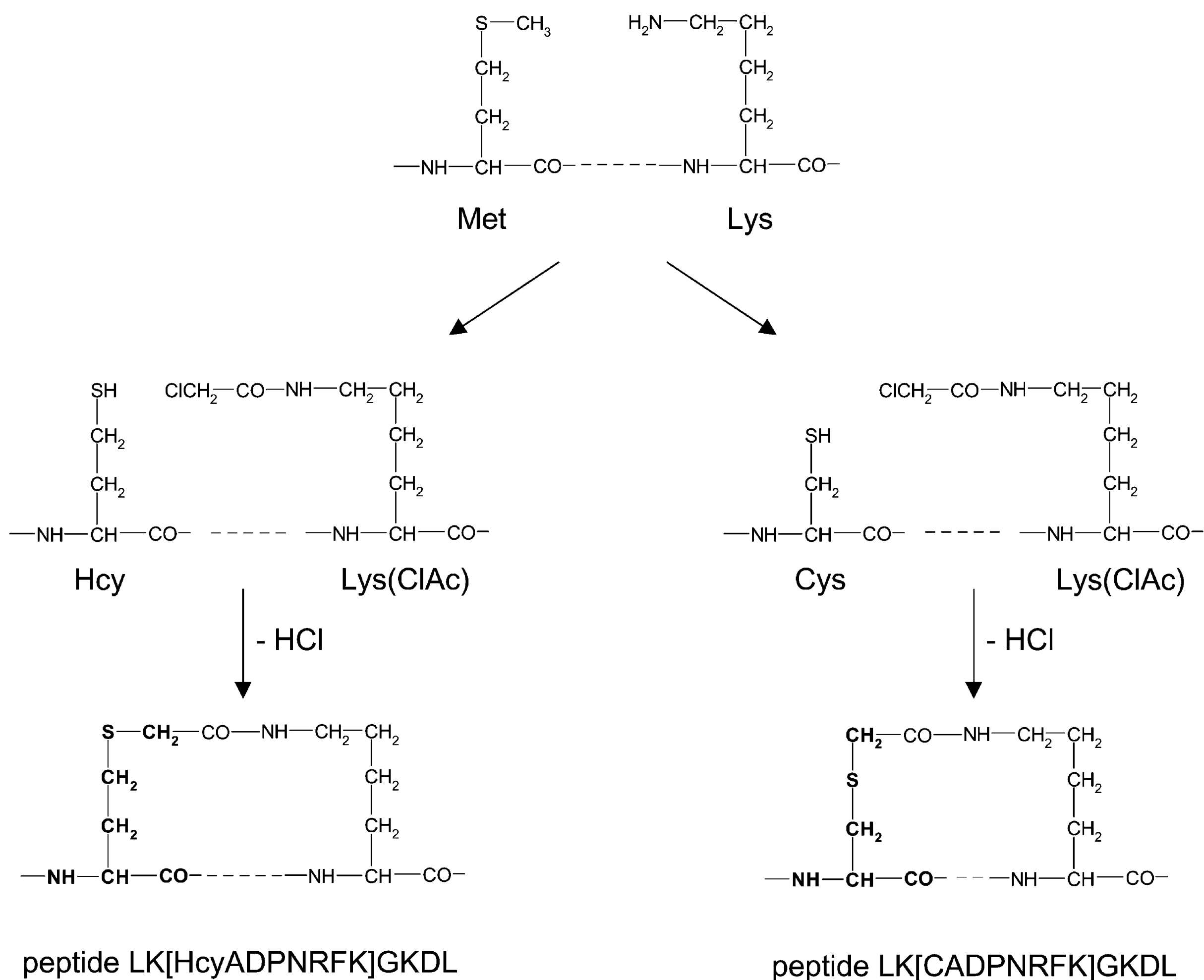

Fig. 1. Design of the cyclopeptides containing thioether bond, between homocysteine or cysteine and lysine.

design of these peptides was based on the following considerations. Firstly, the presence of a C-terminal Asp frequently results in succinimide ring closure side reaction of aspartamide. Therefore, we have elongated the core epitope 9–21 at the C-terminal by Leu also present in the native sequence. Secondly, experimental data proved that the replacement of Met by Nle at position 11 has no influence on the binding of protein-specific Mab A16 [26]. This initiated the selection of position 11 for the introduction of Cys/Hcy as SH-donor component. Finally, we have assumed that the presence of Lys instead of Arg at position 18 will not affect the antibody recognition. In order to prove this we have prepared and tested these two 14-mer analogues (Table 1) The ε-amino group of Lys hosted the chloroacetyl group and provided a partner for the cyclisation. For the formation of cyclic peptides, the thioether bond was introduced in alkaline solution, between a free thiol group and a chloroacetylated side chain of a lysine [21]. In the case of the first model peptide, we prepared a cycle with homoCys in position 11, and its thiol group was coupled to the chloroacetylated side chain of ^{18}Lys. In this case, the substituted side-chain of homoCys mimics the side-chain of the methionine of the native sequence (Fig. 1). In the second case, the use of Cys, instead of homoCys at position 11, has resulted in a cycle shorter by one methylene group than present in the first compound. The reaction in both cases proceeds smoothly under alkaline condition and good yields were obtained (Table 1). Our experience has shown that in the case of both peptides reaction is fast enough to avoid dimerisation of the linear precursor peptides. If the chloroacetylated peptide was added in small portions to the reaction mixture of ammonia and water, the concentration of the non-reacted peptide remains constantly low until the completion of the reaction. In this way, we could also avoid the disulfide bond formation, and at the same time cyclisation is performed at higher concentration than generally applied. The work up of the reaction mixture is simple and it does not require either organic solvents or large volume.

The characteristics of RP-HPLC purified peptides used for our experiments demonstrate the high purity of the produced compounds. The amino acid composition of peptides was determined by amino acid analysis. Considering the presence of Cys/Hcy in the cyclic peptides, two-independent analysis approaches were performed. Data obtained from the analysis of peptide LK[CADPNRFK]GKDL are representative, and shown in Table 2.

3.2. Conformational analysis by CD and FT-IR spectroscopy

The conformation of the cyclic peptides was studied by CD, FT-IR and NMR spectroscopy.

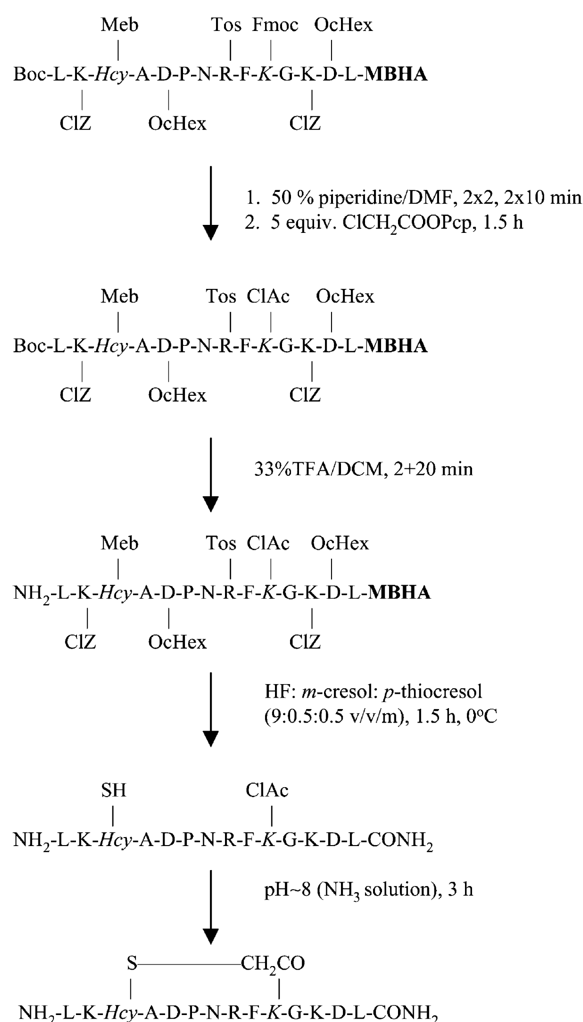

Fig. 2. Outline of the synthesis of HSV gD-1 9–22 cyclic peptide containing a thioether bond.

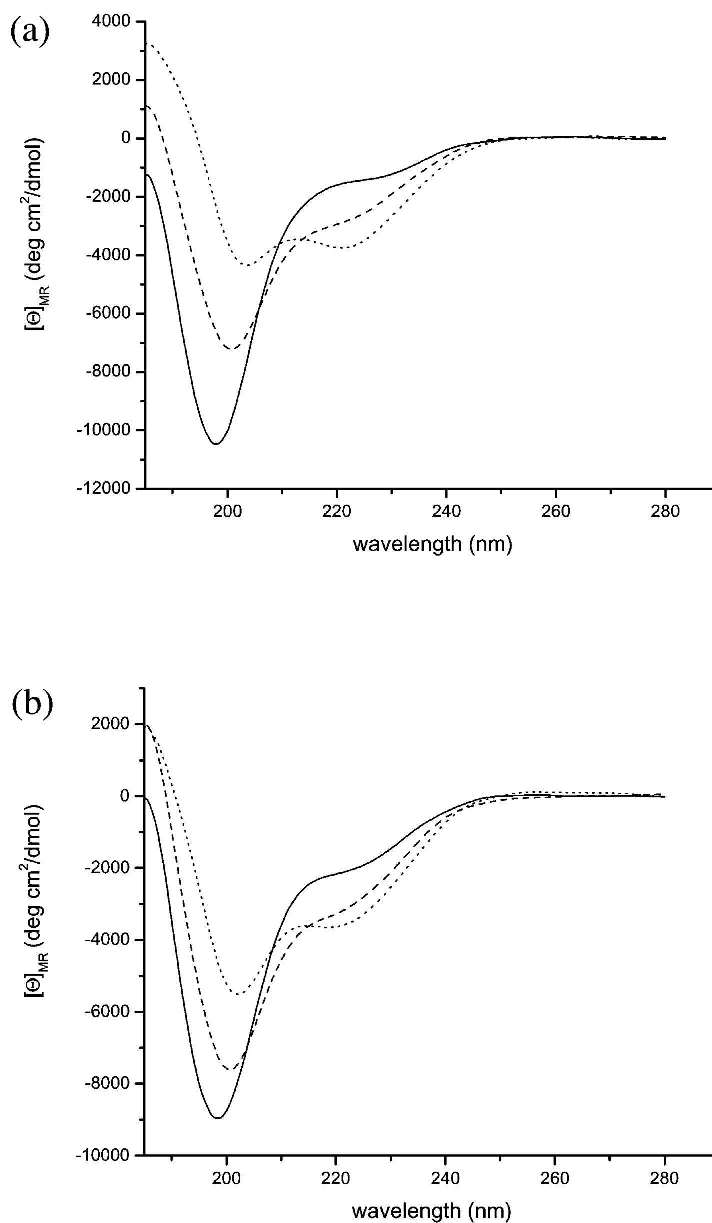

Fig. 3. CD spectra of cyclic peptides. Spectra were recorded in H_2O (——), in the mixture of H_2O:TFE (1:1, V/V) (------), and in pure TFE (......). CD spectra of peptide LK[HcyADPNRFK]GKDL (a), and of peptide LK[CADPNRFK]GKDL (b). Results are expressed as mean ellipticity values.

CD studies were performed either in aqueous solution, in mixture of TFE:water (1:1, V/V) or in pure TFE. The latter type of solvent (TFE) is known to introduce some 'ordering' to structure(s). The CD spectra of both cyclopeptides recorded in water (solid line, Fig. 3a and b) show small differences. Based on an U-type CD-curve with a characteristic negative band at 200 nm, we believe

that both peptides adopt an atypical structure or a mixture of multiple conformers. Compared to pure water, in the mixture of TFE:water the intensity of the $\pi-\pi^*$ transition decreases. Thus, the small, but significant increase of the negative shoulder at 222 nm ($n-\pi^*$ transition) indicates the presence of some 'folded' conformers. The latter tendency is even more plausible when the CD curves obtained in pure TFE are analysed: both spectra show chiroptical features even more characteristic of ordered structure such as β-turns and cyclic peptides. In pure TFE, the $\pi-\pi^*$ band at approximately 203 nm has comparable intensity relative to the $n-\pi^*$ transition (222 nm), and slightly shifted toward a higher wavelength number (from 198 to 203 nm). Peptide LK[HcyADPNRFK]GKDL (Fig. 3a) in pure TFE shows CD spectral features of a more ordered structure compared to that of LK[CADPNRFK]GKDL (Fig. 3b).

In pure TFE, the solution structure of both cyclopeptides was studied by Fourier transform infrared spectroscopy. The interpretation of the spectra for structure analysis is more difficult, because lysine and mainly arginine have intensive absorbtion bands overlapping the amide I region. H-bond acceptor amide carbonyls of β-turns absorb between 1645 and 1635 cm^{-1} in the amide I region in TFE solution. Results of FT-IR studies are summarised in Table 3. Resonance at approximately 1644 cm^{-1} suggests that both the cyclic peptides could adopt a β-turn like structure. However, the shift of the amide I band toward the higher wave number suggests that such a hairpin-like conformation could be atypical or partially distorted. Comparing amide I band intensities, peptide LK[HcyADPNRFK]GKDL could have more β-turn 'character' than LK[CADPNRFK]GKDL.

3.3. Conformational analysis by NMR spectroscopy

As is common for a linear peptide (e.g. a tetradecapeptide such as the precursors of the two cyclic peptides), they adopt in water a conformational ensemble. However, when structural restraints are introduced, such as disulfide bonds, bridged molecular constitution or cyclic backbone, these peptides can adopt a significantly lower number and less diverse backbone structures. Due to these constitutional (sequence-dependent) restrictions, some peptides can exhibit only a small number or even a single molecular conformation stabilised by backbone–backbone, backbone–side-chain or side-chain–side-chain type interactions. The cyclic nature of the above two peptides, where the thiol group of ^{11}Cys (or ^{11}Hcy) is bound to the side-chain of ^{18}Lys via a carboxymethyl linker, does provide some restrictions for both molecules at least for their cyclic part. Undoubtedly, for residues between 11 and 18 approximately twice as many NOE distance restraints were acquired as for the exocyclic parts (Fig. 4a and b). Even though most of these distance restraints arise from intraresidual or from neighbouring NOEs, few medium range NOEs were also assigned. Although the total number of distance restraints is lower than ideal, they make it possible to compute backbone conformation but of limited resolution (Fig. 4a and b). The comprehensive structure analysis of the two-cyclic peptides reveals significant differences. As a result of structural refinement, we found that the major structural difference between LK[CADPNRFK]GKDL and LK[Hcy-ADPNRFK]GKDL is at the ^{12}Ala and ^{13}Asp. These differences are clearly noticeable even by the significant chemical shifts changes of the same

Table 3
Amide I bands in the FT-IR spectra of cyclopeptides

Peptide	ν (cm^{-1}) [%]			
	High-frequency region	Solvated amides	β-turns	γ-turns
LK[HcyADPNRFK]GKDL	1676 (15)	1661 (43)	1644 (10)	1629 (14)
LK[CADPNRFK]GKDL	1674 (20)	1660 (44)	1643 (6)	1629 (18)

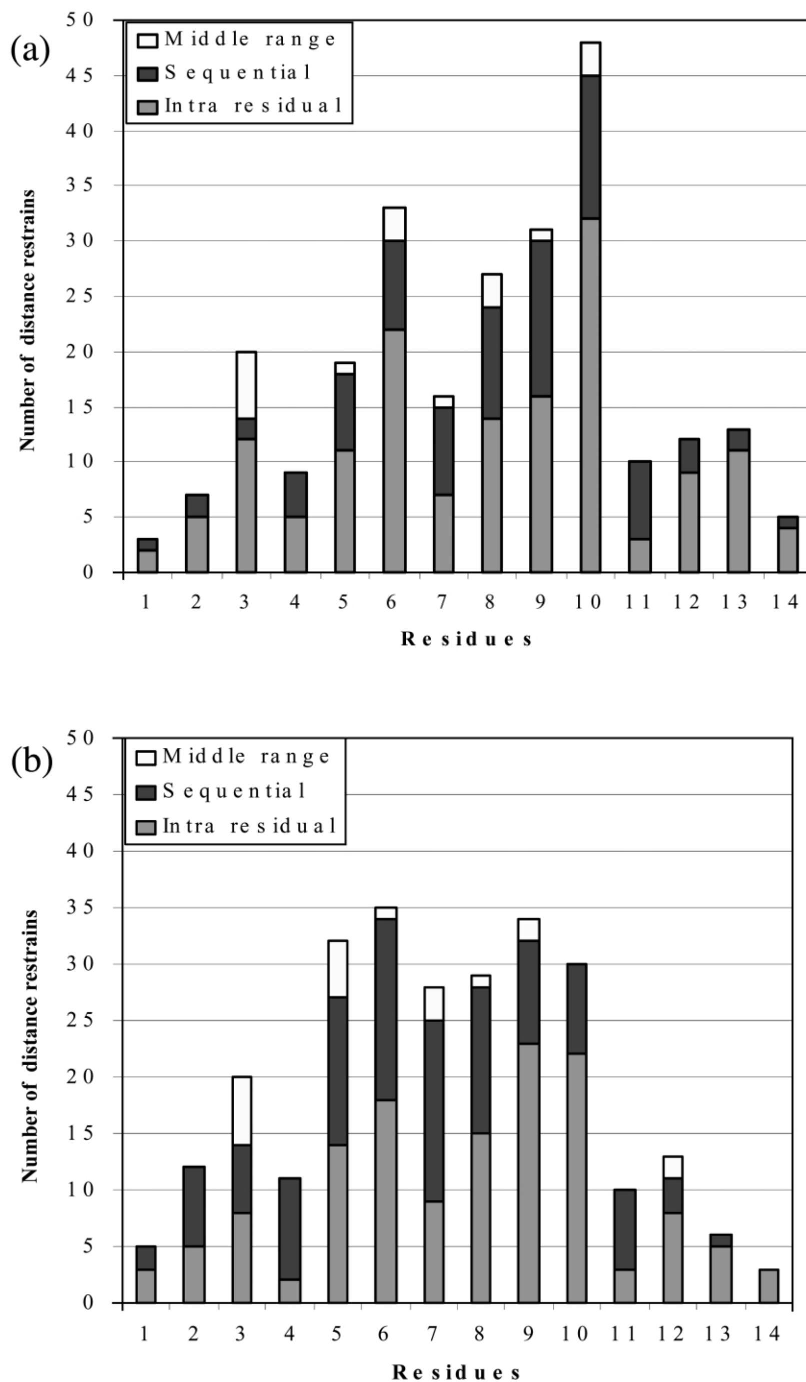

Fig. 4. Distribution of distance restrains per residues of the cyclic peptides. Assigned in a two-dimensional-NOESY spectrum of 140 ms mixing time for peptide LK[HcyADPNRFK]GKDL (a), or in a two-dimensional-NOESY spectrum of 250 ms mixing time for peptide LK[CADPNRFK]GKDL (b).

Table 4
Proton chemical shift values of peptide LK[HcyADPNRFK]GKDL in H_2O:D_2O (9:1, V/V) recorded at 297 K

	NH	Hα	Hβ$_1$	Hβ$_2$	Hγ$_1$	Hγ$_2$	Hδ$_1$	Hδ$_2$	Hε$_1$	Hε$_2$	H(NH_2)
Leu 9		3.88	1.54	1.54	1.25		0.82	0.82			
Lys 10	8.60	4.21	1.57	1.62	1.27	1.27	1.54	1.54	2.85	2.85	7.41
Hcy 11	8.45	4.24	1.86	1.75	2.43	2.43	3.22	3.22			
Ala 12*	**8.46**	**4.06**	**1.26**	**1.26**							
Asp 13*	**8.19**	**4.87**	**2.77**	**2.56**							
Pro 14		4.27	2.43	2.14	1.87	1.87	3.67	3.54			
Asn 15	8.06	4.52	2.66	2.66			6.90	7.58			
Arg 16	7.71	4.02	1.55	1.55	1.25	1.25	2.99	2.99	7.00		7.54
Phe 17	8.28	4.49	2.92	3.00			7.15	7.15	7.22	7.22	6.90
Lys 18	8.04	4.13	1.55	1.65	1.25	1.15	1.34	1.40	3.10	3.10	8.12
Gly 19	7.50	3.72									
Lys 20	8.07	4.16	1.57	1.62	1.27	1.27	1.54	1.54	2.85	2.85	7.41
Asp 21	8.42	4.53	2.65	2.79							
Leu 22	8.16	4.13	1.52	1.52	1.47		0.70	0.72			

* The main differences between chemical shift of the two peptides are highlighted.

amino acid residues of the two peptides (Tables 4 and 5). Undoubtedly, the backbone fold of LK[CADPNRFK]GKDL exhibits a more or less unique conformation with R.M.S.D. value of 0.73 Å for residues 4–10 (Table 6). However, the structural refinement of the other peptide LK[HcyADPNRFK]GKDL resulted in a molecular conformation likely to be composed of two clearly distinguishable backbone folds '*A*' and '*B*', respectively. They differ from each other in the main chain folds of residue ^{12}Ala (Figs. 5–7).

3.4. Reactivity of the peptides with Mab A16

We have studied the antibody binding properties of the cyclic and linear peptides by ELISA experiments, using Mab A16. This antibody is directed against the group VII epitope of glycoprotein D and was obtained by immunizing mice with extract of HSV infected cells.

For the preparation of the two cyclic peptides we have used the native sequence of gD epitope with two modifications. It has been described in

Table 5
Proton chemical shift values of peptide LK[CADPNRFK]GKDL in H_2O:D_2O (9:1, V/V) recorded at 297 K

	NH	Hα	Hβ$_1$	Hβ$_2$	Hγ$_1$	Hγ$_2$	Hδ$_1$	Hδ$_2$	Hε$_1$	Hε$_2$	H(NH_2)
Leu 9		3.94	1.69	1.66	1.40		0.81	0.81			
Lys 10	8.64	4.26	1.71	1.71	1.33	1.33	1.59	1.59	2.90	2.90	7.45
Cys 11	8.52	4.44	2.86	2.89			3.26	3.26			
Ala 12*	**8.62**	**4.16**	**1.32**	**1.32**							
Asp 13*	**8.02**	**4.94**	**2.78**	**2.58**							
Pro 14		4.26	2.15	2.15	1.89	1.89	3.68	3.55			
Asn 15	8.13	4.55	2.73	2.89			6.99				
Arg 16	7.74	4.13	1.58	1.58	1.29	1.29	3.03	3.03	7.04		7.59
Phe 17	8.29	4.52	2.96	3.07			7.20	7.20	7.27	7.27	6.94
Lys 18	8.06	4.17	1.42	1.42	1.59	1.72	1.22	1.30	3.13	3.13	8.16
Gly 19	7.50	3.77									
Lys 20	8.08	4.21	1.71	1.71	1.33	1.33	1.59	1.59	2.90	2.90	7.45
Asp 21	8.44	4.57	2.79	2.89							
Leu 22	8.17	4.20	1.60	1.24	1.42		0.79	0.71			

* The main differences between the chemical shift of the two peptides are highlighted.

Table 6
R.M.S.D. values calculated for the best 17 conformers of peptide LK[CADPNRFK]GKDL (A), for the best 8 '*A*' conformers of peptide LK[HcyADPNRFK]GKDL (B) and for the best 7 '*B*' conformers of peptide LK[HcyADPNRFK]GKDL (C)

Fitted range	Backbone value (Å)	Heavy atom value (Å)
(*A*)		
4–10	0.73 ± 0.26	1.77 ± 0.51
5–9	0.45 ± 0.15	1.63 ± 0.50
(*B*)		
4–10	0.79 ± 0.18	2.12 ± 0.51
5–9	0.64 ± 0.12	1.97 ± 0.42
(*C*)		
4–10	0.87 ± 0.26	2.11 ± 0.58
5–9	0.68 ± 0.24	1.97 ± 0.46

the literature that the replacement of Met at position 11 by Nle has no influence on the antibody binding [26]. In order to provide an amino group for cyclisation, we have substituted Arg at position 18 by Lys. Although, residue 18 is not one of the epitope core residues recognised by Mab A16 [25], this change may have an effect on the binding properties of Mab A16 to the peptides. Therefore, we have studied first the binding properties of the three 14-mer linear peptides LKNle-ADPNRFRGKDL, LKMADPNRFKGKDL and LKNleADPNRFKGKDL. Peptides LKNleADPNRFRGKDL and LKMADPNRFKGKDL comprise the native gD-1 sequence with an Nle/Met or Lys/Arg mutation at position 11 or 18, respectively. The third peptide, LKNleADPNRFKGKDL contains both substitutions. The results of the ELISA experiments are summarised in Table 7. These data show that all the three peptides reacted almost equally well with Mab A16, indicating that the replacement of Arg by Lys in linear peptides essentially does not affect the binding of Mab A16. It should be added that the native gD-1 peptide (LKMADPNRFRGKDL) possesses similar binding properties 31.4 pmol/100 μl in direct ELISA and 4.9 pmol/100 μl in competition ELISA.

Based on this finding, we prepared two cyclic derivatives using the Lys residue at position 18 for cyclisation, and investigated the reactivity of these compounds with Mab by direct and competition ELISA. In the direct ELISA, the cyclic peptides as well as the linear peptide LKNle-ADPNRFRGKDL as control, were used as coating (target) antigens. After incubation with the gD-specific Mab A16, the binding of the compounds was expressed as the amount of peptide needed for coating to obtain an OD_{490} of 1.00. We found significant differences between the two cyclic peptides in antibody binding of the Mab, while the peptides only differ in one methylene group. The

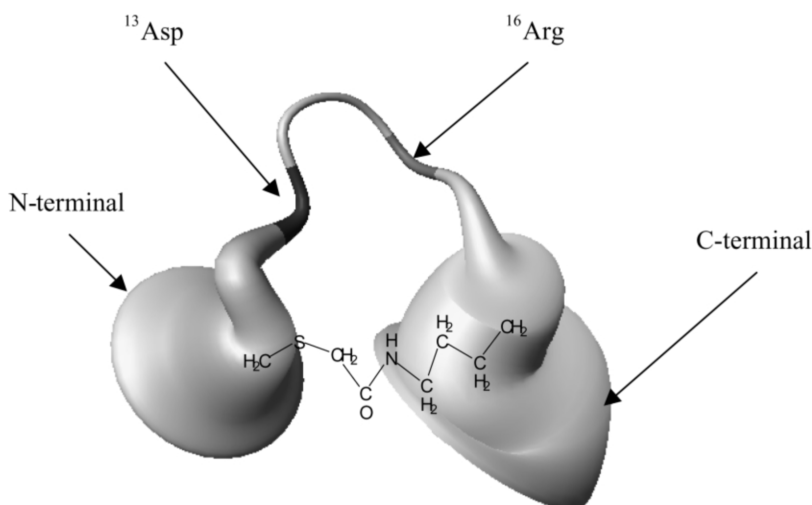

Fig. 5. Mean average NMR structure of peptide LK[CADPNRFK]GKDL balanced with B-factors.

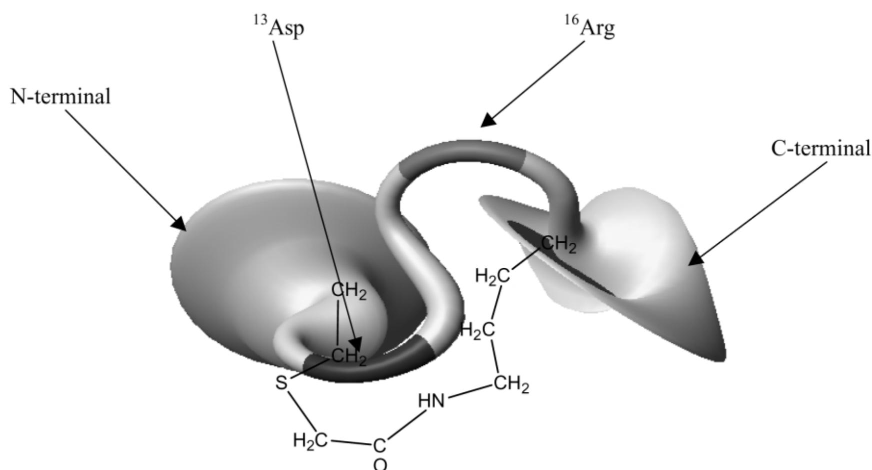

Fig. 6. Mean average NMR structure of conformation '*A*' of peptide LK[HcyADPNRFK]GKDL balanced with B-factors.

data showed, that Mab A16 binds to peptide LK[HcyADPNRFK]GKDL, but that the other cyclic peptide LK[CADPNRFK]GKDL was not recognised under identical conditions (Table 7). However, the reactivity of the cyclic peptide, LK[HcyADPNRFK]GKDL and Mab 16, is much less pronounced than that of the linear peptide LKNleADPNRFRGKDL and the Mab. Binding at an $OD_{490}=1.0$ of Mab A16 required at least coating of 10 μg per well (100 μl) of peptide LK[HcyADPNRFK]GKDL, while 31 ng per well of the linear peptide 9–22[^{11}Nle] was sufficient. The difference in reactivity of the two cyclic peptides in the direct ELISA could be either due

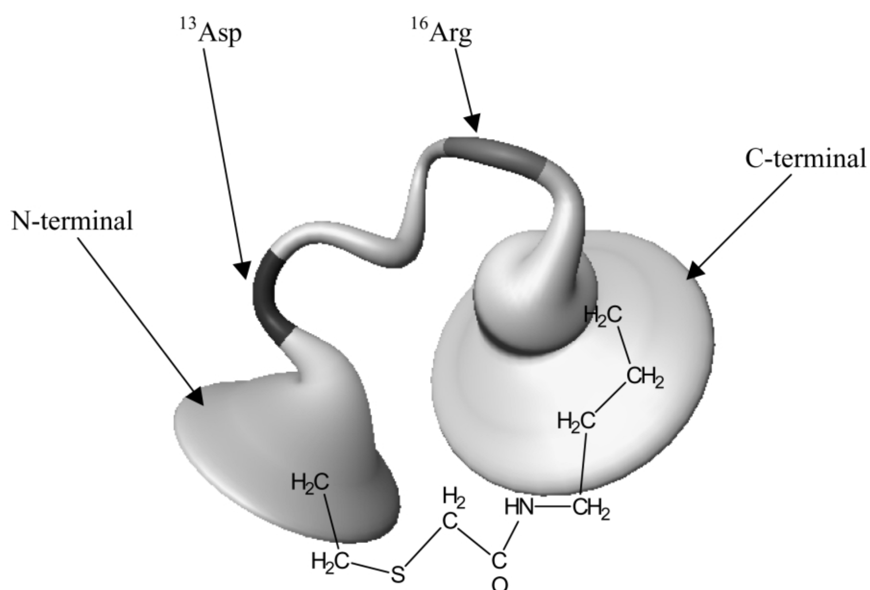

Fig. 7. Mean average NMR structure of conformation '*B*' of LK[HcyADPNRFK]GKDL balanced with B-factors.

Table 7
Reactivity of Mab A16 with linear and cyclopeptides derived from glycoprotein D of HSV type 1

Peptide[a]	Direct ELISA (pmol/100 μl)[b]	Competition ELISA (pmol/100 μl)
[9]LKNleADPNRFRGKDL[22]	18.9	2.8[c]
[9]LKMADPNRFKGKDL[22]	25.9	8[c]
[9]LKNleADPNRFKGKDL[22]	25.4	5[c]
[9]LK[CADPNRFK]GKDL[22]	>6000	7900[d]
[9]LK[HcyADPNRFK]GKDL[22]	4443	2300[d]

[a] Sequence according to the sequence of mature gD-1 without signal sequence. Substitutions are underlined.
[b] Required for coating to obtain an OD of 1.00 at $\lambda=490$ nm with Mab A16.
[c] Amount of the compound required in the pre-incubation step, resulting OD=1.0 at $\lambda=490$ nm when the pre-incubation mixture was added to microtiter plates, using peptide [9]LKNleADPNRFRGKDL[22] as coat.
[d] Amount of the compound required in the pre-incubation step, resulting OD=1.0 at $\lambda=490$ nm when the pre-incubation mixture was added to microtiter plates, using peptide LK[CADPNRFK]GKDL as coat (10 μg/100 μl).

to differences in coating properties of the two compounds or to differences in antibody binding. To discriminate between these two possibilities, we have performed competition ELISA experiments, in which the two cyclic peptides LK[HcyADPNRFK]GKDL and LK[CADPNRFK]GKDL and linear peptide LKNleADPNRFRGKDL, as positive control, were allowed to react with Mab A16 in solution. Serial dilutions of peptides were pre-incubated with a fixed amount of Mab A16 and the residual antibody binding activity was determined on ELISA plates coated with the cyclic antigen (LK[CADPNRFK]GKDL). The results depicted in Fig. 8 show differences between the binding of the two cyclic compounds. In accordance with direct binding experiments, the antibody recognition of peptide LK[CADPNRFK]GKDL was slightly less potent than in case of peptide LK[HcyADPNRFK]GKDL. The latter compound had an 3.1 ± 0.6 higher reactivity with Mab A16 than peptide LK[CADPNRFK]GKDL. It should be also noted that the linear control peptide exhibited pronounced A16 binding (Table 7). The data obtained from competition experiments suggest that the difference in reactivity of two cyclic peptides LK[HcyADPNRFK]GKDL and LK[CADPNRFK]GKDL against Mab A16 most probably could be due to their altered structural features. In addition, the data of the competition ELISA cannot completely exclude the effect of the structural differences on the immobilisation of the two cyclic peptides.

4. Conclusion

In this study, we reported the preparation, structural and binding characterisation of two stable conformational mimetics of a linear antibody epitope sequence derived from glycoprotein D of HSV type 1. For this, two cyclic peptides were synthesised with thioether bond. The two compounds differ only in one methylene group in their cyclic part. In spite of this 'minor' change, their solution structures as well as their antibody recognition, and immobilisation capacity exhibited significant differences. The comprehensive solution structure analysis of these peptides revealed that the inclusion of an extra CH_2 group resulted in a fundamental change in the peptide backbone conformation with altered turn structure. By using NMR spectroscopy, it was possible to distinguish the different conformers and clearly identify the main structural changes: the backbone conformation and side-chain positions were particularly different for residues [12]Ala and [13]Asp. This observation is in accord with the fact that [13]Asp was identified earlier as an essential amino acid for antibody recognition. Solution structure analysis of these peptides in connection with their antibody binding efficiency clearly shows the importance of secondary structure in antibody binding and demonstrates that even a relatively minor modification

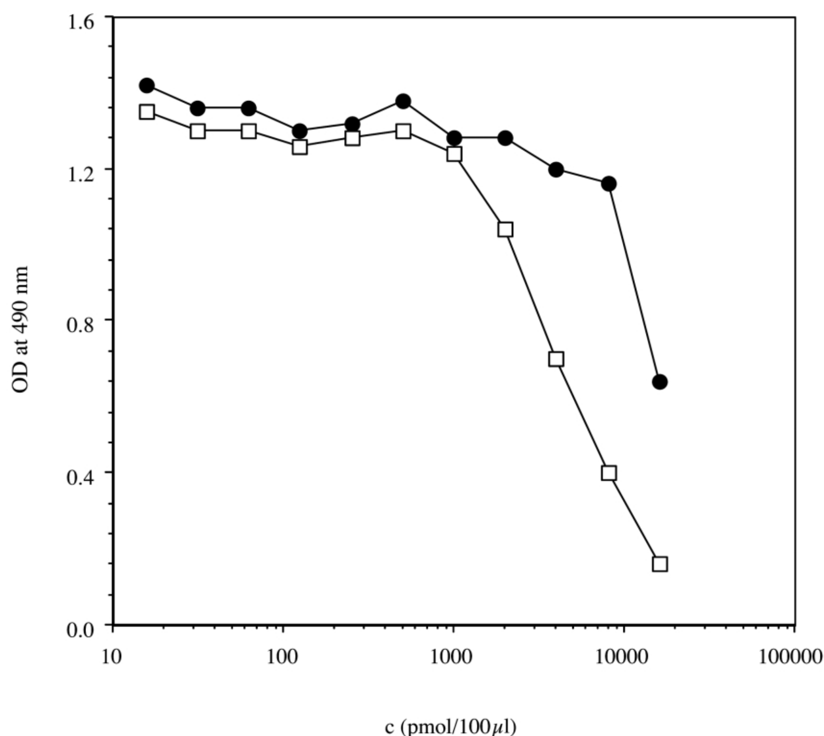

Fig. 8. Antibody binding of cyclic peptides in the competition ELISA. The residual activity of Mab A16 is shown after pre-incubation with cyclopeptides LK[CADPNRFK]GKDL (●) and LK[HcyADPNRFK]GKDL (□).

of the primary structure could influence dramatically the immune recognition of synthetic antigens. These results show that the cyclic peptides had lower affinity to Mab A16 compared to the linear ones, probably due to their restricted flexibility. However, the differences between the structures and antibody recognitions of cyclic peptides show strong correlation providing a basis for a successful design of cyclic peptides with good antibody recognition, which can be potent synthetic vaccines.

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